CHAPTER 4

Synthesis, characterization, single crystal X-ray studies, molecular docking and anticancer activity of novel Bcl-2 inhibitors

4.1 Introduction

Apoptosis is one of the major mechanisms of cell death in response to cancer therapies\textsuperscript{1}. Alterations in susceptibility to apoptosis not only contribute to neoplastic development\textsuperscript{2} but also can enhance resistance to conventional anticancer therapies, such as radiation and cytotoxic agents\textsuperscript{3}. One of the suggested mechanisms of resistance to cytotoxic antineoplastic drugs is the alteration in expression of B-cell lymphoma-2 (Bcl-2) family members. The Bcl-2 family of proteins consists of 25 pro- and anti-apoptotic members, which interact to maintain a balance between newly forming cells and old dying cells. When anti-apoptotic Bcl-2 family members are overexpressed, the ratio of pro- and anti-apoptotic Bcl-2 family members is disturbed and apoptotic cell death can be prevented. Targeting the anti-apoptotic Bcl-2 family of proteins can improve apoptosis and thus overcome drug resistance to cancer chemotherapy\textsuperscript{4-6}. The key players that execute the apoptotic cascade are the initiator and the effector caspases, which are activated by cleavage early in apoptosis\textsuperscript{3,7}. Two major pathways of apoptosis converge on the effector caspases, the intrinsic and extrinsic cell-death pathways. The intrinsic cell death pathway, also known as the mitochondrial apoptotic pathway, is activated by a wide range of signals, including radiation, cytotoxic drugs, cellular stress, and growth factor withdrawal, and involves the release of proteins (including cytochrome c) from the mitochondrial membrane space\textsuperscript{8}. Cytochrome c combines with an adaptor molecule, apoptosis protease-activating factor 1, and also with an inactive initiator caspase, pro-caspase-9, within a multiprotein complex called the apoptosome\textsuperscript{9}. This leads to the activation of caspase-9, which then triggers a cascade of caspase (caspase-3, caspase-6, caspase-7) activation, resulting in the morphologic and biochemical changes associated with apoptosis. By contrast, the extrinsic cell-death pathway can function independently of mitochondria and is activated by cell-surface death receptors, such as Fas and tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) receptors, directly activating the caspase cascade via an “initiator” caspase (caspase-8) within a death-inducing signaling complex\textsuperscript{10}. The intrinsic pathway (via mitochondria) plays a key role in regulating cell death in response
to various stimuli. Mitochondrial outer membrane permeabilization is considered the “point of no return” for apoptotic cell death, triggering release into the cytoplasm of proteins that mediate cell death, such as cytochrome c. Outer membrane permeabilization is mediated by certain Bcl-2 family members that coordinately regulate apoptosis among a series of interacting pro- and anti-apoptotic proteins. Inner membrane permeabilization can be altered by the redox status of mitochondrial protein vicinal thiols and through opening of the mitochondrial permeability transition pore. Although the mitochondrial permeability transition pore complex contains proteins on the outer and inner mitochondrial membranes, there is no clear involvement of inner membrane permeabilization in mitochondrial apoptosis and there is no compelling evidence that inner membrane permeabilization is necessary for outer membrane permeabilization.

4.2 Bcl-2 family of proteins

To date, 25 members of the Bcl-2 family of proteins have been identified. These proteins are localized to mitochondria, smooth endoplasmic reticulum, and perinuclear membranes in hematopoietic cells. Over-expression of several anti-apoptotic Bcl-2 family proteins has been reported in hematologic malignancies. Bcl-2 proteins are characterized by the presence of up to four relatively short sequence motifs, which are <20 amino acid residues in length, termed Bcl-2 homology domains. Bcl-2 family members can be divided into three subfamilies based on structural and functional features. The anti-apoptotic subfamily contains the Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bfl1/A1, and Bcl-B proteins, which suppress apoptosis and contain all four Bcl-2 homology domains, designated Bcl-2 homology 1-4. Some pro-apoptotic proteins, such as Bax, Bak, and Bok, contain Bcl-2 homology 1-3 domains and are termed “multidomain proteins,” whereas other pro-apoptotic proteins, such as Bim, Bad, and Bid, contain only the BH3 domain and are termed “BH3-only” proteins. Figure 1 illustrates the role of the Bcl-2 family of proteins in apoptosis. How Bcl-2 family proteins interact to permeabilize the mitochondrial membrane remains controversial. There are two tentative models explaining how BH3-only proteins can interact with other Bcl-2 family proteins to induce apoptosis. The “direct activation model” suggested by Letai et al. divides BH3-only domain proteins into groups, which are “sensitizers” and “activators.” The activator BH3-only molecules (Bim, Bid) directly bind and oligomerize Bax/Bak, leading
to release of cytochrome c. A study supporting the latter theory showed that a Bid mutant that lacks the ability to interact with Bcl-2 (but maintains interaction with Bax) is still potently pro-apoptotic, suggesting that the interaction with Bax and/or Bak is important in the function of Bid. The sensitizers, such as Bad, Bik, and Noxa, cannot directly activate Bax/Bak, but they inhibit anti-apoptotic Bcl-2 proteins from engaging with activator proteins or Bax/Bak, thereby sequestering activators and Bax/Bak. The engagement of BH3-only proteins with anti-apoptotic Bcl-2 members is common between the two models. Unlike most oncogenes that promote proliferation, Bcl-2 functions by preventing programmed cell death. As the anti-apoptotic Bcl-2 family proteins promote cancer cell survival by antagonizing apoptosis, they provide therapeutic targets, and inhibition of anti-apoptotic Bcl-2 family proteins is expected to predominantly induce apoptosis in cancer cells. Bcl-2 was identified because of a characteristic chromosomal translocation t(14;18) present in 85% of follicular lymphomas and 20% of diffuse B-cell lymphomas, which results in deregulated BCL-2 gene expression at the transcriptional level. The in vivo effects of Bcl-2 were initially investigated in Bcl-2 transgenic mice in which Bcl-2 over-expression was targeted to B and T lymphocytes, which lead to follicular hyperplasia or T-cell lymphomas. Constitutively high levels of Bcl-2 or Bcl-XL have been associated with a more aggressive malignant phenotype and/or drug resistance to various categories of chemotherapeutic agents in hematologic malignancies and solid tumors. As an example, high Bcl-2 levels in primary prostate cancer were associated with high Gleason scores and a high rate of cancer recurrence after radical prostatectomy. Expression of BCL-XL in the NCI 60 cell line panel strongly correlated with resistance to most chemotherapy agents. Overexpression of Bcl-2 RNA and/or protein has been observed in acute myelogenous leukemia (AML) and in acute lymphoblastic leukemia, and the Bax/Bcl-2 ratio inversely correlates with prognosis of AML and acute lymphoblastic leukemia. Bcl-2 overexpression is commonly observed but is highly variable at diagnosis in acute lymphoblastic leukemia. Despite observations that Bcl-2 modulation can increase the sensitivity to chemotherapeutic agents in vitro, the level of Bcl-2 expression has not been observed to impact event-free survival or aggressiveness of acute lymphoblastic leukemia, possibly owing to the complexity of interactions among Bcl-2 family members. Thus, it has been speculated that the balance between anti- and pro-apoptotic
Bcl-2 family members, rather than mere overexpression of Bcl-2, regulates the death of cancer cells\textsuperscript{33}.

\textbf{Figure 1:} The apoptotic pathway to cell death from the perspective of the Bcl-2 proteins. (Adapted from\textsuperscript{19})

4.3 \textbf{Bcl-2 as a target in cancer}

In recent years, it is increasingly being realized that over-expression of Bcl-2, Mcl-1, Bcl-XL and other members contributes to cancer progression and confers resistance to apoptosis induced by standard anticancer therapies\textsuperscript{34,35}. This is more common in genetically complex and inherently resistant cancers such as pancreatic, ovarian, prostate and metastatic breast cancers that are known to over-express at least one family member of anti-apoptotic proteins. Currently available anticancer therapies are based on targeting cancer cell DNA integrity or replication, which indirectly trigger apoptosis in tumor cells\textsuperscript{36}. Tumors expressing high levels of Bcl-2, Mcl-1 or Bcl-XL are often found to be resistant to chemotherapeutic agents or radiation therapy\textsuperscript{37}. There is an urgent medical need for the development of novel
strategies that can circumvent the observed resistance developed towards currently available chemotherapeutics. Therefore, inhibition of the function of the anti-apoptotic members represents a novel and promising strategy for designing new classes of anticancer drugs that can overcome the resistance of cancer cells to chemotherapy or radiation.

4.4 Existing treatments and advancements in the development of Bcl-2 targeted therapy

Initial advancements in this area were based on silencing strategies using antisense technology that in principle could inhibit Bcl-2 expression levels, which formed the basis of the concept behind Bcl-2 antisense therapy\textsuperscript{38-43}. A promising Bcl-2 antisense oligonucleotide G3139 was in several clinical trials for treatment of different cancers\textsuperscript{44-48}. Phase III studies suggest that this approach is reasonably successful, at least, in myeloid leukemia patients in whom robust intracellular concentrations of G3139 could be achieved in vivo in bone marrow (range, 3.4 – 40.6 pmol/mg protein) and PBMCs (range, 0.47 – 19.4 pmol/mg protein) that were directly related to Bcl-2 mRNA downregulation\textsuperscript{49}. Among such Bcl-2 antisense, oblimersen is already in Phase III clinical trials. However, antisense oligonucleotides have short half-life and are prone to rapid enzymatic degradation and turnover. This is certainly a hindrance in the success of antisense therapy and, therefore, researchers are focusing on the development of better chemical modifications of such antisense oligonucleotides to increase resistance to nuclease digestion, prolong tissue half-lifes and improve scheduling\textsuperscript{50}. Another attractive approach to block the activity of Bcl-2 is the use of antibody directed against Bcl-2. The concept that antibodies might be effective for the treatment of cancers originated more than a century ago with Paul Ehrlich's hypothesis that it would someday be feasible to develop a ‘magic bullet’ that has an affinity for ‘parasites’ sparing normal tissues. However, since then, a hundred years have elapsed before antibodies could actually be developed as effective agents for the treatment of cancer. An intracellular anti-Bcl-2 single chain antibody has been shown to increase drug-induced cytotoxicity in the MCF-7 breast cancer cell lines as well as other cancers\textsuperscript{51}. Other approaches include the use of a ribozyme against Bcl-2 and, more recently, a synthetic, cell permeable Bak BH3 peptide that binds to Bcl-2 has been shown to be partially successful both in vitro and in vivo against myeloid leukemia growth\textsuperscript{52}. Like antisense therapy, the use of antibody, ribozymes or peptides as therapeutic strategy is
hindered by the lack of stability and effective delivery. To overcome this issue, a chemical strategy has also been pursued by some researchers using hydrocarbon stabling to generate stapled BH3 peptide with increased pharmacological properties \(^{53,54}\). The stapled peptides, called ‘stabilized α-helix of Bcl-2 domains’ (SAHBs), are helical, protease-resistant and cell-permeable molecules that bind with increased affinity to multi-domain Bcl-2 member pockets. Such a SAHB of the BH3 domain from the Bid protein was shown to specifically activate the apoptotic pathway to kill leukemia cells. Furthermore, other stapled Bid-BH3 peptides have also been synthesized that have shown to have better apoptotic potential than parent peptide alone.

4.5 Current research goal in Bcl-2 inhibition

The last 2 decades have witnessed numerous advancements in our understanding of the apoptotic machinery and many approaches have been designed towards targeting the Bcl-2 family members. Even though partially successful, none of these approaches has been proven to be useful in the clinic, and thus attention has been focused on newer agents with better clinical outcome such as non-peptidic Small Molecular weight Inhibitors (SMI). Researchers over the years have realized that peptide and enzyme based approaches may not be successful due to stability issues. Therefore, the current goal of researchers is to devise newer approaches that could be more stable and also overcome the membrane barrier. To this end, an important step has been taken, that is, the development of SMIs in the inhibition of bcl-2 proteins.

4.6 Scientific rationale for bcl-2 inhibition

Due to limited success of antisense, oligonucleotide and antibody-based approaches, the researchers changed their center of attention towards a different strategy that was focused on antagonizing the function of Bcl-2 rather than to reduce its levels. This was approached mechanistically following the delineation of the crystal structure of BclXL, which revealed that the BH1 – 3 domains formed a hydrophobic groove\(^{55}\), where the α-helix of the BH3-only proteins bind\(^{56}\). The structural analysis of BclXL bound to the Bak BH3 peptide was a proof-of-concept experiment indicating that it could be possible to create small molecules that bind to the hydrophobic groove of Bcl-XL, thereby, inhibiting its anti-apoptotic function. SMIs are organic molecules of low molecular mass (usually < 750 Dalton). Their small size makes their
use in vivo even more practical, and possibly more cost-efficient, compared to oligonucleotides or other small peptides. The anti-apoptotic function of Bcl-2 is attributed, at least in part, to the ability to hetero-dimerize with pro-apoptotic members such as Bim, and it has been hypothesized that SMIs that bind to this BH3-binding site could in theory be capable of blocking the hetero-dimerization of Bcl-2 with pro-apoptotic members of the Bcl-2 protein family, such as Bid and Bim. Drug occupation of the hydrophobic groove is thus thought to abrogate the anti-apoptotic function of Bcl-2 (and others) and induce apoptosis. This decade has witnessed a tremendous enthusiasm in the area of SMI design and development initiated by half a dozen groups of scientists who have taken up the challenge to develop SMIs. In order to understand the structural basis of (−)-gossypol binding to Bcl-2, Wang et al. have performed computational docking of (−)-gossypol into the BH3-binding groove in Bcl-2. This group has also modeled the binding of a Bim BH3 peptide in a complex with Bcl-2 as this Bim BH3 peptide binds to Bcl-2 with a high affinity. Based on their predicted binding model, (−)-gossypol forms a hydrogen bonding network with residues Arg146 and Asn143 in Bcl-2 through the aldehyde group and the adjacent hydroxyl group on the right naphthalene ring. This mimics the hydrogen bonding network formed by Asp99 and Asn102 in Bim, and Arg146 and Asn143 in Bcl-2. The isopropyl group on the same naphthalene ring inserts into a hydrophobic pocket in Bcl-2, in part mimicking the Phe101 in the Bim peptide. The left half of the (−)-gossypol molecule interacts primarily with Bcl-2 through hydrophobic contacts, mimicking Ile97 in the Bim peptide. This binding model suggests that the two halves of (−)-gossypol interact differently with Bcl-2 and provided clues as well as structural basis for the design of novel SMIs of Bcl-2 such as ApoG2 and TW-37. The following paragraphs summarize the currently studied SMIs, their mode of action and provide a brief description of their success in the clinic.

### 4.7 Currently available Small Molecular Weight Inhibitors (SMIs) of Bcl-2 proteins

Over the last 10 years, the field has witnessed emergence of numerous SMIs of Bcl-2. These inhibitors belong to different class of compounds as well as source or origin. The early inhibitors were originally discovered from natural sources and then later modified for optimal activity and reduced toxicity. The second generation witnessed rapid development of synthetic SMIs each designed to target individual or the entire Bcl-2 family of proteins.
The field has progressed quickly and competitively and some of the latest discoveries on individual inhibitors are described below:

A) **Gossypol and related small molecule analogs**

Prior to chemically synthesizing SMIs against Bcl-2, researchers extensively searched for natural compounds that could function as cell permeable, small molecule mimetic of the crucial Bcl-2 BH3 domain. A number of natural agents were discovered using a library screening process that includes tetrocarcin A\(^5^8\), antimycin\(^5^9\) and gossypol\(^6^0\). Gossypol, also known as BL-193, is a natural compound isolated from cottonseeds and roots\(^6^1\), which was the first compound to reach the clinic, but its mechanism of action at the time was not known\(^6^2\). It has been studied since the 1980s for its contraceptive properties\(^6^3\), DNA damaging capacity\(^6^4;6^5\), ability to generate ROS and cytochrome c release\(^6^6\) and many other modulatory properties that cumulatively were believed to be responsible for its anticancer effects. There are two iso-forms of gossypol, which include (−)-BL-193, (+)-BL-193, while (±)-BL-193 is a racemic mixture of the two. The (−)-BL-193 has been shown to be more potent than its other isoforms as inhibitors of cell growth. Multidimensional NMR methods have shown that (−)-BL-193 binds the hydrophobic groove of Bcl-2 and Bcl-XL\(^5^7\), and is currently in Phase III testing as a Bcl-2 inhibitor. Apart from apoptotic cell death, other mechanisms of growth inhibition have been attributed to gossypol. Recently, Voss et al. have shown that in malignant glioblastoma, (−)-gossypol triggers autophagic cell death through neutralization of Mcl-1\(^6^7\). Therefore, in view of the multiple non-apoptotic effects of gossypol, it is suggested that further studies are needed to completely decipher its anticancer potential. Studies have indicated that in addition to apoptosis, gossypol can also induce autophagy by disrupting the interaction between beclin-1 and Bcl-2 proteins \(^6^8\). In this elegant study, Gao et al. have shown that gossypol induces both beclin-1-dependent and -independent autophagic response in breast cancer cells. However, the same study concluded that the observed autophagy was cytoprotective rather than aiding the apoptotic pathway.

B) **TW37**

The second generation SMI ‘TW37’ is a benzenesulfonyl derivative and was derived from gossypol\(^6^9;7^0\). The drug has both pro-apoptotic\(^7^1\) and anti-angiogenic effects\(^7^2\). It was originally designed to target the BH3-binding groove in the Bcl-XL and has shown to have high affinities for Bcl-2 as well as Bcl-XL, and unlike most SMIs, also targets Mcl-1. Sarkar
et al have extensively studied this SMI for its apoptotic action in leukemia, lymphoma and pancreatic cancers. The third generation SMI ‘apogossypolone’ (ApoG2) is a derivative of gossypol that was designed by Ascenta in order to reduce the nonspecific reactivity and toxicity of gossypol and is currently in the preclinical phase of testing. This modification involved the removal of two reactive aldehyde groups on the polyphenolic rings of gossypol. Studies have shown that ApoG2 blocks binding of Bim and Bcl-2 and induces apoptosis in lymphoma cell lines with minimal toxicity. Furthermore, it has also been shown that ApoG2 induces apoptosis in follicular small cleaved cell lymphoma model, pre-B-acute lymphoblastic leukemia, mantle cell lymphoma, marginal zone lymphoma, as well as chronic lymphocytic leukemia. Therefore, ApoG2 could potentially be a more effective drug in the lymphoma clinic spanning a greater array of patients. An important feature of ApoG2 is that it can effectively target the crucial Mcl-1 that is also emerging to be a key player in the pro-survival machinery.

C) **AT-101**

As mentioned earlier, natural gossypol is a racemic mixture of (−)-gossypol and (+)-gossypol. The (−)-gossypol is the most clinically acceptable form of gossypol and has been brand named as AT-101 (Ascenta). AT-101 is in Phase III clinical trials for chronic lymphocytic leukemia (in combination with rituximab) and in hormone refractory prostate cancer (in combination with docetaxel). AT-101 exhibits submicromolar binding affinity for Bcl-2 and Mcl-1. This SMI has partial gastrointestinal toxicity that was a dose-limiting factor in a Phase I–II clinical trial in prostate cancer patients. Based on its toxicity, previously discussed gossypol analog, apogossypol, may be a better alternate but is still in preclinical development.

D) **The ABT series of Bcl-2 inhibitors**

Of the compounds discovered to date, one of the most promising candidates that selectively kills cancer cells through direct interaction with the Bcl-2 family is the BH3 mimetic ABT-737 developed at the Abbott Laboratories, Abbott Park, IL, USA. This is a Bcl-2 specific SMI, which was confirmed by the observation that ABT-737 has been effective at activating apoptosis in cells doubly deficient of Bax and Bak. ABT-737 was discovered by Abbott using structure–activity relationship (SAR) by NMR strategy that combines nuclear
magnetic screening coupled with structure-based design and combinatorial synthesis\textsuperscript{79}. The SAR by NMR strategy led to the discovery of lead compound ABT-737 that mimicked the BH3 domain of BAD and bound selectively to Bcl-2, Bcl-XL and Bcl-W. Most interesting was the observation that the binding affinity of ABT-737 to different anti-apoptotic members was in the nanomolar range making this an ideal clinical candidate. ABT-737 is fairly specific to Bcl-2, BclXL and Bcl-w as it binds with poor affinity to Mcl-1 and Bfl-1 with a dissociation constant in the micro-molar range\textsuperscript{79}. ABT-263 is the orally applicable version of ABT-263 that has been extensively studied, which inhibits the antiapoptotic proteins Bcl-2, BclXL and Bcl-w, and has shown single-agent efficacy in numerous small cell lung carcinoma (SCLC) and leukemia/lymphoma cell lines in vitro and in vivo\textsuperscript{80-82}. It is currently in clinical trials for treating patients with SCLC and various leukemia/lymphomas. A very recent study by Tahir et al. has utilized a systems approach and identified the expression of Bcl-2 family genes that correlated best with sensitivity to ABT-263 in a panel of 36 SCLC and 31 leukemia/lymphoma cell lines\textsuperscript{83}. The group has examined global expression differences to identify gene signature sets that correlated with sensitivity to ABT-263 to generate optimal signature sets predictive of sensitivity to ABT-263. In their study, independent cell lines were used to verify the predictive power of the gene sets and to refine the optimal gene signatures. When comparing normal lung tissue and SCLC primary tumors, the expression pattern of these genes in the tumor tissue was found to be similar to sensitive SCLC lines, whereas normal tissue response was similar to resistant SCLC lines. Most of the genes identified using global expression patterns were related to the apoptotic pathway. This study leverages global expression data to identify key gene expression patterns for sensitivity to Bcl-2 targeted drugs, which may provide guidance in the selection of patients in future clinical trials using different cancer patients.

E) Obatoclax

Obatoclax is a synthetic derivative of prodiginines (GX015 – 070) from Gemin X Biotech (Avenue du Parc, Montreal, Quebec, Canada) and is a pan-Bcl-2 inhibitor with lower binding affinity to Bcl-2 family members than ABT-737\textsuperscript{84,85}. Recently, it has been shown that in cholangiocarcinoma, obatoclax induces Bax-mediated apoptosis\textsuperscript{86}. However, similar to many other putative BH3 mimetics, obatoclax is not entirely dependent on Bax and Bak and can induce cancer cell death independently\textsuperscript{87,88}. In this regard, it has been shown earlier that
obatoclax can also antagonize Mcl-1 and overcome the resistance observed through Mcl-1. Unfortunately, this compound may need to be redesigned for pharmacokinetic reasons especially because a recent study has shown that the treatment of mice with bolus injection of GX-015 – 070 failed to reach pharmacologically effective levels in the blood; and dose escalation was limited by significant neurologic toxicity.

**F) HA14 – 1**

The new compound ethyl-2-amino-6-cyclopentyl-4-(1-cyano-2-ethoxy-2-oxoethyl)-4Hchromone-3-carboxylate (HA14 – 1) is the first reported small molecule antagonist for Bcl-2 protein and was identified by Wang et al. This simple chemical structure is a putative Bcl-2 inhibitor, which was identified from in silico screens. HA14 – 1 disrupts the binding interaction of the Bak BH3-domain peptide with Bcl-2 and Bcl-xL proteins, and strongly inhibits the Bcl-2–Bax interaction, and also inhibits the interaction between Bcl-2 and BH3-only protein Bim. Since its discovery, HA14 – 1 has been shown to enhance the cytotoxic effects of a variety of anticancer agents. HA14 – 1 has also been shown to act as a chemosensitizer with selectivity to enhance the apoptotic effect of cisplatin by modulating Bcl-2 family members in MDA-MB-231 breast cancer cells. However, HA14 – 1 has shown redox reactivity and instability for which newer stable versions of sHA14 – 1 have been developed that are less redox active.

**4.8 Bcl-2 family inhibitors as sensitizers to chemotherapy**

The BH3 mimetic are often not very effective as single agents especially against some of the epithelial and genetically complex cancers, such as the pancreatic, ovarian and breast cancers. The observed resistance to Bcl-2 family targeted drugs is due to multiple factors that include redundancies in crosstalk between major signaling pathways, lack of target validation and acquired resistance to chemotherapy. In the last few years, results from our laboratory and those of others have proven that an important niche for Bcl-2 family SMIs is in combination therapy with standard chemotherapeutics such as cisplatin, gemcitabine or other therapies such as TRAIL. This is especially exemplified in pancreatic cancer where chemotherapy has been shown to induce prosurvival molecule NF-κB and Bcl-2, and the SMI serves to inhibit Bcl-2-mediated resistance, enabling killing by conventional chemotherapy. Numerous examples exist in the literature in support of the enhanced apoptotic response when the BH3 mimetics are combined with traditional therapies to treat various
cancers such as melanoma. Thus, it seems that the SMIs are capable of priming the cancer cells to make them vulnerable to be killed by conventional chemotherapeutics especially those cancers that are drug resistant due to genetic complexity. Indeed, recent reports have shown the use of the BH3 mimic ABT-737 for overcoming both de novo and acquired resistance for the killing of cancer cells. It was effective at re-sensitizing drug-resistant breast cancer cell lines to apoptosis induced by paclitaxel emphasizing the potential effectiveness of the use of BH3 mimetics in combination therapy.

4.9 Potential developmental issues and hindrances in clinical progress of Bcl-2 family inhibitors

Although a number of different classes of SMIs have been developed over the last decade, only five are at present in clinical trials: genasense (G3139), TW-37, AT-101, obatoclax and ABT-263 (Figure). The progress in clinical trials has been hindered by the observed toxicity with most of these inhibitors. In preclinical in vitro experiments, ABT-737 as a single agent showed toxicity to leukemia and lymphoma, while at higher doses it induces apoptosis in multiple cancer cell lines. Numerous studies have shown that primary cells from patients with chronic lymphocytic leukemia, acute myeloid leukemia and acute lymphocytic leukemia are extremely responsive to ABT-737, with widespread apoptosis at nano-molar concentrations. To enhance its clinical potential, ABT-737 has been modified (ABT-263) to make the drug orally available. ABT-263 binds to serum proteins resulting in a longer oral half-life. The tumoricidal action of ABT-263 was shown in xenograft models of small cell lung cancer, where ABT-263 caused complete regression of the tumors and is now in clinical trials. Similarly, obatoclax, the pan-Bcl-2 inhibitor, shows single agent efficacy in the killing of multiple myeloma, acute myeloid leukemia cell lines and primary tumor cells. However, it also causes cell cycle arrest independent of apoptosis. Sarkar and co-workers have shown that TW-37 causes a reduction in cell proliferation of pancreatic and B-cell lymphomas, and is capable of reducing the tumor size in xenograft models. TW-37 shows efficacy in cell lines and patient derived cells and is effective in a spectrum of B-cell tumors, irrespective of their proliferative and differentiation status. Finally, AT-101 from Ascenta is in multiple clinical trials including an open-label, multi-center Phase I/II single agent study in castrate resistant prostate cancer patients. This brings up the issue of specificity of these SMIs. For a drug to be approved clinically, it has to be specific with lesser side effects or
beneficial off target effects. Even though SMIs were originally designed to specifically hit a single target, researchers have constantly observed that these Bcl-2 family SMIs also modulate other key molecules that are considered secondary or off targets. Studies from our laboratory and others have revealed that different Bcl-2 family SMIs modulate numerous key molecules such as NF-κB, Notch and Par-4. Whether modulation of secondary targets is beneficial for the overall activity of these Bcl-2 family targeting agents or the off targets mask the SMI potential is a very important question that needs to be addressed for SMIs to achieve success as novel drugs in the clinical setting.

Figure 2: Current Bcl-2 inhibitors

4.10 Nature and scope of present investigation

Previous attempts to target Bcl-2 therapeutically using antisense technology to inhibit protein translation so far have not significantly improved outcomes for cancer patients, although improved oligonucleotide design may enhance efficacy. The development of orally bioavailable Bcl-2 inhibitors, such as ABT-263, with the ability to inhibit specifically BH3-Bcl-2 protein-protein interactions at low nanomolar concentrations, potentially marks a significant development in cancer therapy. Phase I studies of ABT-263 are currently ongoing in both lymphoid malignancies and SCLC, both of which have been shown to be sensitive to the compound in vitro, although in the latter case only in cell lines and not in primary material. Whether compounds such as ABT-263 find their way into routine clinical use will depend not only on efficacy, but also manageable, acceptable toxicities. Thrombocytopenia
for example may limit the value of ABT-263 in patients with heavy bone marrow infiltration; the long-term toxicities of profound Bcl-2 suppression remain to be determined. Identification of proteins that mediate resistance to ABT-263 may allow the development of rational combinations. After many years of development, it now seems likely that the rational design of specific compounds that inhibit specific protein-protein interactions may lead to significant therapeutic advances. In view of current synthetic bcl-2 inhibitors we have designed novel sulfonamide conjugates as anticancer agents acting through apoptosis pathway. Our literature review indicated that sulfonamide moiety can serve as a lead molecule for development of potent bcl-2 inhibitor compounds since

1. Combination of Sulfonamide with Gallate pharmacophore could possibly enhance activity of the parent compound.

2. Second strategy could be the combination of the potent sulfonamide appended with known pharmacophores such as chromone, coumarin and indoles.

Hence in present investigation, we have synthesized and characterized hybrids of sulfonamides, gallates and other potent pharmacophores and have evaluated them against panel of cancer cells including triple negative breast cancer, prostate cancer and pancreatic cancer cells.

**Scheme 1**

![Scheme 1](image)

**Scheme 2**

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4.11 Experimental

Solvents employed for all reactions were purified by standard methods prior to use. Reaction progress was monitored using thin layer chromatography (TLC) on pre-coated silica gel on aluminium plates (Merck). Sulfacetamide, Sulfanilamide, Sulfathiazole, Sulfadiazine, 2,3,4-trihydroxy benzaldehyde, 3-acetyl coumarin, 3-formyl,6-methyl chromone, 3-acetyl indole were purchased from Sigma-Aldrich and used without any further purification.

4.12 Results and Discussion

A) Compositional Studies

The condensation of various sulfonamides with trihydroxy benzaldehyde and sulfacetamide with various pharmacophores like chromone, coumarin and indole in dry methanol at 55-60°C in presence of catalytic amount of trifluoro-acetic acid yielded corresponding Schiff Bases with good yield. The progress and completion of the reaction was monitored by thin layer chromatography on pre-coated TLC plates with CHCl₃-MeOH as developing solvent. The products were purified by column chromatography using silica gel as stationary phase and CHCl₃-MeOH as mobile phase. All purified compounds were shades of yellow-orange with yields of 58-74%.
B) Characterization details

i) SNLTHB

Yield 81%. IR (cm\(^{-1}\)): 3578 (-OH), 1595 (C=N), 1328 (-SO\(_2\)-R).

\(^1\)HNMR (400 MHz, DMSO-D6) (δ, ppm) 7.359 (s, 2H), 8.814 (s, 1H), 8.533 (s, br, 1H), 9.836 (s, br, 1H), 13.203 (s, br, 1H), 7.864 (d, 2H Ar), 7.505 (d, 2H Ar), 6.455 (d, 1H Ar), 6.992 (d, 1H Ar).

\(^13\)CNMR (125 MHz DMSO) (δ, ppm) 7.359 (s, 2H), 8.814 (s, 1H), 8.533 (s, br, 1H), 9.836 (s, br, 1H), 13.203 (s, br, 1H), 7.864 (d, 2H Ar), 7.505 (d, 2H Ar), 6.455 (d, 1H Ar), 6.992 (d, 1H Ar).

ESI-MS: M+1 peak at 309 (M=308 in accordance with MF C\(_{13}\)H\(_{12}\)N\(_2\)O\(_5\)S

ii) STMTHB

Yield 85%. IR (cm\(^{-1}\)): 3398 (-OH), 1590 (C=N), 1340 (-SO\(_2\)-R).

\(^1\)HNMR (400 MHz, DMSO-D6) (δ, ppm) 1.892 (s, 3H), 12.054 (s, 1H), 8.805 (s, 1H), 8.558 (s, br, 1H), 9.857 (s, br, 1H), 13.045 (s, br, 1H), 7.936 (d, 2H Ar), 7.526 (d, 2H Ar), 6.457 (d, 1H Ar), 6.997 (d, 1H Ar).

\(^13\)CNMR (125 MHz DMSO) (δ, ppm) 23.2, 108.3, 112.3, 121.6, 124.7, 129.7, 132.4, 136.1, 151.3, 152.6, 165.4, 168.8, 193.2.

ESI-MS: M+1 peak at 351 (M=350 in accordance with MF C\(_{15}\)H\(_{14}\)N\(_2\)O\(_6\)S

iii) STZTHB

Yield 72%. IR (cm\(^{-1}\)): 3394 (-OH), 1523 (C=N), 1279 (-SO\(_2\)-R).

\(^1\)HNMR (400 MHz, DMSO-D6) (δ, ppm) 12.729 (s, 1H), 7.249 (d, 1H), 6.824 (d, 1H), 7.827 (d, 2H Ar), 7.450 (d, 2H Ar), 8.783 (s, 1H), 8.524 (s, br, 1H), 9.832 (s, br, 1H), 13.164 (s, br, 1H), 6.971 (d, 1H Ar), 6.441 (d, 1H Ar).

\(^13\)CNMR (125 MHz DMSO) (δ, ppm) 108.1, 108.2, 112.3, 121.4, 124.5, 127.2, 132.3, 139.4, 150.9, 151.1, 151.3, 164.8, 168.8.

ESI-MS: M+1 peak at 392 (M=391 in accordance with MF C\(_{16}\)H\(_{13}\)N\(_3\)O\(_5\)S\(_2\)
iv) SDZTHB

Yield 64%.  **IR (cm$^{-1}$):** 3396 (-OH), 1575 (C=N), 1339 (-SO$_2$-R).

$^1$H NMR (400 MHz, DMSO-D6) ($\delta$, ppm) 12.297 (s, 1H), 8.83 (d, 2H), 6.85 (m, 1H), 7.782 (d, 2H Ar), 7.900 (d, 2H Ar), 8.930 (s, 1H), 8.245 (s, br, 1H), 9.416 (s, br, 1H), 13.624 (s, br, 1H), 6.272 (d, 1H Ar), 6.297 (d, 1H Ar).

$^{13}$C NMR (125 MHz DMSO) ($\delta$, ppm) 110.2, 110.6, 112.8, 124.2, 126.4, 128.6, 138.2, 140.2, 152.4, 153.6, 156.6, 156.2, 157.5, 170.3

ESI-MS: M+1 peak at 387 (M=386 in accordance with MF C$_{17}$H$_{14}$N$_4$O$_5$S

v) STMDHB

Yield 70%.  **IR (cm$^{-1}$):** 3384 (-OH), 1542 (C=N), 1327 (-SO$_2$-R).

$^1$H NMR (400 MHz, DMSO-D6) ($\delta$, ppm) 1.889 (s, 3H), 12.083 (s, 1H), 7.237 (m, 2H Ar), 7.889 (d, 1H Ar), 7.519 (d, 1H Ar), 8.381 (s, 1H), 6.107 (s, 1H Ar), 9.364 (s, br, 1H), 10.075 (s, br, 1H), 6.876 (m, 1H Ar), 6.593 (d, 1H Ar).

$^{13}$C NMR (125 MHz DMSO) ($\delta$, ppm) 22.3, 116.2, 118.1, 123.8, 124.1, 127.8, 129.2, 136.6, 148.2, 150.6, 156.2, 161.1, 183.7.

ESI-MS: M+1 peak at 335 (M=334 in accordance with MF C$_{15}$H$_{14}$N$_2$O$_5$S

vi) STMCHR

Yield 70%.  **IR (cm$^{-1}$):** 3390 (C=N), 1340 (-SO$_2$-R), 1645 (C=O).

$^1$H NMR (400 MHz, DMSO-D6) ($\delta$, ppm) 2.296 (s, 3H), 11.996 (s, 1H), 7.878 (d, 2H Ar), 7.553 (d, 2H Ar), 5.798 (s, 1H), 7.347 (d, 1H Ar), 6.994 (d, 1H Ar), 1.915 (s, 3H), 7.617 (s, 1H Ar).

$^{13}$C NMR (125 MHz DMSO) ($\delta$, ppm) 22.3, 24.2, 108, 116.7, 122.8, 123.5, 128.2, 130.4, 133.2, 135.2, 138.4, 151.4, 153.2, 154.6, 156, 182, 186.

ESI-MS: M+1 peak at 385 (M=384 in accordance with MF C$_{19}$H$_{16}$N$_2$O$_5$S
vii) STMCMR

**Yield** 62%.  **IR (cm⁻¹):** 1585 (C=N), 1332 (-SO₂-R), 1641 (C=O)

**¹H NMR** (400 MHz, DMSO-D6) (δ, ppm) 3.02 (s, 3H), 11.6 (s, 1H), 7.925 (s, 1H Ar), 7.428 (m, 2H Ar), 7.517 (s, 1H Ar), 8.630 (s, 1H), 2.78 (s, 3H), 7.723 (s, 1H), 6.592 (d, 2H Ar), 7.428 (m, 2H Ar).

**¹³C NMR** (125 MHz DMSO) (δ, ppm) 23.1, 29.9, 112.2, 116.1, 118.1, 123.8, 124.4, 124.8, 129.7, 130.7, 134.4, 147.0, 153.5, 154.5, 158.3, 168.2, 195.0

**ESI-MS:** M+1 peak at 385 (M=384 in accordance with MF C₁₉H₁₈N₂O₅S

viii) STMIND

**Yield** 68%.  **IR (cm⁻¹):** 1578 (C=N), 1329 (-SO₂-R).

**¹H NMR** (400 MHz, DMSO-D6) (δ, ppm) 1.860 (s, 3H), 12.116 (s, 1H), 8.067 (d, 2H Ar), 7.517 (d, 2H Ar), 8.266 (s, 1H), 7.399 (s, 1H), 6.606 (d, 2H Ar), 7.517 (m, 2H Ar).

**¹³C NMR** (125 MHz DMSO) (δ, ppm) 21.4, 25.1, 112.4, 112.9, 119.4, 120.3, 122.4, 122.8, 126.6, 128.9, 130.5, 135.1, 137.5, 155.4, 164.7, 179.2.

**ESI-MS:** M+1 peak at 356 (M=355 in accordance with MF C₁₈H₁₇N₃O₃S

C) **FTIR Spectroscopy**

In all the sulfonamide conjugates two distinct peaks for primary amino group (-NH₂) at 3405 and 3465 cm⁻¹ disappeared distinctively. This shows that –NH₂ group has undergone chemical transformation during the synthesis of conjugates of trihydroxy aromatic aldehydes as well as carbonyl compounds containing chromone, coumarin and indole. Whereas the peak for imine bond (-CH=N-) appeared in range of 1523 cm⁻¹ to 1591 cm⁻¹ for all the compounds which confirms this transformation.

In addition, all compounds exhibited a band in the range of 1278-1340 cm⁻¹ which is characteristic of –SO₂-R group, confirming presence of sulfone group. In addition, the compounds containing trihydroxy groups exhibited band in the region of 3379 cm⁻¹ to 3398 cm⁻¹ confirming the presence of –OH functionality in the compounds. The representative FTIR spectra are shown in **Figure 4 to Figure 7.**
Figure 4: FTIR spectrum of STMTHB

Figure 5: FTIR spectrum of STZTHB
D) $^1$H and $^{13}$C NMR spectroscopy

The $^1$H- and $^{13}$C-NMR spectra of the test compounds are shown in Figure 8 to Figure 15. In case of $^1$H NMR spectra, the singlet peak observed in the region 8.131-8.814 ppm can be attributed to –imino group confirming Schiff base formation. In case of trihydroxy
compounds, three separate broad singlets appear in the range of 8.533-13.045 ppm confirming the presence of three distinct hydroxyl groups. The singlet for methyl protons in case of sulfacetamide conjugates appear in the range of 1.889-3.02 ppm. The aromatic protons for all compounds were found to give signal in the region of 6.455-8.067 ppm which was attributed to the aromatic protons from sulfonamide moiety along with aromatic protons from chromone, coumarin, indole and trihydroxy benzaldehyde moieties. The singlet for –SO$_2$-NH- proton appeared downfield in the region of 11.6-12.7 ppm due to de-shielding effect of highly electronegative sulfonyl group.

$^{13}$CNMR of all compounds show the aliphatic carbon atoms appearing in the range of 23.13 ppm to 29.9 ppm for methyl groups of sulfacetamide, acetyl coumarin and chromone ring. Olefinic and aromatic carbons atoms appear in the range of 102 ppm to 160 ppm and the azomethine carbon appeared in the range of 164-168 ppm for all the conjugates.

Figure 8: $^1$H NMR spectra of SNLTHB
Figure 9: $^{13}$C NMR spectra of SNLTHB

Figure 10: $^1$H NMR spectra of STMTHB
Figure 11: $^{13}$C NMR spectra of STMTHB

Figure 12: $^1$H NMR spectra of STZTHB
Figure 13: $^{13}$C NMR spectra of STZTHB

Figure 14: $^1$H NMR spectra of STMCNR
E) Mass Spectroscopy

The mass spectral (MS) data for the ligand SNLTHB, STMCHR, STMTNB showed molecular ion peak at 309.1, 385.09 and 351.1 respectively corresponding to their M+1 peak. Similarly the MS data for other ligands STZTHB, SDZTHB, STMDHB, STMCNR, and STMIND revealed peaks at 392.4, 387.8, 335.3, 385.1 and 356.2 respectively confirming formation of the respective Schiff bases. The representative ESI-MS spectra for the test compounds are provided in the following figures (Figure 16-18).
Figure 16: Mass spectra of STMCHR

Figure 17: Mass spectra for SNLTHB
F) Single crystal X-ray diffraction studies

i) X-ray crystal structure of ligand Sulfanilamide-gallate conjugate (SNLTHB)

A suitable crystal of SNLTHB was selected and oil on loop on Oxford Diffraction Super Nova diffractometer. The crystal was kept at 110 K during data collection. Using Olex2, the structure was solved with the XS structure solution program using Direct Methods and refined with the XL refinement package using Least Squares minimisation. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed geometrically and held in the riding mode during the final refinement.

The molecular structure for the ligand (SNLTHB) is shown in Figure 19, while the crystallographic parameters, bond distances and bond angles are summarized in Tables 1 and 2 respectively. The ligand SNLTHB crystallized as a monomeric species having triclinic space group P-1 with crystal parameters a/Å 7.7940(10), b/Å 12.932(2) c/Å 14.1876(14), respectively. The C=N– azomethine bond length [N (2)–C (7)] is 1.320(4) Å in SNLTHB has a double bond character, whereas the bond angle for C(4)-N(2)-C(7) is 127.4 (3). Other details regarding crystal structure parameters have been provided in following tables.
Figure 19: ORTEP diagram for SNLTHB

Note: ellipsoids are represented at 50% probability.
Table 1: Bond lengths [Å] and angles [deg] for SNLTHB.

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<th>Length/Å</th>
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Table 2: Crystal data and structure refinement for SNLTHB

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<td>Empirical formula</td>
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<td>Temperature/K</td>
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<td>Space group</td>
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<td>Unit Cell Dimensions</td>
<td>a/Å 7.7940(10) b/Å 12.932(2) c/Å 14.1876(14) α° 90.375(11) β° 100.509(9) γ° 107.119(13)</td>
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<td>Volume</td>
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4.13 Molecular Docking

Apoptosis represents an efficient mechanism adopted by multicellular organisms in order to eliminate superfluous or damaged cells\textsuperscript{108}. Disruption of this mechanism is implicated in several human malignancies \textsuperscript{109,110}. Central regulators of the apoptotic pathway are proteins belonging to Bcl-2 (B-cell lymphocyte/leukemia-2) family. Anti-apoptotic Bcl-2 proteins, such as Bcl-xL, are over-expressed in most human cancer types and therefore are very attractive targets for the development of anticancer agents \textsuperscript{111-113}. While Bcl-xL and its closest relatives such as Bcl-2, Bfl-1, Mcl-1, Bcl-W and Bcl-B, promote cell survival, the structurally similar pro-apoptotic members such as Bak, Bax, Bad, Bim or Bid, promote cell death. In the present study we have chosen the Bcl-xl protein cavity as the docking site for our synthetic inhibitors.

Docking studies were carried out on compounds 1-8 along with the standard inhibitor TW-37 in Bcl-Xl protein cavity (PDB ID: 1YSI)\textsuperscript{79}. The high resolution 1YSI.pdb of Bcl-xl was selected for docking and the active pocket was considered to be the site where

\[ n-\{4'-fluoro-1,1'-biphenyl-4-yl\}carbonyl\}-3-nitro-4-\{2-(phenylsulfanyl)ethyl\}amino\}benzenesulfonamide \]

was complexed in 1YSI pdb (Figure 20). The active pocket consisted of amino acid residues surrounding co-crystallized ligand is shown in Figure 21. The synthesized ligand molecules having 2D structure were converted to energy minimized 3D-structures and were used for \textit{in silico} docking with protein cavity modeling software by using Autodock vina\textsuperscript{114}. 
Figure 20: Secondary Structure of Bcl-Xl protein complexed with acyl-sulfonamide ligand.  
(PDB ID: 1YSI)  

Figure 21: PDB sum’s Ligplot Results for 1YSI, Showing Amino Acid Residues of Active Pocket
Table 3: Docking Results and Consensus Scores of novel sulfonamide conjugates and standard inhibitor TW-37 in Bcl-Xl cavity.

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<th>Bond Length (Å)</th>
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<td>-7.9</td>
<td>2</td>
<td>2.6 2.3</td>
<td>TYR105 ARG143</td>
</tr>
</tbody>
</table>

B.E. = binding energy (Kcal/mol), H bond = hydrogen bond, H bonding residues = hydrogen bonding residues

All compounds were found to be docked at the active site of Bcl-Xl with good fit confirming that these sulfonamide derivatives could play an active role in the inhibition of this protein. (Table 3 and Figure 22). The compounds were ranked according to their binding energies. The docking results show that the standard inhibitor TW-37 has the lowest energy (-9.2 K.Cal/mol) indicating that it is the best fitting molecule in this series of test compounds. However it is able to form only one H bond interaction with ARG 143 amino acid residue. Amongst the synthetic derivatives almost all the compounds exhibited comparatively good fit into the protein cavity with comparable binding energies. Compound SDZTHB (B.E. = -7.9Kcal/mol) could form 3 H bonds with GLY142, ASN201 and TYR199, whereas STMCMR (B.E.=-7.9Kcal/mol) could form 2H bond interactions with TYR105 and ARG143.
Additionally STZTHB (-7.6Kcal/mol) also formed 2 H bonds with TYR105 and ARG143 amino acid residues. Other compounds also show good fit in the cavity. Interestingly it was seen that amino acid residues of ARG143 and TYR105 are common to most of the test compounds indicating some similarities in the binding modes of these compounds as well as importance of these residues in the protein activity. Thus on the basis of docking studies it was concluded that the novel sulfonamide conjugates have the potency to inhibit the function of Bcl-XI protein.
Figure 22: Docking figures of sulfonamide-gallic acid conjugates in Bcl-XI protein cavity
4.14  **Anticancer activity**

The anticancer potency of the novel sulfonamide conjugates were evaluated against a panel of three cancer cell lines including Triple negative breast cancer (MDA-MB-231) cells, prostate cancer (PC3) cells and pancreatic cancer (BxPc-3) cells. All compounds exhibited moderate toxicity towards the cell lines. The IC\textsubscript{50} values for all conjugates against the three cell lines were in the range of 25-50 µM (Table 4). Amongst all the conjugates STMTHB and STZTHB were found to be comparatively more potent than other test compounds. One probable reason for such activity may be due to presence of gallic acid moiety (trihydroxy benzaldehyde pharmacophore) in the compound. Also it was interesting to observe that the novel compounds exhibited moderate selectivity towards triple negative breast cancer and pancreatic cancer cells, whereas the compounds were somewhat less toxic towards prostate cancer cells. The compounds containing sulfonamide group attached to different pharmacophores including chromone, coumarin and indoles were however not very effective in inhibiting the proliferation of cancer cells. However the compounds containing gallate moiety and sulfonamide were found to be more potent against breast, prostate and pancreatic cancers.

The two most potent compounds were further studied for their ability to downregulate the protein expression of Bcl-2 by western blot experiment (Figure 23). The results clearly indicated that at moderate molar concentrations (~25 µM), the compounds had a considerable effect on the protein expression. As expected at higher concentrations (~50 µM), the expression had almost disappeared. This suggests that the test compounds are exhibiting anticancer activity through apoptosis pathway and down-regulation of anti-apoptotic protein Bcl-2.
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</tr>
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<tr>
<td>STMCNR</td>
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**Table 4**: MTT results for the synthesized bcl-2 inhibitors (after 72 hr)
Figure 23: Western Blot Analysis of STMTHB and STZTHB in MDA-MB-231 cells (72 h)
4.15 Conclusion

In summary, the sulfonamide-gallate conjugates represent a promising class of bcl-2 inhibitors which exhibit moderate antiproliferative activity against breast cancer, prostate cancer and pancreatic cancer cells. Our molecular docking studies show that the compounds fit well in the bcl-XI protein cavity and interact with the amino acid residues. Although the compounds show cancer cell inhibition at higher concentration, we would certainly modify the compounds to increase the potency against bcl-2 expressing cancer cells. The results of the present study indicate the potential of bcl-2 as target in cancer therapy and prevention using our newly synthesized sulphonamide-polyhydroxy gallate compounds.
4.16 References


24, Tsujimoto, Y.; Cossman, J.; Jaffe, E.; Croce, C. M. Science 1985, 228, 1440.


29, Campos, L.; Sabido, O.; Sebban, C.; Charrin, C.; Bertheas, M. F.; Fiere, D.; Guyotat, D. Leukemia 1996, 10, 434.


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97, Kutuk, O.; Letai, A. Cancer Res. 2008, 68, 7985.


