MOLECULAR MECHANISM OF DRUG INDUCED APOPTOSIS and CHEMORESISTANCE in ESTROGEN RECEPTOR alpha +/- BREAST CANCER CELL LINES: MCF-7 AND MDA-MB-231

by

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Abstract

It was recently shown that inhibition or downregulation of Bcl-2 represents a new therapeutic approach to by-pass chemoresistance mechanism in cancer cells. Therefore, we explored the potential of this approach in breast cancer cells; MCF-7 (drug-sensitive; p53 wild type) and MDA-MB-231 (drug-insensitive; p53 mutant). Cisplatin and paclitaxel induced apoptosis in a dose-dependent manner in both cell lines. Furthermore, silencing of Bcl-2 remarkably increased cisplatin and paclitaxel induced apoptosis. Dose dependent induction of apoptosis by cisplatin and paclitaxel was enhanced by the pre-treatment of these cells with HA14-1, a Bcl-2 inhibitor. Although the effect of cisplatin on cell death was significant in MCF-7 and MDA-MB-231, paclitaxel was less potent only in MDA-MB-231 cells.

To further understand the distinct role of drugs in breast cancer cells which were pre-treated with HA14-1, changes in mitochondrial membrane potential, caspase activation, and Bcl-2 family protein levels, generation of reactive oxygen species and lipid peroxidation were studied. The apoptotic effect of cisplatin with or without HA14-1 pre-treatment was shown to be caspase-dependent in both cell lines. While pro-

apoptotic Bcl-2 proteins (Bax, Puma, Bad) were found to be up-regulated, Bcl-2 and Bcl-x_L were down-regulated when cells were pre-treated with HA14-1 followed by cisplatin or paclitaxel. MCF-7 and MDA-MB-231 cells overexpressing Bcl-2 displayed different responses upon drug-treatment. Although cisplatin could still induce apoptosis in Bcl-2 overexpressing MCF-7 cells by promoting pro-apoptotic Bcl-2 family members, Bcl-2 overexpression abrogated paclitaxel induced apoptosis in MCF-7 and MDA-MB-231 breast cancer cells, respectively.

In conclusion, our findings suggest two important implications for understanding cisplatin and paclitaxel induced apoptosis mechanism and the potential role of Bcl-2 in this apoptotic pathway. First, the potentiating effect of Bcl-2 inhibitor (HA14-1) is drug and cell type specific and may not only depend on the inhibition of Bcl-2. Importantly, alteration of other pro-apoptotic or anti-apoptotic Bcl-2 family members may dictate the apoptotic response when HA14-1 is combined with chemotherapeutic drugs. Second, cisplatin activated a p53- regulated pro-apoptotic pathway to overcome Bcl-2 mediated resistance. These insights may be useful for the development of novel treatments for cancer cells overexpressing anti-apoptotic Bcl-2 proteins.

ÖSTROJEN RESEPTÖR alfa +/- MCF-7 ve MDA-MB-231 MEME KANSERİ HÜCRELERİNDE İLAÇLARCA TETİKLENEN APOPTOZ VE İLACA DİRENÇ MOLEKÜLER MEKANİZMASI

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Özet

Çok yakın bir zamanda Bcl-2'nin aktivitesinin azalması veya protein anlatımının azaltılması yeni bir terapotik yaklaşım olarak gösterilmiş ve böylece kanser hücreleri tarafından ilaçlara gösterilen cevapsızlık mekanizmasının aşılabileceği ortaya konulmuştur. Bu nedenle, bu yaklaşımın potansiyel etkileri meme kanseri hücrelerinde (MCF-7, p53 doğal tip ve ilaca duyarlı; MDA-MB-231 p53 mutant, ilaca duyarsız) araştırılmıştır. Sisplatin ve paklitaksel hücrelerde farklı şekillerde ölümü tetikleyen kemoterapötik ajanlar olup, MCF-7 ve MDA-MB-231 hücrelerinde sırası ile doza bağlı sitotoksik ve apoptotik etkiler göstermişlerdir. Buna ilaveten, her iki hücre hattında da geçici Bcl-2 siRNA uygulamasının ardından sisplatin ve paklitaksel uygulandığında, Bcl-2 siRNA uygulaması yapılmamış kontrol hücrelere göre, sisplatin ve paklitaksel daha fazla apoptotik etkiye neden olmuştur. MCF-7 ve MDA-MB-231 hücrelerine Bcl-2 inhibitörü, HA14-1 ön uygulamasını takiben değişik dozlarda ilaç uygulaması yapılmıştır. HA14-1 ön uygulamasının sisplatin ve paklitaksel tarafından tetiklenen apoptotik etkiyi arttırdığı tespit edilmiştir. Ancak bu etki MCF-7 meme kanseri hücrelerinde her iki ilaç için daha anlamlı bulunurken, HA14-1 ön uygulamasının

ardından paklitaksel uygulaması yapılan MDA-MB-231 hücrelerinde daha az etki tespit edilmiştir.

İlaçlar tarafından tetiklenen apoptoz mekanizmasındaki farklılıkları anlamak üzere hücrelerde mitokondri zar potansiyelindeki azalmada görülen değişimler, kaspaz aktivitesi, Bcl-2 ailesinin anlatımlarındaki değişiklikler, reaktif oksijen türlerinin ve lipid peroksidasyonu hücrelere sisplatin ve paklitaksel uygulaması sonrasında belirlenmiştir. Sisplatin Bcl-2 inhibitörü varlığında veya tek başına uygulandığı zaman kaspaz aktivitesine bağlı olarak apoptotik yolağı tetiklediği gösterilmiştir. Kemoterapötik ilaçların meme kanseri hücrelerinde pro-apoptotik proteinlerin anlatımlarını arttırırken (Bax, Puma, Bad), anti-apoptotik proteinlerin (Bcl-2 ve Bcl-x_L) anlatımlarının ise kontrol hücrelere oranla azaldığı tespit edilmiştir. Bcl-2 anlatımı arttırılmış MCF-7 ve MDA-MB-231 hücrelerinde sisplatin apoptozu 48 saat içinde tetiklerken, paclitaksel ve HA14-1 bu etkiyi göstermemişlerdir. Bcl-2 anlatımı fazlalaştırılmış MDA-MB-231 hücrelerinin ise MCF-7 hücrelerine gore sisplatine daha dirençli oldukları saptanmıştır.

Sonuç olarak, bulgularımız sisplatin ve paklitaksel tarafından tetiklenen apoptoz mekanizması ve apoptotik yolakta Bcl-2 in rolü üzerine iki önemli çıkarımı önermektedir: Birincisi, Bcl-2 inhibitörünün (HA14-1) olası etkisi ilaç ve hücre tipine özgüdür ve sadece Bcl-2 inhibisyonuna bağlı etki göstermeyebilir. En önemlisi, Bcl-2 inhibitörü ile kombine edilen ilaçlarca tetiklenen apoptoz mekanizmasında diğer Bcl-2 ailesi üyelerinde görülen değişimlerin belirleyici olabileceğidir. İkinci durum ise, sisplatin p53 tarafından düzenlenen bir pro-apoptotik yolağı aktive etmekte ve Bcl-2 tarafından oluşturulmuş direnci aşabilmektedir. Bu bilgiler Bcl-2 anti-apoptotik proteinlerinin aşırı anlatımı görülen kanser hücrelerinde yeni uygulama tiplerinin gelişmesinde yararlı olabilir.

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ABBREVIATIONS

ADP: Adenosine di-phosphate

ANT: Adenine nuclear translocator

AIF: Apoptosis inducing factor

AIP: Apoptosis inducing protein

Apaf-1: Activator protease activating factor-1

Asp: Asparagine

ATP: Adenosine tri-phosphate

ATR: *Ataxia telangiectasia* mutated and Rad3-related kinase

Bak: B-cell homologous antagonist/killer

Bax: Bcl-2 associated protein X

Bcl-2: B-cell lymphocyte/leukemia-2

Bcl-x_L: B-cell lymphocyte/leukemia-2 like protein 1

BH: Bcl-2 homology

Bid: BH3 interacting domain death agonist

tBid: Truncated BH3 interacting domain death agonist

Bik: Bcl-2 interacting killer

Bok: Bcl-2 related ovarian killer

C-terminus: Carboxyl terminus

CAD: Caspase-activated DNase

CED: Cell death effective

Cisplatin: cis-diamminedichloroplatinum II

Cyc d: Cyclin D

Cyt c: Cytochrome c

DCFH-DA: Dichlorodihydrofluorescein diacetate

DISC: Death inducing signaling complex

DIABLO: Direct inhibitor of apoptosis binding protein with low PI

DED: Death effector domain

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DD: Death domain

DR: Death receptor

Endo G: Endonuclease G

ER: Endoplasmic reticulum

ERα: Estrogen receptor alpha

ERK: Extracellular signal-regulated kinase-1

FADD: Fas adaptor death domain protein

FasL: Fas ligand

FasR: Fas receptor

FBS: Foetal bovine serum

FLICE: FADD-like IL-1β-converting enzyme

Gly: Glycine

GST: Glutathion S-transferase

HA14-1: Ethyl-2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate

HER2: Human epidermal growth factor receptor-2

HIF-1: Hypoxia induced factor-1

IAP: Inhibitor of apoptosis protein

ICAD: Inhibitor of caspase-activated DNase.

JNK: c-Jun-N-terminal kinases

MAPK: Mitogen activated protein kinase

miRNA: Micro RNA

MMP: Mitochondria membrane potential

MOMP: Mitochondria outer membrane potential

MW: Molecular weight

NAC: N-acetyl cystein

NMR: Nuclear magnetic resonance

N-terminus: Amino terminus

UPR: Unfolded protein response

PAK2: p21-activated kinase family

PARP: Poly-(ADP-ribose) polymerase

Phe: Phenylalanine

PI3K: Phosphatidylinositol-3 kinase

PKA: Protein kinase A

PTP: Permeability transition pore

RISC: RNA-induced silencing complex

RNA: Ribonucleic acid

RNAi: RNA interference

ROS: Reactive oxygen species

siRNA: Small interfering RNA

shRNA: Short hairpin RNA

SMAC: Second mitochondria-derived activator of caspases

TBARS: Thiobarbituricacid substances

TIM: Translocator inner membrane

TM: Transmembrane

TOM: Translocator outer membrane

Trp: Tryptophan

VDAC: Voltage dependent anion channel

XIAP: X-linked Inhibitor of Apoptosis Protein

3D: Three dimensional

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CHAPTER 1

1 INTRODUCTION

Cancer formation or malignant cell turnover requires the acquisition of six fundamental properties: self-sufficient proliferation, insensitivity to anti-proliferative signals, evasion of apoptosis, unlimited replicative potential, the maintenance of vascularization, and, for malignancy, tissue invasion and metastasis. Therefore cancer is a really complex problem and solutions seem to like fuzzy logic. In fact, fuzzy logic approach reveals that cells have inner workers which cross talks with each other. This is also referred as signaling network which has its own set of rules that determine how it responds to a particular stimulus. Despite the many hundreds of molecules involved in carcinogenesis, there are several families of star players in the story of carcinogenesis.

One of the goals of cancer research is to develop new effective and non-toxic cancer therapies. It is shown that the ability to trigger death in tumor cells is an important strategic design for cancer therapeutics. This is supported by the fact that many successful conventional therapies work by triggering apoptosis albeit indirectly. Molecular oncology mainly can be defined as using knowledge of the molecular players in the cell death pathway which enable us to design direct and the most appropriate apoptotic inducers. According to histopathological and clinical observations, it is obvious that more complex therapy tools and monitoring substances at molecular level with better understanding of their network signaling mechanisms are required. However, chemoresistance mechanism is the major obstacle in cancer therapy. Generally, classical chemotherapeutics are extracted from natural sources or designed

specially to target a specific place. Although presence of new scientific tools, typical drug discovery process takes 10 to 15 years which has high costs varies from US$ 897 million to US$ 1.9 billion. To develop smart drugs, a series of stages should be followed by application of multi-disciplinary knowledge. Thus, instead of cancer researchers, Bioinformatians, computer science workers, pure physicist and chemistry researchers, pharmaceutical academics and manufacturers, different kinds of engineering branches may carry out the different stages at different facilities.

Since Bcl-2 is overexpressed in a broad range of tumors, inhibition of its expression by any tool provide a therapeutic strategies. Furthermore, over-expression anti-apoptotic Bcl-2 proteins has been associated chemotherapy resistance. Therefore Bcl-2 family can be referred as a star player and targetting each member is an important target for the design of apoptotic drugs. Three main strategies have been used to modulate their expression: a. antisense RNA or oligonucleotides, b. small molecules to inhibit protein function and protein-protein interactions of anti-apoptotic molecules, drugs, c. drugs that induce the activity of pro-apoptotic molecules. Following death stimuli, these proteins are modulated and death decision is finalized.

In this study, our aim was to characterize the possible role of Bcl-2 in drug-induced apoptosis mechanism. Here, we demonstrated that transiently silencing of Bcl-2 increased the apoptotic effect of cisplatin and paclitaxel in both cell lines. Chemical inhibition of Bcl-2 by specific small molecule, HA14-1 enhanced the cisplatin and paclitaxel apoptosis in MCF-7 cells. Pre-treatment of HA14-1 significantly enhanced the cisplatin induced apoptosis in MDA-MB-231 cells, but paclitaxel was found to be much less potent. This selective sensitizing effect of HA14-1 was shown to be via modulation of other pro or anti-apoptotic Bcl-2 family members. Cisplatin activates a pro-apoptotic pathway that bypasses Bcl-2 mediated protection against apoptosis in Bcl-2 over-expressing MCF-7 cells. However, cisplatin did not induce apoptosis in Bcl-2 over-expressing MDA-MB-231 cells. Interestingly, paclitaxel treatment did not lead to apoptosis in Bcl-2 over-expressing MCF-7 cells. Lastly reactive oxygen species (ROS) generation and lipid peroxide levels were determined in MCF-7 and MDA-MB-231 cells to assess anti-antioxidant role of Bcl-2 in drug-induced apoptosis mechanism.

A chapter for the background follows introduction section which explains current studies and baseline information about our hypothesis. This chapter is followed by Chapter 3, which explains material and methods utilized in this study in a detailed fashion. The results are presented in Chapter 4. Discussion part is placed in Chapter 5 in the light of current and past literature. Finally, Chapter 6 gives brief synopsis and conclusions of this study along with future perspectives.

CHAPTER 2

2 BACKROUND

2.1 Cancer

Cancer can be simply characterized by uncontrolled cell growth and spread of abnormal cells. Generally two basic reasons trigger the malignant cell transformation. One of them is external factors such as smoking, diet, life style, chemicals, infections, pollution or radiation. Intrinsic factors such as hereditary factors, deleterious mutations, immune system capability, hormones are also important determinatives in malign cell transformation. Therefore eliminating some external factors such as smoking, low carbohydrate diet or doing regular exercise might be important in prevention of cancer during lifespan. Although cancer is a multi-step disease and one of these factors can be enough to promote disease.

A confluence of discoveries in the mid- and late nineteenth century led to our understanding of how tissues and complex organisms arise from fertilized eggs. The most fundamental of these was the discovery that all tissues are composed of cells and cell products, and that all cells arise through the division of pre-existing cells. That means the egg is the source of all cell lineages. Therefore in principle, each cell carries some clues about its origin. According to histopathological investigations of tumor sections it is possible that the source of tumor cells can be traced back and origin of the

malign formation can be identified. Moreover, pathological and biochemical approaches provide detailed understanding of clinical behavior of tumor. Tumors are segregated into two broad classes depending on their degree of aggressive growth. Those that grew locally without invading adjacent tissues were classified as benign. Other that invaded nearby tissues and spawned metastases were termed malignant [1].

These malfunctioning cells are starting point of disorganized tissue architecture of tumors. Tumors are classified into four groups according to their origin (epithelial, mesenchymal, hematopoetic and neuroectodermal). Virtually all cell types in the body can give rise to cancer, but the most common human cancers are of epithelial origin-carcinomas. A number of carcinomas separated into two categories: squamous cell carcinomas which arise from epithelia, while adenocarcinomas arise from secretory epithelia. Non-epithelial malignant tumors include sarcomas which originate from mesenchymal cells, hematopoietic cancers, which arise from cells of the circulatory and the immune systems and neuroectodermal tumors, which originate from components of the nervous system. However some tumors which are said to be anaplastic do not fit this classification scheme [1]

Although the incidence of some cancers is comparable worldwide, many of them vary dramatically by country and therefore cannot be due simply to a normal biological process gonee awry by chance. Differences in heredity or environmental might well explain these differences; in fact, epidemiologic studies have shown that environment is the dominant determinant of the country-based variations in cancer incidence [1]. Cancer is the second leading cause of death after cardiovascular diseases in economically developed countries and causes one of eight deaths worldwide. This ratio is greater than AIDS, tuberculosis and malaria. In developing countries, cancer is third cause of death after cardiovascular and gastrointestinal infections. In 2007, more than 12 million new cancer cases were estimated worldwide, of which 5.4 million in economical developed countries and 6.7 million in developing countries. Again in 2007, it was estimated 20000 deaths per day. By 2050, the global burden is expected to grow to 27 million new cancer cases and 17.5 million cancer deaths simply due to the growth and aging of the population (Figure 2.1) [1].

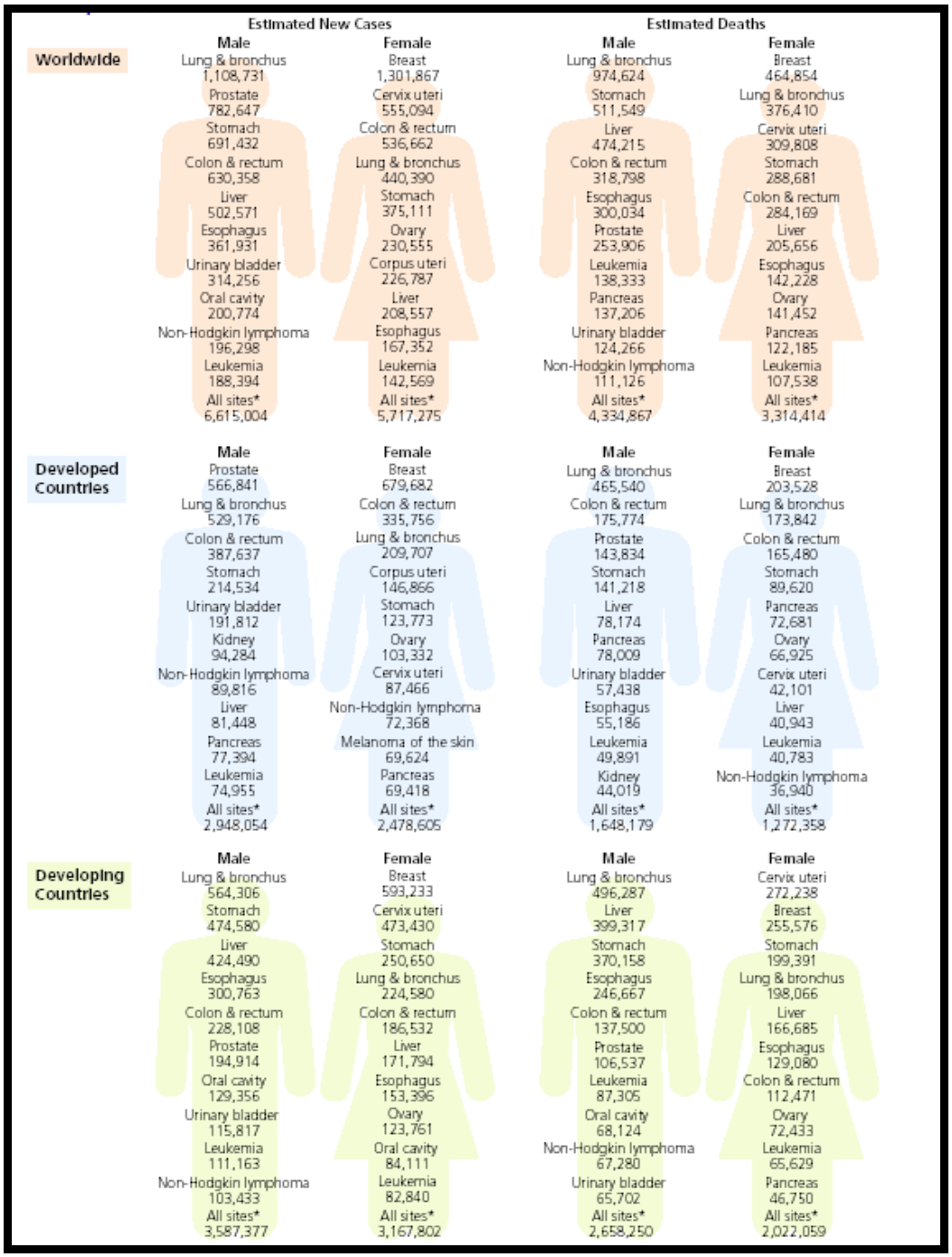

Figure 2.1 Leading sites of new cancer cases and deaths worldwide by level of economic development, 2007. Estimates were produced by applying age-specific cancer rates of a defined geographic region (worldwide, developed and developing countries) from GLOBOCAN 2002 to the corresponding age-specific population for the year 2007 from the United Nations population projections (2004 version). Therefore, estimates for developed and developing countries combined do not sum to worldwide estimates. *Excludes non-melanoma skin cancer. Adopted from [1].

In economically developed countries, the three most commonly diagnosed cancers are prostate, lung and bronchus, and colorectal among men and breast, colorectal, and lung and bronchus among women. Whereas, in economically developing countries, the three most commonly diagnosed cancers are lung and bronchus, stomach, and liver in men, and breast, cervix uteri, and stomach in women. In both economically developed and developing countries, the three most common cancer sites are also the three leading causes of death. This statistical data verifies that why lung, bronchus, breast and colorectal cancers are the most common cancer types due to Western style eating habits with high carbohydrate and lipid uptake and smoking [1].

2.1.1 Breast cancer

Breast cancer represents the most common cancer and the second cause of cancer death in women in the Western world [2]. According to National Cancer Institute statistical prediction, approximately 1.5 million new cases and a half million mortality rates were expecting in women as a result of breast cancer (Figure 2.1). Moreover, The National Cancer Institute estimates that approximately 2.4 million women with a history of breast cancer were alive in January, 2004. Most of them were cancer free [1]. Although mortality rates have recently been decreasing because of earlier diagnosis and adjuvant therapies, once metastases develop the disease is incurable. Metastatic breast cancer has a median survival of just 15 to 20 months. Cytotoxic chemotherapy is generally the treatment of choice for women with hormone insensitive, extensive visceral or rapidly progressive disease [3].

This disease is controlled by surgery and radiotherapy, and is commonly supported by adjuvant chemotherapies or hormonotherapies [4]. It is well established that the ovarian hormones, estrogen and progesterone, are essential for the growth and maintenance of the mammary ductal tissue [5]. During postlactational regression of breasts, extensive apoptosis of ductal cells is required, whereas myoepithelial cells and basal lamina persist, and are reused during the resumption of extensive cell proliferation. Breast tumor cells are also heavily dependent on estrogen and progesterone hormones for their maintenance and growth [6]. Fortunately, very effective antagonists for these hormones exist, such as tamoxifen, which is widely used for the

treatment of these tumor types usually subsequent to surgical resection [7]. However, occasional loss of receptors due to genetic lesions in tumor cells and overexpression of drug efflux pumps lead to resistance towards hormone-mimetic drugs as well as other chemotherapeutic agents. Although second-generation selective estrogen receptor modulators such as raloxifene and second-line treatment options such as the aromatase inhibitors (letrozole and anastrazole) are somewhat effective, they are primarily useful against tumors that have a positive hormone receptor status. The challenge, thus, lies in the emergence of hormone-refractory tumors which no longer respond to the anti-hormone therapy.

2.1.2 Chemotherapeutics

2.1.2.1 Cisplatin

The treatment of anti-estrogens for estrogen-responsive breast cancers has been used for almost three decades. However, a major problem with the use of anti-estrogens is that most patients with advanced breast cancer eventually develop resistance to the compounds. Several mechanisms have been proposed to understand the molecular basis of resistance, including loss of estrogen receptor-α (ERα) expression, posttranslational modifications of the estrogen receptor, and changes in signal transduction [8].

Cisplatin (*cis*-diamminedichloroplatinum II) is a platinum compound used for treatment of a variety of cancers. It was reported that cisplatin is active in breast carcinoma given alone or in combination with other chemotherapeutic drugs. In women with human epidermal growth factor-2 (HER2)-positive metastatic breast cancer, treatment with cisplatin, or the related carboplatin, in combination with taxanes and trastuzumab (Herceptin) shows promising results. Cisplatin induces intrastrand and interstrand cross-links in DNA, resulting in DNA adducts, which is followed by induction of cell death [9]. Once cisplatin enters a cell its chloride ligands are replaced by water molecules generating a positively charged aquated species that can react with nucleophilic sites on intracellular macromolecules to form protein, RNA and DNA adducts [10]. Cisplatin activates caspases through different signaling pathways, including stabilization of tumor suppressor protein p53 and release of cytochrome *c*

(Cyt c) from mitochondria [11]. Evidence of alternative death programs has emerged during the past few years, where it has become clear that chemotherapeutic compounds, including cisplatin, also can trigger, e.g., lysosomal membrane permeabilization, resulting in release of lysosomal proteins to the cytosol [12, 13]. However, intrinsic or acquired resistance to cisplatin is major limitations in use of this drug in cancer chemotherapy. Alterations in the expression of Bcl-2 family members during apoptosis can alter the sensitivity of the cells to apoptosis following cisplatin treatment. Thus higher concentration of drug might be effective in Bcl-2 overexpressing cells. It was reported that decreased Bax expression was observed in cisplatin resistant cells, whereas induced Bax expression sensitize the cells to cisplatin. However, it is clear that one of the cisplatin resistance responsible evidence is Bcl-2 and Bcl-x_L over-expression [10]. Thus, cisplatin induced apoptosis mechanism should be further studied in *in vitro* and *in vivo* models to understand resistance mechanism related with different cell signaling pathways.

2.1.2.2 Paclitaxel

Over the last few years, breast cancer mortality has been steadily decreasing due to a variety of reasons, including the availability of newer cytotoxic agents. Among the most active newer agents, the taxanes, which target the microtubules, have emerged as a new cornerstone in the treatment of advanced breast cancer and, increasingly, of early disease as well [14]. Taxanes are natural products derived from trees of the genus *Taxoidaceae*. The first taxanes introduced in cancer therapy was paclitaxel, isolated from *Taxus brevifolia* [15, 16]. As well as paclitaxel, docetaxel is the most widely used agent in cancer therapy. These natural products have shown important clinical benefits in the adjuvant and in the metastatic setting, with objective response rates of 32 % – 68 % when used as single agents [17]. However, this clinical success has been accompanied by significant side effects and primary as well as acquired (secondary) resistance. The mechanisms of resistance to taxanes are not fully understood and, as with many other agents, are likely to be multifactorial, including the overexpression of P-glycoprotein, the presence of β-tubulin mutations, and high microtubule-associated protein expression [14].

In earlier studies, the microtubule network appeared as the main target of paclitaxel. In fact, taxanes binds to β-tubulin subunits, thereby disrupting normal turnover of the microtubules [16]. The validation of the microtubules as a cancer target has led to the development of newer agents that target the microtubules. Microtubules play a fundamental role in diverse cellular functions such as cell division, growth, and motility, the development and maintenance of cell shape, and the trafficking of vesicles, organelles, and proteins. Microtubules are filaments formed with the polymerization of heterodimeric α/ß tubulin subunits. A very complex dynamic process of polymerization and depolymerization is critical for microtubule homeostasis, which finally leads to the formation and functioning of the mitotic spindle. The taxanes interact with the tubulin units, resulting in alterations in the polymerization and depolymerization process. If this function is disrupted, a cell cycle arrest at the G_2/M phase occurs, which in turn results in apoptosis [14]. Along with arrest in M phase of the cell cycle, taxanes have also been reported to induce post-translational serine phosphorylation of the Bcl-2 protein. Disagreement exists on the levels of Bcl-2 and resistance to taxanes [16]. A strong suggestion for a direct role of Bcl-2 in mediating paclitaxel sensitivity comes from observation that a cell line not expressing Bcl-2 is resistant to paclitaxel-induced apoptosis [18, 19]. Further support for this observation that paclitaxel is able to entrap, from a random peptide library, a panel of peptides showing a high degree of structural homology with disordered loop of Bcl-2, thereby indicating the latter as a motif for direct paclitaxel binding. In addition, it was shown that Bcl-2 or Bcl-x_L over-expression protected ovarian cancer cells from paclitaxel-induced apoptosis. As well as other cellular signal molecules, Bcl-2 has a functional status in death machinery and modulation of Bcl-2 family members determines the cell fate following drug treatment [16]. Thereby, paclitaxel sensitivity should be discussed with all anti-apoptotic Bcl-2 family members. Chemotherapy by microtubule-interfering agents is also limited by the emergence of drug resistance owing to mutations in the target, microtubules/tubulin, overexpression of drug efflux pumps, and many other mechanisms [18]. Taken together, despite the currently used treatment modalities, there is still no effective cure for patients with advanced stages of breast cancer, especially in cases of hormone-refractory cancer.

Therefore, the discovery and/or the development of drugs that combat hormone-insensitivity and display better therapeutic indices would have an important effect on breast cancer morbidity and mortality [6].

2.2 Cell Death

Apoptosis and necrosis are two morphologically distinct forms of cell death that are important for maintaining of cellular homeostasis [20-22]. Almost all agents can provoke either response when applied to cells; but the duration of treatment and the dose of the used agents determine which type of death (apoptosis or necrosis) is initiated. Necrosis occurs when cells are exposed to extreme variance from physiological conditions (e.g., hypothermia, hypoxia) which may result in damage to the plasma membrane. Under physiological conditions direct damage to the plasma membrane is evoked by agents like complement and lytic viruses. Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response [20-22].

Apoptosis, in contrast, is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide"). It is also referred as programmed cell death which was first time described by Lockshin and Williams in 1964 [23]. It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine-dependent tissue atrophy.

Cells undergoing apoptosis show characteristic morphological and biochemical features. These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and

nuclear material. *In vivo*, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells. Due to this efficient mechanism for the removal of apoptotic cells *in vivo* no inflammatory response is elicited. *In vitro*, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyses. This terminal phase of *in vitro* cell death has been termed "secondary necrosis" (Table 2.1).

Table 2.1 Apoptosis *versus* necrosis, biochemical and morphological features

Morphological features		
	Apoptosis	**Necrosis**
Outset	Shrinking of cytoplasm and condensation of nucleus	Swelling of cytoplasm and mitochondria
Plasma membrane	Blebbing of plasma membrane without loss of integrity	Loss of membrane integrity
Chromatin	Aggregation of chromatin material in nuclear membrane	
Organelles	Mitochondria become leaky due to pore formation involving proteins of the bcl-2 family.	Disintegration (swelling) of organelles
Vesicles	Formation of membrane bound vesicles (apoptotic bodies)	No vesicle formation, complete lysis
Terminal	Fragmentation of cell into smaller bodies	Total cell lysis
Biochemical features		
Regulation	Tightly regulated process involving activation and enzymatic steps.	Loss of regulation of ion homeostasis.
Energy input	Energy adenosine tri-phosphate (ATP)-dependent (active process, does not occur at 4°C)	No energy requirement (passive process, also occurs at 4°C)
DNA	Non-random mono- and oligonucleosomal length fragmentation of DNA (Ladder pattern after agarose gel electrophoresis)	Random digestion of DNA (smear of DNA after agarose gel electrophoresis)
Timing	Pre-lytic DNA fragmentation	Postlytic DNA fragmentation (late event in cell death)
Biochemical events	Release of various factors (cytochrome C (Cyt c), apoptosis initiation factor (AIF)) into cytoplasm by mitochondria. Activation of caspase cascade. Alterations in membrane asymmetry (translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane)	

Autophagy is an ancient mechanism by which starved cells produce energy and stave off death by gradually targeting their organelles and cytoplasmic elements to lysosomes for digestion. It is referred as lysosomal cell death or excessive self-cannibalization [24]. It is a tightly-regulated process that plays a normal part in cell growth, development, and homeostasis, helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products. It is a major mechanism by which a starving cell reallocates nutrients from unnecessary processes to more-essential processes.

A variety of autophagic processes exist, all having in common the degradation of intracellular components via the lysosome. The most well-known mechanism of autophagy involves the formation of a membrane around a targeted region of the cell, separating the contents from the rest of the cytoplasm. The resultant vesicle then fuses with a lysosome and subsequently degrades the contents. It was shown that deficient autophagic pathways result in cancer formation [21, 25].

2.2.1 Apoptosis

Apoptosis, or programmed cell death, evolved in metazoans as a means to maintain tissue homeostasis by eliminating unwanted, damaged or infected cells in multicellular organisms. Apoptosis is very important step in body structure modeling during embryogenesis. As important as cell division and cell migration, programmed cell death allows the organism to tightly control cell numbers and tissue size, and to protect itself from rogue cells that threaten homeostasis. Therefore, it is an active process of cellular self-destruction with distinctive morphological and biochemical features. This type of cell death is classically defined by a pattern of molecular events and morphological changes, including condensation of cytoplasm, rounding up, loss of mitochondrial membrane potential, chromatin condensation to compact and simple geometric figures, nuclear fragmentation, blebbing with maintenance of membrane integrity (zeiosis) and finally loss of plasma membrane asymmetry, coupled to the display of phagocytosis markers on the cell (Table 2.1).

Apoptosis term originally comes from old Greek word which means the loss of petals and leaves in plants. The complicated process was discovered and rediscovered by cytologists and developmental biologists at several times during past 200 years and took several names. Finally the term adopted is apoptosis by Kerr and his collegues in 1972 in order to describe a morphologically distinct form of cell death [26].

Since the beginning of the 1990s, many human diseases have been associated with too much or too little apoptosis, such as degenerative diseases and cancer, respectively [27]. In their influential paper, Hanahan and Weinberg defined the ability to evade programmed cell death as a hallmark of most human cancers and pointed to the fundamental interplay between apoptosis and cell proliferation [20]. Impaired apoptosis mechanism also renders the tumor cell more resistant to conventional cytotoxic therapy [28]. Consequently, an attractive approach for anticancer therapeutics is to overcome this inherent resistance to apoptosis by directly activating the normal cell death machinery [29].

Two major apoptotic pathways through which caspases become activated in mammalian cells: the death-receptor pathway and the mitochondrial pathway. These apoptotic signaling pathways congregate at the mitochondria, where signals are processed through a series of molecular events ending in the release of potent death factors that trigger either caspase-dependent or -independent apoptosis.

In fact, depends on the death signal type, apoptosis is regulated between three main parts of the cell. Plasma membrane is the starting point of receptor-mediated apoptotic pathway. Death stimulus starts when death ligands bind to the death receptor, resulting in their oligomerization. This leads to recruitment of adaptor proteins via death domain (DD) to the receptor. A C-terminus of adaptor proteins contains a death effector domain (DED), which interacts with pro-caspase-8, forming a complex called FLICE. Pro-caspase-8 is activated within this complex, and active caspase-8 can in some cells (type I) directly cleave and activate pro-caspase-3, which cleaves many structural proteins and proteins involved in DNA maintenance (e.g. by Inhibitor of caspase-activated DNase (ICAD)). Upon this cascade of reactions, Caspase-activated DNase (CAD) is released and induces cleavage of DNA, resulting in nuclear apoptotic

morphology. In type II cells, caspase-8 is also capable to initiate mitochondria-mediated apoptotic signaling through cleavage of Bid with formation of truncated Bid (tBid) which interconnects death receptor pathway to mitochondria. The second place is mitochondria which covers energy metabolism within the cells. Mitochondria mediated pathway, the Bcl-2 family proteins are perhaps the most important key players. These proteins can also be responsible for bridging signals from the death-receptor pathway to mitochondrial pathway. The Bcl-2 family of proteins consist both anti-apoptotic and pro-apoptotic members. While pro-apoptotic members serve as sensors to death signals and executors of the death program, the anti-apoptotic members inhibit the initiation of the death program. The mitochondria-mediated apoptotic signaling might be triggered by diverse stimuli and resulted in release of several apoptogenic factors, i.e., cytochrome c, Smac/DIABLO, AIF, Endonuclease G, and Omi/HtrA2 from the intermembrane space of mitochondria into cytosol. This release is regulated by the Bcl-2 family proteins. Bcl-2 and Bcl-x_L block this process, whereas Bak and Bax promote it. Cytosolic Cyt c forms with Apaf-1 and pro-caspase-9 so called the apoptosome complex. As a result, pro-caspase-9 is activated and subsequently initiates the caspase cascade, including the activation of pro-caspase-3. The activation/activity of pro-caspase-9 and -3 is inhibited by inhibitor of apoptosis protein (IAP), which in turn is regulated by Smac/DIABLO also released from the mitochondria. AIF and Endo G released from mitochondria translocate to the nucleus and cause chromatin condensation and DNA fragmentation. Heat shock proteins (Hsps) or Aven may block apoptotic signaling at several levels, including apoptosome formation, activation of caspases, and redistribution of Bid to mitochondria. Genotoxic stress inducers trigger nucleus. In this process, pro-caspase-2 is activated and involved in transducing the apoptotic signal from nuclei to mitochondria (Figure 2.2).

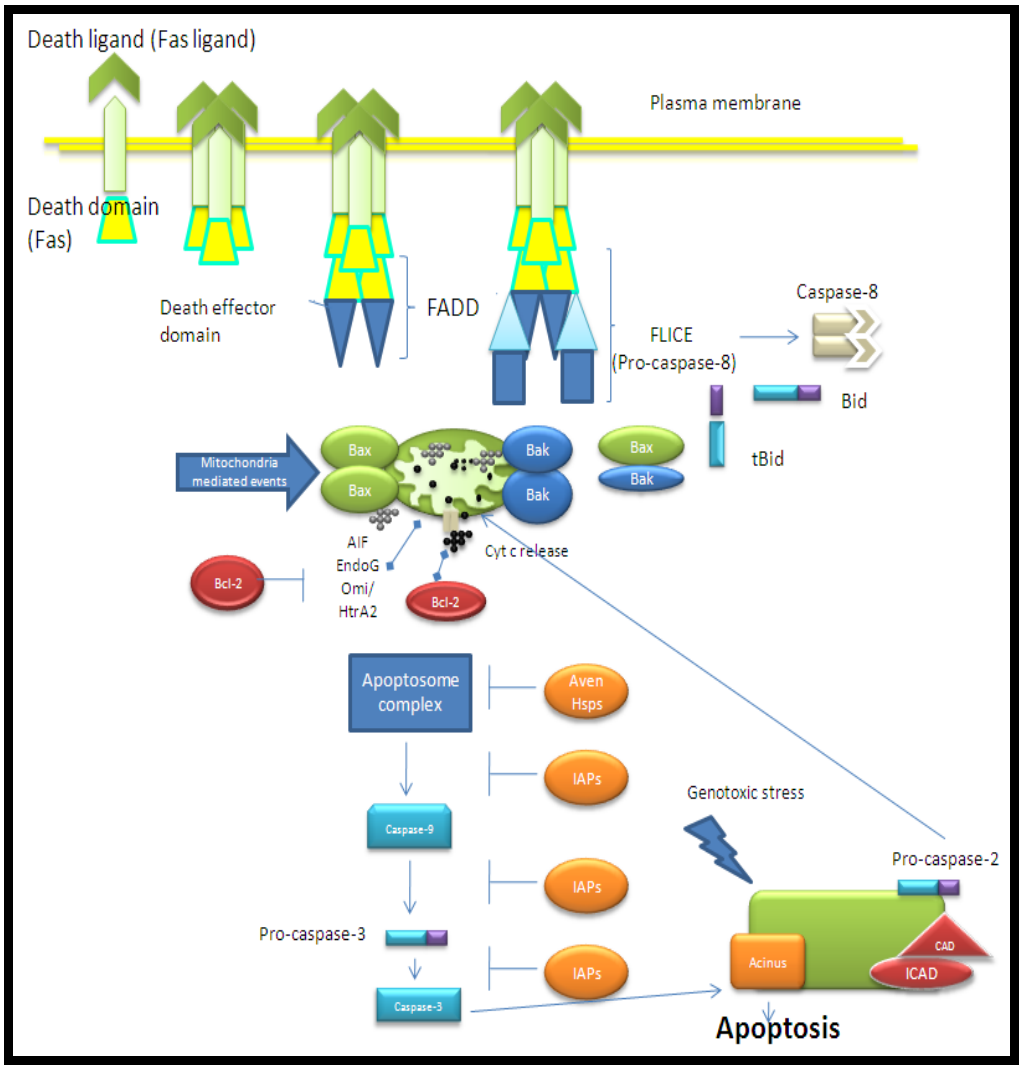

Figure 2.2 Cross-talk between plasma membrane, mitochondria, and nuclei in apoptotic signaling. Adopted from [30].

Accumulating evidence also shows other intracellular compartments and/or organelles such as the nucleus, the endoplasmic reticulum (ER), and the lysosomes all participate in apoptotic signaling [13, 30]. How the signals emerging from these organelles bifurcate into extrinsic and intrinsic apoptotic signaling pathways is of major importance for cancer therapy but has been only partly revealed as of yet. Therefore intensive research activity is required within the apoptotic field [30].

2.3 Modulators of Apoptotic Signalling

2.3.1 Caspases

Programmed cell death depends on a family of aspartate-spesific cystein proteases (caspases) that cleave certain vital structural proteins such as lamins, gelsolin and preteolytically activate latent enzymes (nucleases) that contribute to cell destruction [31]. Of note, not all caspase family members participate in apoptosis. For example caspase-1 and caspase-11 are predominantly involved in the processing of pro-inflammatory cytokines (interleukins 1 and 18) [32]. Their mechanistic action is first time described in *Caenorhabditis elegans and* well described in *Drosophila melanogaster*. Caspases are highly conserved through evolution, and can be found from humans all the way down to insects, nematodes and hydra. More than half number of identified caspases has been suggested to function in apoptosis [31, 33].

Caspases can be divided into two groups with respect to their structure. Caspases with long pro-domain have structural motifs: caspase activation and recruitment domain or death effector domains. These domains provide interaction with other proteins. Short domain caspases such as caspase-3, 6, 7, 14 are activated by proteolytic cleavage by other caspases. The rest of fourteen identified human caspases belongs to long pro-domain caspase subgroup. Caspases can also be grouped based on their function as apoptotic or inflammatory. The apoptotic group can be divided in two subgroups: initiator and effector caspases. Initiator caspases; caspase-2, 8, 9, 10, 12 activate effector caspases (caspase-3, 6, 7, 14) or indirectly interact through another pathway [29, 34]. Furthermore, as indicating before caspase-8 and caspase-10 are activated by death receptors in humans [35]. Contrary extrinsic pathway, mitochondrial pathway requires different initiator caspases and their adaptors; caspase-9 and its adaptor Apaf-1 are involved in this pathway but not essential all cell types [36, 37]. Effector caspases are usually activated proteolytically by an upstream caspase, whereas initiator caspases are activated through regulated protein-protein interactions. The actual molecular mechanisms mediating initiator caspase activation are still unclear.

All known caspases possess an active-site cystein and cleave substrates at Asp-Xxx bonds after aspartic acid residues. A caspase's distinct substrate specificity is determined by four residues amino-terminal to the cleavage site [37]. Zymogens are the inactive form of caspases in healthy cells and they are activated through interaction with specific adaptor proteins that enhance conformational change and autocatalytic processing [31]. The inert zymogens are composed of three domains: an N-terminal pro-domain, p20 and p10 domains. The mature enzyme is a heterotetramer which contains two p20/p10 heterodimers and two active sites. Thus they have two active sites (Figure 2.3).

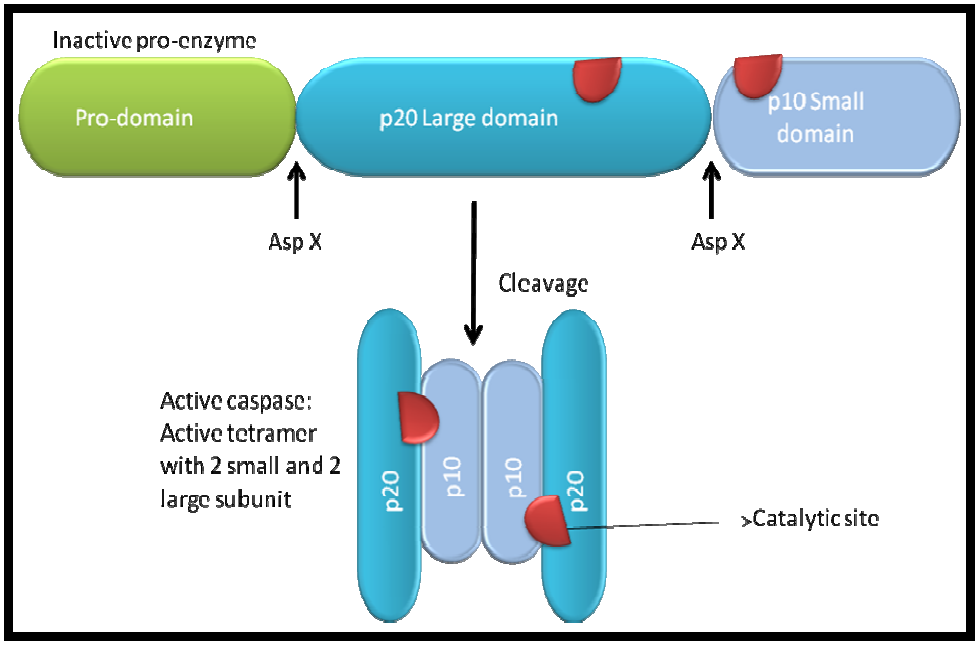

Figure 2.3 Pro-caspase is cleaved at Asp X sites and result in active-caspase which contains two active sites.

Caspases selectively cleave a limited set of target proteins, usually at one or at most a few positions in the primary sequence (always after aspartate residue). Moreover, they can indirectly activate a set of protein by cleaving off a negative regulatory domain or inactivating regulatory domain [37]. One decade ago, an important target was identified. Caspases was shown to activate nucleases which cut genomic DNA between nucleosomes, to generate DNA fragments [38]. Now, this approach is used for determination of apoptosis. DNA ladder nuclease CAD was shown as inactive

complex with an inhibitory subunit, ICAD in healthy cells. Activation of CAD occurs by caspase-3 mediated cleavage of the inhibitory subunit. Following cleavage, catalytic subunit released and then activated [39].

During apoptosis, caspases cleave nuclear lamins which are required for nuclear shrinking and budding. Moreover, in order to loss of cell shape, cytoskeletal proteins are cleaved. Finally, active blebbing is occurred following PAK2 cleavage which is a member of p21-activated kinase family. PAK2 activated by caspase-mediated cleavage of negative regulatory submit. Thus active blebbing is observed in apoptotic cells [37].

Mechanisms of caspase activation include proteolytic cleavage by an upstream caspase. Generally, activation of downstream, effector caspases is regulated by this way. It is probably also used for induction of apoptosis by non-caspase proteases, such as granzyme B. The second mechanism is recruitment of aggregation of multiple pro-caspase-8 molecules into close proximity. The last proposed mechanism is activation of caspase-9 by means of conformational change, not proteolysis. Thus, holoenzyme formation, Cyt c and adenine tri-phosphate (ATP)-dependent oligomerization of Apaf-1 allows recruitment of pro-caspase-9 into the apoptosome complex [37]. Of note, although it is clear that mitochondria membrane permeabilization (MMP) is involved in the activation of caspases, there has been considerable debate over whether MMP can occur independently of caspase activity [40]. It is clearly known that pro-caspase-8 activation upon death receptor stimuli induces MMP by cleaving Bid into tBid [40, 41]. However, it was shown that in some cases caspase inhibitors could not prevent Cyt c release upon genetoxic stress induction. Although silencing of caspase-2 blocked Cyt c and Smac release from mitochondria following genotoxic stress inducers, caspase-2 was insensitive to pan-caspase inhibitors and directly evoke Cyt c and Smac release from isolated mitochondria.

Furthermore, it was reported that loss of MMP, increased production of reactive oxygen species (ROS) and concominant lipid peroxidation and disruption of mitochondrial morphology by pan-caspase inhibitors. However, mutation studies showed that Cyt c release occurred normally in the presence of caspase inhibitors. These findings indicated that, Cyt c release occurred without disruption of respiratory

processes which indirectly supply ATP for various apoptotic processes while caspases are being activated in the cytosol. Following Cyt c release, caspases might enter mitochondria through partially permeabilized outer mitochondria membrane and shut down energy production to finalize apoptotic processes. Then sequentially, other intermembrane space proteins are released, ROS generation is induced and lipid peroxidation is increased for morphological disruption [40, 42].

2.3.2 Bcl-2 family proteins

The integrity of mitochondrial outer membrane (MOM) is regulated by an evolutionary conserved group of proteins known as Bcl-2 family [27, 43]. Therefore, Bcl-2 family is central to both regulation and execution of most intrinsic apoptotic pathways. The Bcl-2 family proteins consists more than 30 different members that fulfil anti- or pro-apoptotic functions. All Bcl-2 family members hold at least one of four conserved Bcl-2 homology domains (BH1 to BH4). The family is comprised of three groups which are classified according to their content of BH-domains. To date, the human repertoire of multidomain members comprises six anti-apoptotic members (Bcl-2, Bcl-x_L, Bcl-w, Nrh/Bcl2l10/Bcl-B and Bfl-1) which contain four BH- domains, except Mcl-1 and Bfl-1/A1 [44], defined by their similarity among the members of the family. The pro-apoptotic Bcl-2 family is divided into the multidomain pro-apoptotic members (e.g. Bax, Bak, Bok) which contains BH1-3 domains, and the BH3-only pro-apoptotic members (e.g. Bid, Bim, Bik, Blk, Hrk, Noxa, and Puma) (Figure 2.4).

It seems that the so-called BH3-only molecules such as Bid, Bim and Bad are sensors for the peripheral death signals and are able to activate the multidomain executioner molecules, Bax or Bak [45]. BNIP is usually included into the BH3-only group based on its limited homology with BH3 domains [43]. Beclin, a Bcl-2 binding protein that promotes autophagy, and the cytosolic fragment of Erbb4 have also been proposed to be BH3-only proteins [46].

Most multidomain members and several BH3-only proteins contain a hydrophobic segment at their C-termini and this transmembrane domain (TM) is important for subcellular localization and/or activity. Some Bcl-2 family members such as Bcl-2 and

Bak are constitutively localized to the mitochondrial membrane, whereas others such as Bax and Bid reside in the cytosol and translocate to the mitochondria during apoptosis. It is worth highlight that Bcl-2 family members have been found in other organelles such as the ER and the nuclear envelope in addition to their localization in mitochondria.

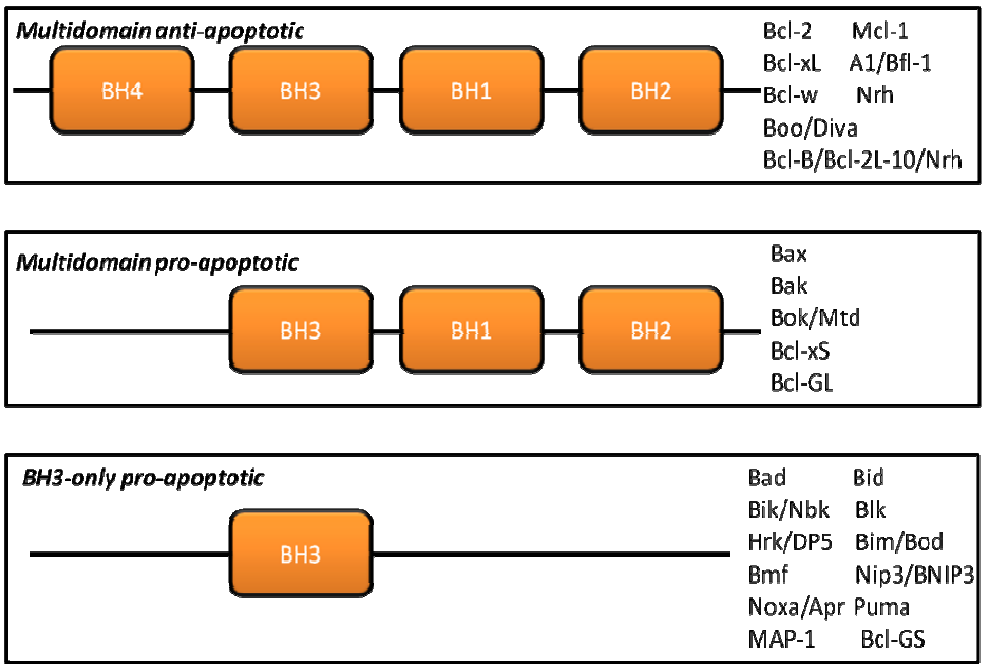

Figure 2.4 Schematic representation of three groups of Bcl-2 family members. (modified from [47]).

2.3.2.1 Functional Bcl-2 homology

Mutagenesis studies have revealed that BH-domains are important for the various molecular functions and for protein interactions among the family members. The BH1 and BH2-domains are necessary for the death repression function of the anti-apoptotic molecules, and the BH4-domain has been suggested to be important for anti-apoptotic activity [48], BH3-domain is essential and sufficient for pro-apoptotic effect [49]. Despite opposite biological functions and wide differences in amino acid sequences, three dimensional (3D) structures and secondary structure predictions suggested that all multidomain Bcl-2 family members share a similar helical bundle structural fold, resembling the pore-forming domains of bacterial toxins. Based on this finding, at least

four members of Bcl-2 family members were determined to produce ion-conducting pores in synthetic lipid membranes *in vitro* [50]. This activity may relate to function of these proteins in regulating MMP.

One of the major structural differences between multidomain proteins and BH3-only proteins is that, a hydrophobic groove formed on the surface of anti-apoptotic Bcl-2 proteins by the combination of their BH1, BH2 and BH3 regions is the binding site for the amphiphatic, α-helical BH3-domain of the pro-apoptotic family members. These findings suggest that BH3-only proteins may function as a donor in interaction with the multidomain proteins, whose hydrophobic of pocket can serve as an acceptor. BH3-only proteins, with the notable exception of Bid, have unrelated secondary structures and do not share pore forming helical bundle class [43, 47, 51]. The Bim was determined to be disordered in absence of interaction partners and to experiment a conformational change in its BH3 region upon binding to an anti-apoptotic member. In contrary, Bid is unique among the BH3-only proteins due to similarity to multidomain members of Bcl-2 family. Different from other pro-apoptotic members, Bid interconnects death receptor apoptosis pathway to mitochondrial apoptosis pathway, and play important role in the control of cell cycle progression. Structural studies revealed that Bid has conserved structure before activation. However, activation of Bid by proteolysis can cause the exposure of its BH3-domain for its killing function. Contrary, the anti-apoptotic members usually have their BH3-domain buried, which may explain why they are not apoptotic. Recent findings showed that C-terminal of hydrophobic helix occupies the BH3-binding pocket. Therefore, displacement of the C-terminal tail from the hydrophobic groove is probably key event involved in the activation and targeting of Bax to the mitochondrial membrane upon apoptotic stimuli. The critical role of BH3-domain as a mediator of cell death was also identified in studies on the molecular interactions between Bak and Bcl-x_L, which revealed a unique requirement of BH3 for the interaction with Bcl-x_L as well as for cell killing [49]. In the meanwhile, other pro-apoptotic members were analyzed for their sequence similarity between their homologous domains were indentified and resulted in discovery of BH3-only proteins such as Bik/Nbk and Bad [52].

The anti-apoptotic members Bcl-2, Mcl-1, Bcl-x_L, Bcl-w have similar overall structure as Bax and Bak, but with the exception of Mcl-1 and Bfl-1/A1 which contain a BH4-domain located toward their N-terminus [47]. Therefore structural studies are important to reveal their functional properties. Although, Bax was found very similar to Bcl-x_L, it is not clear how Bax functions in opposition to the anti-apoptotic members. One clue from the structural study is that the full length Bax actually has a conformation similar to that of C-terminal-truncated Bcl-x_L binding to a Bak BH3 peptide. The transmembrane domain of Bax is actually occupying its own hydrophobic pocket. It is known that Bax needs to change its conformation to be activated. Therefore solution structure is different in quiescent Bax than active Bax [47, 53].

The other clue is that BH4-domain is directly involved in heterodimerization, but it may be decisive for the distinction between anti- and pro-apoptotic functions. Indeed, caspase-mediated cleavage of BH4 from Bcl-2 and Bcl-x_L, thus the conversion of Bcl-2-like proteins to Bax-like proteins, has been shown to result in Bcl-2 and Bcl-x_L-derived pro-apoptotic forms [54]. It was shown that BH4-domain is being required for stabilization of MMP and Cyt c release [44]. The BH4-domain mediated anti-apoptotic activity was found independent from BH3-mediated heterodimerization [44].

2.3.2.2 The network of Bcl-2 protein family members-how does it work?

Bcl-2 is the most famous member of the Bcl-2 family members and it was first described in 1984 by Tsujimoto *et al.* [55]. It was initially cloned from the t(14;18) breakpoint in human follicular lymphoma as a proto-oncogene and result in its up-regulation. Since this discovery, many malignancies have been shown to overexpress Bcl-2 and other anti-apoptotic members. This overexpression results in constitutive block to Bax and Bak oligomerization, thereby preventing translation of the upstream death signals to outer mitochondria membrane permeabilization (MOMP). Although its proto-oncogene character was quickly realized, its biological function as an anti-apoptotic gene was not realized until some years later [47, 53]. A number of proteins were soon discovered that share sequence homology with Bcl-2, but only some engage in anti-apoptotic activities; others actually promote apoptosis. The Bcl-2 family proteins are evolutionary conserved. Interestingly, a number of viruses were shown to be able to

encode Bcl-2 homologs. Most of these viral homologs are anti-apoptotic, probably because viruses need to keep the infected cells alive for latent and persistent infection. Perhaps the most understood pathway is that in the worm *Caenorhabditis elegans,* where detailed genetic studies have shown that two Bcl-2 related proteins anti-apoptotic CED-9, and a BH3-only death agonist, EGL-1 are essential for controlling developmentally programmed somatic cell deaths [56]. Expression of EGL-1, the death trigger, is induced by damage signals. Binding of EGL-1 to CED-9, the worm Bcl-2 ortholog, releases the adapter protein CED-4 from CED-9. Once released, CED-4 binds to and activates caspase CED-3 to cause cellular demise [57].

BH-domains of anti-apoptotic members seem to interact with the BH3-domain of pro-apoptotic members. The first identified pro-apoptotic Bcl-2 family member, Bax, was cloned based on its interaction with Bcl-2. The functional inhibition of Bax by Bcl-2 relies, in part, in the ability of a hydrophobic cleft to bind to the conserved BH3 domain of pro-apoptotic Bcl-2 members. The proposed interaction allowed that Bcl-2 engages a group of pro-apoptotic Bcl-2 family members, the BH3-only proteins and prevent the BH3-domains of functional subset of proteins such as Bid, Bim by physically sequestering them from directly activating Bax. The directly interaction of these proteins with Bax, prevent it from exerting its deleterious effect on its activation by an apoptotic stimulus [58]. However interaction of proteins can be broken down by mutations which can occur at one of the domains.

Previously it was shown that the regions outside of the BH-domain may be required for interactions, such as the interaction between BNIP1 and Bcl-x_L [43]. Critical amino acids have been defined in each BH-domain, such as Gly^{145} in the BH1-domain, Trp^{188} in the BH2-domain of Bcl-2, and Gly^{94} in the BH3-domain of Bid. Although Bcl-x_L can bind to BH3-only and multidomain pro-apoptotic members, certain amino acids (Phe^{131} and Asp^{133}) seem to be important for binding to BH3-only molecules, but not to Bax. Moreover, variations in certain key amino acids could result in different affinities in binding to the same molecule, which occurs with two Bcl-2 isoforms in binding to Bak or Bad-derived BH3-peptides [27, 43, 57].

Many other Bcl-2 family members were cloned based on this type interaction. A number of methods such as yeast-2-hybrid, co-immunoprecipitation and GST pull down assays were also utilized to find such interactions between pro-apoptotic and anti-apoptotic members. However, identification of this kind of interactions is not easy *in vitro* conditions and should be proofed by *in vivo* experiments. Based on these analyses, several interaction types are defined [30]. These models also provide information how to BH3-only proteins activate Bax and Bak. Rheostat model focuses on the balance of anti-apoptotic and pro-apoptotic Bcl-2 proteins which determines the cellular fate. This mechanism is based on the assumption that the activities of pro-apoptotic Bcl-2 proteins are kept in check by the anti-apoptotic Bcl-2 family members, which are often permanent constituents of the mitochondrial membrane and which can bind and neutralize Bax and Bak. When any stress stimuli occurs, pro-apoptotic Bcl-2 proteins are induced or activated to trigger MOMP. In contrast, growth factors promote cellular survival by increasing the amount of anti-apoptotic Bcl-2 proteins or decreasing pro-apoptotic members. For the interaction of Bcl-2 proteins, the BH3-domain seems to be required and binds into a hydrophobic pocket of anti-apoptotic Bcl-2 proteins [44, 59]. However, not all anti-apoptotic members can interact with all pro-apoptotic members. The selectivity between two kinds of proteins suggests that specific amino acids required for particular interactions may only exist in some but not all of the family members. The second type of interaction (neutralization model) is shown between two pro-apoptotic members, usually one BH3-only and one multidomain molecule (e.g. Bid to Bax or Bak) [44, 60]. According to neutralization model, BH3-only molecules directly bind to multi-domain anti-apoptotic members and neutralize their activity. This interaction leads to release of Bax and Bak. However the specificity of BH3-only proteins differs for each anti-apoptotic protein. For example, Noxa only binds Mcl-1 and A1, and Bad engaged only Bcl-2, Bcl-x_L and Bcl-w [61]. The last model is direct activation which is similar to neutralization model. According to this model, BH3-only proteins are functionally subdivided into activators and sensitizers [44]. Activation of Bax and Bak require direct association with activator BH3-only proteins. However, these proteins are normally bound and kept in check by anti-apoptotic Bcl-2 proteins. Bax and Bak are activated when activator BH3-only proteins are released from anti-apoptotic Bcl-2 proteins through their binding to sensitizer BH3-only proteins (Figure 2.5). These sensitizers cannot directly activate Bax and Bak. The main statement of the

last model is however still controversial as Bax and Bak were recently shown to mediate apoptosis also without association to BH3-only proteins. In Figure 2.5a, the indicated activator BH3-only proteins, via their BH3-domain (blue triangle), directly engage Bax and Bak and activate them, whereas sensitizer BH3-only proteins (e.g. Bad or Noxa), which can only bind the pro-survival proteins, serve only to displace activators from the pro-survival proteins. In the indirect activation model (Figure 2.5b), the BH3-only proteins only bind the pro-survival proteins. Because the promiscuous binders (Bim, tBid, and Puma) can neutralize all pro-survival proteins, each can readily trigger Bax/Bak activation, whereas any selective binder (e.g. Bad) must be co-expressed with a complementary binder (e.g. Noxa) to do so. [59, 62].

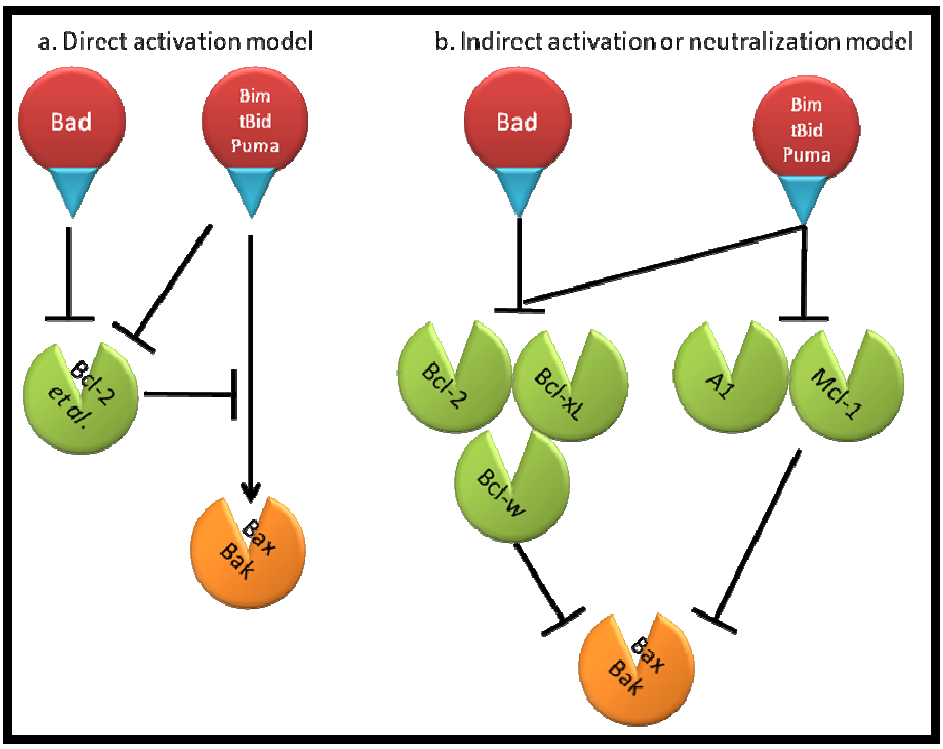

Figure 2.5 Two models for how BH3-only proteins activate Bax and Bak. Modified from [59, 62].

Furthermore, a number of cellular factors outside Bcl-2 family may also directly interact with Bcl-2 proteins to modulate their action. p53 can directly activate Bax and Bak, or block Bcl-2 and Bcl-x_L, acting as an activator and de-repressor, respectively (Figure 2.6). p53 is found as mutated over half of cancer types. Therefore its transcriptional control is very important in normal cell behavior. A large number of pro-apoptotic Bcl-2 family proteins and apoptosis related genes are regulated by p53. Among these proteins are Bax, Puma, Noxa, IGF-BP3, DR5/KILLER, Fas/Apo-1, the PIGs, PERP, Apaf-1 and p53-AIP. During apoptosis, some of these proteins translocate to mitochondria and promote release of mitochondria intermembrane proteins to the cytosol. Previous report showed that when recombinant p53 is added to mitochondria, results in Bak oligomerization and release of Cyt c. Moreover, it was shown that p53 accumulation in cytosol was inefficient in $Bax^{-/-}$ knock-out mice models and treatment of purified mitochondria from $Bak^{-/-}$ MEFs with recombinant p53 did not induce Cyt c release [30, 63, 64].

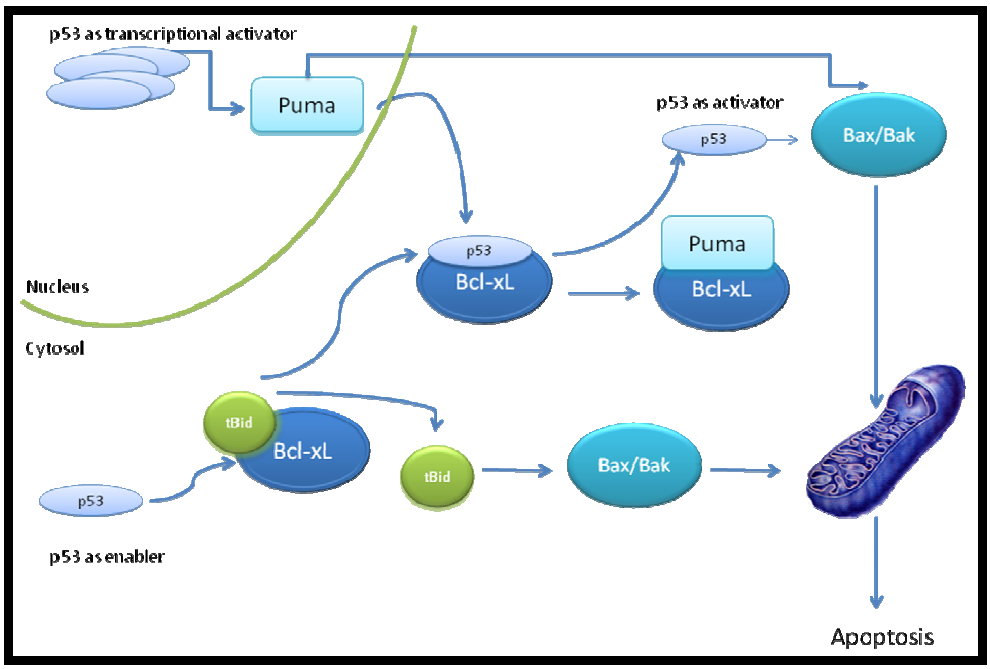

Figure 2.6 The impact of p53 on the mitochondrial pathway in apoptosis. The trancriptional-independent and transciptional-dependent (Puma induction) functions of p53 are depicted (modified from [64]).

2.3.2.3 Functional regulation of BH3-only proteins

There are main three types of regulation for BH3-only proteins at cellular level. First, selective phosphorylation of proteins at different residues might modulate different molecular and cellular responses. One of the example is that Bad phosphorylation on Ser-112, Ser-136 and Ser-155, which leads to the sequestration and inactivation of Bad by 14-3-3 proteins [65]. Moreover, phosphorylation of Ser-170 was shown to decrease pro-apoptotic activity of Bad [66]. The second factor is transcriptional control of BH3-only proteins. Puma and Noxa are transcriptional targets for p53 [67]. Puma is transcriptionally induced by the chemotherapeutics in a p53-dependent or independent fashion (Figure 2.6). Puma is localized to mitochondria where it interacts with Bcl-2 and Bcl-x_L. While the pro-apoptotic effect of Puma depends on Bax multimerization, induction of Noxa did not show any relevance to subcellular localization of Bax. Noxa selectively binds to Bcl-2, Bcl-x_L and Mcl-1. The third regulation mechanism is cleavage. After death signal reception, the full length Bid (22 kDa) is cleaved within its unstructured loop and a 15kDa truncated form of Bid is created, tBid [68]. tBid is selectively transferred to mitochondria and induces oligomerization of Bax and Bak.

2.3.2.4 Pro-apoptotic Bcl-2 proteins trigger apoptosis independently of BH3-domain

Bcl-2 family proteins can interact with each other and also with several other proteins. However, increasing evidence indicates that pro-apoptotic function of Bcl-2 proteins can also be independent of a functional BH3. Bok/Mtd is a structural homolog of Bax and Bak and predominantly detected in brain, liver and lymphoid tissues. While Bok/Mtd can bind Mcl-1 and Bfl-1 (but not Bcl-2 and Bcl-x_L), Bax and Bak can not bind to these anti-apoptotic proteins. Mutational studies showed that BH3-mutant Bok can behave as a pro-apoptotic member [44, 69]. BNIP3 is structurally related to the BH3-only proteins. In normal cell, BNIP3 loosely associates with mitochondria and integrates deeper upon induction of cell death, while its N-terminus remains in cytoplasm. Complete deletion of BH3 could not diminish the killing activity of BNIP3 not as same as other pro-apoptotic members. It act independent from Apaf-1, caspase-

activation or Cyt c release but associated with an opening of the MMP. BNIP3 was shown as a critical for hypoxia-induced autophagy, and its expression and induction of autophagy upon hypoxia was blocked in hypoxia induced factor-1 (HIF-1) deficient cells [44]. Spike is a novel BH3-only protein but it is associated with endoplasmic reticulum instead of mitochondria and showed interaction with Bap31 protein. There is no evidence about its interaction with anti-apoptotic members but induce other pro-apoptotic pathways. Hence it is independent of a BH3- mediated interaction [70].

2.3.2.5 Bcl-2 family members and mitochondria

Mitochondria are also referred as a powerhouse or gatekeeper or gardens of cell death with dual function in survival and death decision. The recent evidence is that passive release of apoptotic molecules into cytosol upon MOMP. Moreover in many proposed scenarios MMP appears to have a better prognostic value for cell demise, than other events such as caspase activation which is induced by any of number of apoptotic insults, including ligation of death domain receptors, DNA-damaging agents, growth factor withdrawal or irradiation. It is not clear whether mitochondria can act as enhancer of caspase activity, or downstream caspases can contribute to Cyt c release or AIF release and loss of MMP.

MMP is determined by two factors: outer membrane stability and inner membrane structure. If inner membrane partakes in outer membrane of mitochondria, a permeability transition pore (PTP) opens following by osmotic flux of water and molecules up to 1.5 kDa. Equilibration of ions between the matrix and cytoplasm occurs. PTP complex consists of adenine nuclear translocator in inner membrane, cyclophilin D in the matrix and VDAC and peripheral benzodiazepin outer membrane (Figure 2.7.). Sustained opening of PTP leads to MMP loss and osmotic swelling of the matrix, often sufficient to distort the structure of cristae and to rupture the outer mitochondrial membrane [63].

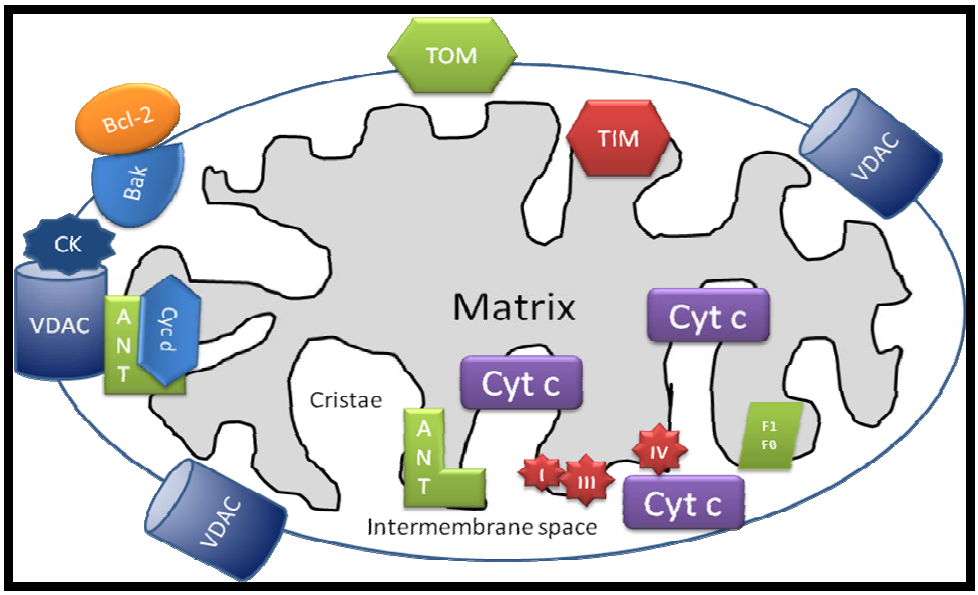

Figure 2.7 Structure of mitochondria. The translocator outer complex (TOM) is localized in the outer membrane and two translocases have been characterized in the inner membrane (TIM). VDAC: Voltage dependent anion channel, CK: creatine kinase, ANT: Adenine nuclear translocator, Cyt c: Cytochrome c, Cyc d: Cycline D.

In spite of the exact process accounting for MOMP, three discernible and not mutually exclusive mechanisms were proposed to understand how cell death process occurs [63]:

1. Release of mitochondria-residing factors that promote caspase-dependent (holocytochrome c, Smac/DIABLO and Omi/HtrA2) or caspase independent (AIF, Endonuclease G and Omi/HtrA2) cell death. This mechanism still is not clear. There are controversial studies whether these proteins are co-released during apoptosis.

2. Loss of mitochondrial functions imperative for cell survival. It is well known that Cyt c hold a fundamental role in respiration, transfer of electrons from complex III to complex IV. Although mitochondrial damage is considered as the point of no return of death machinery, it is an accumulating process and not all or nothing phenomenon. When respiratory functions are disabled during early apoptosis can be overcome upon addition of exogenous Cyt c.

3. Generation of ROS. Mitochondria are the place for generation of ROS which leads lipid peroxidation, calcium mobilization, mitochondrial permeability transition, ATP depletion, protein oxidation, loss of electron transport and/or DNA damages. Their over-existence than required concentration triggers cell defense and the last death mechanism. Contrary, ROS can induce nuclear factor kappa B (NFκB) activation or inhibition of caspases to protect cells.

The mitochondrial pathway is thought to be controlled by the relative balance of opposing Bcl-2 family members and their mutual interactions. However, despite extensive investigation, the precise biochemical mechanisms by which the three subgroups of Bcl-2 family members control mitochondrial apoptosis are still unclear [63]. BH3-only proteins function upstream of the multidomain proteins and act as sensors connecting multiple cytotoxic signals to the core apoptotic pathway. It was shown that Bax and Bak knock-out cells do not undergo MOMP and become resistant to various death stimuli. Mechanistically, Bax and Bak are thought to homooligomerize into pores in the MOM and cause Cyt c and other proteins release into cytosol [30, 63].

In healthy cells, Bak is held in an inactive and it is placed into MOM at monomeric state thorough its association with VDAC-2. However, Bax may lay dormant in the cytosol through interactions with several proteins, including Ku-70, 14-3-3 and humanin peptide [40, 71]. Following apoptotic stimuli, BH3-only protein dependent translocation of Bax is induced and then placed into MOM. Bax and Bak homooligomers are formed here and change the intermembrane space proteins conformation. Bcl-2 and Bcl-x_L are localized on mitochondria and antagonize the functions of Bax, Bak and other BH3-only proteins. It was shown that increased association between Bcl-2 and Bak is observed following binding of tBid to Bak. Hence, it is proposed that Bcl-2 may play a role in regulation of Bax and Bak oligomerization. It is probable that anti-apoptotic proteins regulate mitochondrial apoptosis not only via interaction with BH3-only proteins, but also through heterodimerization with Bax and Bak and prevention of their insertion or/and oligomerization within MOM. Following this action, mitochondria outer membrane permeablized and intermembrane space proteins leak out. However, induction of

intermembrane space proteins occur one step before membrane permeabilization and these proteins differs upon upstream check point signals [40].

Briefly, Bcl-2 family proteins are key regulators of the mitochondrial or intrinsic apoptotic pathway, including or preventing the release of Cyt c, AIF, Smac/DIABLO, Endonuclease G, translocates to the nucleus and causes oligonucleosomal DNA fragmentation, and Omi/HtrA2 that reside in the intermembrane space of mitochondria in healthy cells. Mitochondrial proteins cause caspase-dependent cell death include Cyt c which triggers caspase-9 activation by binding and activating the Apaf-1, and Smac/DIABLO, which potentiates caspase activation by binding IAP and blocking their caspase-inhibitory activity. However, the existence of a redundant molecule or molecules capable of compensating for the loss of Smac function has been suggested by gene deletion studies [62, 63].

Instead of mitochondria, Bax, Bak and Bcl-2 can localize to the ER and can affect ER Ca^{2+} homeostasis and Ca^{2+} uptake by mitochondria which are regulating mitochondrial permeability transition pore formation and intermembrane space protein release. ER is normally an organelle wherein chaperone-assisted polypeptide folding and modification ensure the proteins obtain their mature conformation [30]. If unfolded protein number increases in ER, a conserved alarm system is activated and unfolded protein response (UPR) is triggered. UPR stop protein synthesis and increases ER localized chaperons, which allow the cells for correction. When the damage is severe and proper protein folding is not restored, unfold protein response triggers apoptosis [30]. It was shown that caspase-12 and caspase-4 primarily activated within the ER. Since mitochondria and the ER are interconnected physically and physiologically, it is very likely that apoptotic signaling, when initiated in ER, might relay to mitochondria. When the cells treated with ER stress inducers such as tunicamycin or brefeldin A, Cyt c is released and MMP occurs. These events block Bcl-2 localization in ER. Of note, it was shown that apoptotic response were not observed following in Bax and Bak deficient cells [30]. Bax and Bak deficiency were also characterized impaired Ca^{2+} release [30]. Recently, it is shown that ER-mediated stress activate Bim in two ways: enhanced transcription by CHOP(C/EBP) transcription factor and prevention of Bim ubiquination and degredation by protein phosphatase-2A [72]. This finding indicates

that ER pathway act predominantly upstream of mitochondria rather than through an independent path. However, more evidence is required to prove this fact. Hence, it is proposed that Bcl-2 proteins may activate various pathways in the mitochondria and ER during apoptosis [73].

2.3.2.6 Atypical domain structures of pro-apoptotic Bcl-2 family proteins

2.3.2.6.1 Bcl-rambo

This atypical protein includes all four BH-motifs as well as the TM, but it was referred as pro-apoptotic protein. BH-domains of Bcl-rambo are separated from TM by a unique 250 amino acids long sequence with no relation BH-domains. The pro-apoptotic function of Bcl-rambo is dependent on this sequence which works independently from BH-domains [44].

2.3.2.6.2 Bcl-G

This protein encodes two pro-apoptotic proteins as a result of alternative splicing. The short one Bcl-G_s has similarities with BH3-only proteins, but long form, Bcl-G_L is defined by a novel combination of BH3 with BH2 which is a repressing control domain of this protein. Mutational studies showed that when BH2-domain deleted, pro-apoptotic function of Bcl-G_L is enhanced [44].

2.3.2.6.3 Bfk

This protein also contains BH3 with BH2-domain like Bcl-G_L. It is found in cytosol not on organels. As Bcl-G_L, Bfk does not bind to other Bcl-2 proteins. BH2-domain again suppresses the pro-apoptotic function of BH3-domain of Bfk [74].

2.3.2.7 Alternative splicing of Bcl-2 proteins

Several proteins in Bcl-2 family arise from alternative splicing, and many splice products are still waiting for characterization. There are also identified splicing products such as Bim_s, Bim_L, Bim_{EL} and Bax has eight splice variants with party deviating

domain structures. A well known is Bcl-x gene, which has four identified isoforms with different activities. Bcl-x_L and Bcl-x_{ES} are anti-apoptotic, whereas Bcl-x_S and Bcl-x_{AK} are pro-apoptotic. Bim alpha and gamma were also shown in apoptotic process and still they are investigated to understand relations with other Bcl-2 family members. Recently two variants of Noxa is identified [75].

2.3.2.8 Targetting Bcl-2 Family Members

The *bcl-2* gene and other anti-apoptotic members of Bcl-2 family members are widely expressed in human cancers. Pathogenesis of disease and resistance to chemotherapeutics are linked with their over-expression. Several strategies either to reactivate pro-apoptotic Bcl-2 members or to suppress the anti-apoptotic ones have been developed with the aim of increasing anticancer treatment responses. Thus, modulation of pro-apoptotic members of the Bcl-2 family (mainly Bax and Bid) is becoming therapeutically relevant.

2.3.2.8.1 Gene therapy and antisense oligonuclotides

One current approach is delivery of Bax. Recombinant adenoviruses encoding for Bax are highly toxic, even to healthy cells. Although gene therapy tools need to optimization, Bax adenovirus transfected mice xenografts responded to therapy [76]. However till today, there are still problems with side-effects of gene therapy.

Oblimersen (G3139, GC3139, oblimersen sodium) is an antisense oligonucleotide developed by Genta for systemic use as an injection. It comprises a phosphorothioate backbone linking 18 modified DNA bases. Oblimersen targets the first six codons of Bcl-2 mRNA to form a DNA/RNA complex. The duplex is subsequently recognised as a foreign message and is cleaved enzymatically, thereby destroying the Bcl-2 message. The Bcl-2 protein, which is a potent inhibitor of apoptosis, is overexpressed in many cancers, including follicular lymphomas, breast, colon and prostate cancers, and intermediate-/high-grade lymphomas. By reducing the amount of Bcl-2 protein in cancer cells, oblimersen may enhance the effectiveness of conventional anti-cancer treatments. Genta has reported results from randomised phase III trials of oblimersen in

four different indications: malignant melanoma, chronic lymphocytic leukemia, multiple myeloma and acute myleoid leukemia. However there are some negative opinions for indicated diseases therapy. Therefore drug is not approved for many of them in USA and European countries. Phase I and II trials are also underway or have been completed for a range of other cancer types. Overall in the clinical setting, oblimersen usually has statistically significant results but it has medically unimportant benefit. Therefore, the enthusiasm is diminished for the drug, especially when the side effects are re-considered. Specifically, the unmethylated CpG oligodinucleotides (and/or the phosphorothioate group) activates the immune system, but this potentially important anti-cancer effect is lost when the immune cells undergo premature apoptosis apparently because their Bcl-2 levels have been lowered by the antisense effect of G3139. While this effect on immune cells is usually undesirable, it is exactly what would be useful for activating immune cells, initiating provirus transcription in retrovirus-infected cells, and facilitating selective apoptosis of these infected cells. In general, G3139 might have benefit in clearing chronic infections by intracellular parasites including viruses (HIV, SIV, HTLV, HBV, coronavirus, etc.). Indeed, G3139 has been shown to cause apoptosis in EBV-infected cells leading to clearance of the virus [77].

2.3.2.8.2 RNA interference strategy to down-regulate Bcl-2 protein levels

A recent technology based on RNA interference (RNAi) is selectively and postranscriptionally block homologous gene expression, and it has revolutionized approaches in the study of gene function. RNAi is considered to have begun as an evolutionarily ancient mechanism for protecting organisms from viruses. Many viruses have RNA, rather than DNA, as their genetic material and go through at least one stage in their life cycle in which they make double stranded RNA. Perhaps not surprisingly, all multicellular organisms have evolved a well conserved protein apparatus that destroys double stranded RNA but this has also been found to play a role in maintenance of the organism's own genome stability by suppressing the movement of mobile genetic elements, such as transposons and repetitive sequences [78].

Briefly, explanation of RNAi is that a double stranded RNA (dsRNA) introduced or naturally expressed in a cell and triggers posttranscriptional gene silencing which results in degredation of specific mRNA target [79].

A ribonuclease III, Dicer, is activated on dsRNA and convert it to short interfering RNA (siRNA). siRNA molecules are generally 21-27 nucleotides long. RNA inducing silencing complex (RISC) cleaves target mRNA which has a complementary region to the siRNA sequence. Each sequence has a 5' phosphate group and a 3'hydroxyl group. Although systems can produce siRNA molecules *in vivo*, these molecules can be also exogenously introduced into cells by various transfection method to bring about the specific knock-down of a gene interest. However, artificially introduction of specific siRNAs into cells can be problematic due to transient knock-down efficiency. One way of overcoming this challenge is to modify the siRNA in such a way as to allow it to be expressed by an appropriate vector. This is done by the introduction of a loop between the two strands, thus producing a single transcript, which can be processed into a functional siRNA [79].

However, applying RNAi *via* siRNAs to living animals, especially humans, poses many challenges. Experimentally, siRNAs show different effectiveness in different cell types in a manner as yet poorly understood: some cells respond well to siRNAs and show a robust knockdown, whereas others show no such knockdown (even despite efficient transfection).

Given the ability to knock down essentially any gene of interest, RNAi *via* siRNAs has generated a great deal of interest in both basic and applied biology. There are an increasing number of large-scale RNAi screens that are designed to identify the important genes in various biological pathways. Because disease processes also depend on the activity of multiple genes, it is expected that in some situations turning off the activity of a gene with an siRNA could produce a therapeutic benefit.

Further studies demonstrated that Bcl-2 siRNA enhances the cytotoxic and apoptotic effect of conventional chemotherapeutics such as etoposide and doxorubicine

in MCF-7 breast cancer cells. *In vivo* studies showed that following Bcl-2 siRNA treatment, tumor volume and growth were diminished.

Recently in addition to siRNA, RNAi can also involve small RNA-mediated gene silencing *via* microRNA (miRNA). Contrary to siRNA, miRNAs are coded by endogeneous genes. *miR-15* and *miR-16* were shown to down-regulate Bcl-2 and triggered apoptosis. Interestingly, these miRNAs were frequently deleted or down-regulated in chronic lymphoid leukemia. Therefore, extend research would be helpful to understand their functional properties in apoptosis machinery with other genes which are directly or indirectly involved in the control of apoptosis [80].

2.3.2.8.3 Synthetic BH3-mimetics or peptide-based Bcl-2 targetting

According to the Bcl-2 activation models, BH3-only members of the Bcl-2 family bind to anti-apoptotic members, counteracting their protective effect by freeing multidomain proapoptotic members Bax and Bak. Therefore BH3-only proteins have become an attractive strategy for the development of new anti-cancer therapies. Their small structure (14-24 amino acids) can be easily synthesized as peptides or pharmacological active small molecules.

It is apparent that overexpression of BH3-only molecules induce apoptosis. Recently it was shown that soluble BH3-peptides produce Cyt c release from isolated mitochondria [22, 81]. Their enhanced stability and easy entrance to cells is promising for potential use in cancer therapy as therapeutic tools. For example, stapled BH3-peptides with enhanced stability like BID-SAHB peptide which is derived form of Bid enhance apoptosis in Jurkat cells and inhibit xenograft growth in leukemia models. A Bad-BH3 peptide with a high cell permeable moiety induces apoptosis in HL-60 leukemia cells in a caspase dependent way and delayed growth of myeloid leukemia in mice models. These findings show that BH3-peptides are promising in cancer therapy models [82].

2.3.2.8.3.1 ABT-737

Computer analysis has lead to discovery of BH3-mimicking small compounds which bind to the hydrophobic groove in the surface of anti-apoptotic proteins. One of leading compound is ABT-737 (Abbott Laboratories, USA) was identified using nuclear magnetic resonance (NMR)-based screening tool. ABT-737 potently inhibits the Bcl-2, Bcl-x_L and Bcl-w but not Mcl-1 or A1/Bfl-1 [83]. It was shown that ABT-737 is a functional Bad-BH3-mimetic but it is not structural similar to Bad-BH3-domain. Therefore its anti-apoptotic function is not sufficient but it has synergistic potential with other drugs. It is worthy to underline that ABT-737 is not efficient in normal cells but enhance the apoptotic effect of other drugs in malign cells [81, 83].

2.3.2.8.3.2 Obatoclax (GX15-070)

Obatoclax is a BH3-mimetic and a derivative of indol bipyrrol (Gemin-X Biotechnologies, Canada). In contrast to ABT-737, obatoclax seems to be a pan Bcl-2 inhibitor that which is a target of Mcl-1. It has high efficiency in hematological neoplasmas, breast cancer and even in cells that are resistant to melphalan, ABT-737 or bortezomib. Recently, it is being tested in Phase I clinical trials, alone or in combination with bortezomib, docetaxel, topotecan or rituximab in patients with relapsed or refractory mantle cell lymphoma, chronic lymphocytic leukemia, non-small lung cancer and other solid tumors [84, 85]. There are also Phase II trials in patients with Hodgkin's lymphoma, myelofibrosis with myeloid metaplasia, myelodysplasia and follicular lymphoma. Obatoclax can also distupt the Mcl-1/Bak interaction [84, 85]. However there is no structural proof of obatoclax binding in the hydrophobic groove of anti-apoptotic Bcl-2 family proteins [53].

2.3.2.8.3.3 HA14-1

Ethyl 2-amino-6-cyclopentyl-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromone-3-carboxylate (HA14-1), the first reported small molecule antagonist for Bcl-2 protein, was identified by Wang et al. [82]. This simple chemical structure is a putative Bcl-2 inhibitor, which was identified from *in silico* screens [86]. HA14-1 disrupts the binding

interaction of the Bak BH3 domain peptide with Bcl-2 and Bcl-x_L proteins [82] strongly inhibits the Bcl-2/Bax interaction [87] and prevents the interaction between Bcl-2 and the BH3-only protein Bim [88]. Since its discovery, HA14-1 has been shown to enhance the cytotoxic effects of variety of anticancer agents (Figure 2.8).

HA14-1 involves changes in Ca^{2+} homeostasis, inhibition of mitochondrial potential, Bax translocation, reactive oxygen species generation (ROS), cytochrome c release and caspase-9/-3 activation, and subsequently poly(ADP-ribose) polymerase (PARP) cleavage [82, 89, 90]. The therapeutic effect of HA14-1 has been described in a variety of tumor cells. HA14-1 cooperates with other drugs such as flavopiridol, bortezomib, dexamethasone, doxorubicin and cytrabine [91, 92]. Additionally, HA14-1 could potentiate non-toxic MAPK inhibitors as lethal agents in the cell culture environment [93]. Although these results indentify HA14-1 as a good candidate for anti-cancer treatment, some concerns have been recently raised about this compound [94]. First, it is very unstable and decomposes very rapidly under physiological conditions. Second, although the biological activity of HA14-1 was supposed to depend on its interaction with Bcl-2, recent findings show that ROS generation could be main reason of cell death induced by this compound [53].

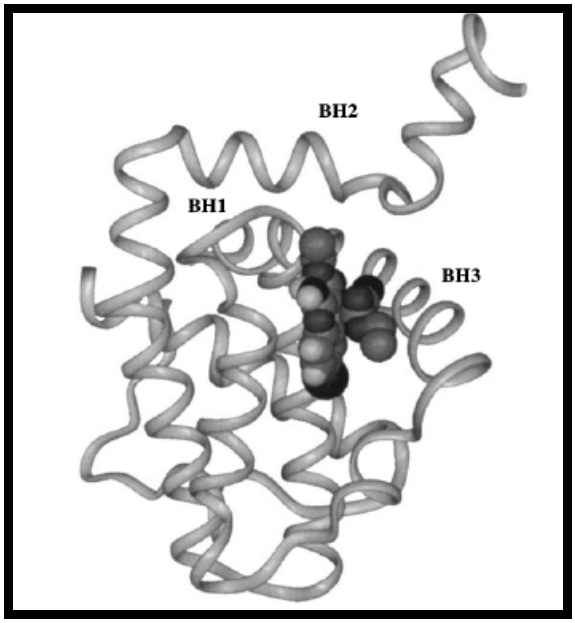

Figure 2.8 The structure of HA14-1 bound to the surface pocket of Bcl-2 as predicted by computer docking studies. Adopted from [86].

2.3.2.8.3.4 TW-37

Recently, a small molecule pleiotropic BH3-mimetic, TW-37 was designed with high affinity to Mcl-1 in addition to Bcl-2 and Bcl-x_L. Since TW-37 discovery, there was no effective synthetic inhibitor for Mcl-1. TW-37 and a MEK inhibitor synergistically killed aggressive melanoma cells with minimal toxicity for normal skin cells [95]. In addition, preclinical studies suggest that the combination of TW-37 and cyclophosphamide-doxorubicin-vincristine-prednisone may be promising in the treatment of diffuse large B cell lymphoma [96].

2.3 Reactive oxygen species and mitochondria

Mitochondria are an important source of ROS within most mammalian cells. This event contributes to mitochondrial damage in a range of pathologies and is also important in redox signalling from the organelle to the rest of the cell [97]. Recent knowledge about how mitochondria produce ROS is vital to understand a range of currently important biological and biomedial topics. The first report about ROS production due to respiration at mitochondria was shown in 1966 [98]. Following this discovery, Chance and colleagues showed that isolated mitochondria produce H_2O_2 [99]. Later, it was confirmed that this H_2O_2 arose from the dismutation of superoxide ($O_2^{\bullet-}$) generated within mitochondria . The parallel discovery that mitochondria contain their own superoxide dismutase, MnSOD, confirmed the biological significance of mitochondrial $O_2^{\bullet-}$ production [100]. Within mitochondria, $O_2^{\bullet-}$ is produced by the one-electron reduction of O2. Therefore it is the kinetic and thermodynamic factors underlying the interaction of potential one-electron donors with O2 that control mitochondrial ROS production [97].

Intracellular generation of ROS *per se* is an inevitable (and sometimes physiologically important) process. To counter it, mitochondria, and cells in general, possess numerous ROS defense systems. It should have been implicit that the true source of oxidative stress is not the ROS generation *per se* but spatiotemporal

imbalance of ROS production and detoxification, and, yet, until recently, even the capacity of mitochondrial ROS defence was unknown.

In fact ROS are not all accidental and unwanted byproducts. This cellular process orchestrate many critical events in the cells. One of the main event is the modulation of cell death decision. Mitochondria is one of the central place which regulate cell death and survival decision under the control of Bcl-2 family members. Excessive production of ROS is a typical sign of apoptosis. Thus, numerous studies have documented cellular changes resulting from oxidative stress induced in cells following exposure to cytotoxic drugs. Although cytotoxic agents such as doxorubicin, daunorubicin, mitoxantrone, bleomycin and cisplatin used in these studies were structurally dissimilar and acted on different cellular targets, they induced oxidative stress by generation of ROS in tumour cells. Formation of ROS increased the cytotoxic activity of the drugs in cancer cells via inducing apoptosis [101].

The sustained mitochondrial inhibition due to apoptotic induction would also potentiate ROS formation, leading to the depletion of antioxidant defenses (thiols such as gluthation) and initiation of lipid peroxidation. Lipid peroxidation could cause structural damage to membranes, including those which form mitochondria, and potentiate their dysfunction. This sequential process can modulate several proteins and structures to finalize cell death decision. H_2O_2 generation which triggers ROS generation and lipid peroxidation was contributed to apoptosis induced by physiological concentrations of several drugs. Therefore, there are a number of studies supporting the hypothesis oxidative stress might modulate the cytotoxicity of several cytotoxic agents. However, the mechanisms by which ROS mediate the apoptotic effect of drugs and the identity of specific ROS involved in the process are unclear [101]. For example, the role of specific ROS and antioxidant systems in the regulation of Bcl-2 and apoptosis induced by drugs are need to be clarified. Previous studies have shown that O_2^- plays a role in Bcl-2 regulation. Downregulation of superoxide dismutase by antisense gene inhibition decreases Bcl-2 expression and increases cell death induced by cisplatin [102]. Since superoxide dismutase acts by dismutation of $O_2\bullet^-$ to form H_2O_2 and since H_2O_2 is a major source of $OH^\bullet$ production, i.e., *via* Fenton-like reactions, it is possible that other oxidative species may play a role [103]. It was proposed that H_2O_2 may be a common regulator of Bcl-2 expression under diverse pathologic and treatment conditions. Since aberrant expression of Bcl-2 has been associated with several human

cancers, our findings on the regulatory mechanism of Bcl-2 by ROS could be important in the understanding of carcinogenesis and in the development of novel therapeutic strategies that could overcome cancer chemoresistance.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, media components and antibodies

Chemicals, media components and antibodies that are used are listed in Appendix A.

3.1.2 Molecular biology kits

Molecular biology kits which are used for cell viability determination, apoptosis screening, gene transfection, RNAi transfection, and protein analysis are listed in Appendix B. Other special materials including DNA, RNA and protein markers are indicated in Appendix C.

3.1.3 Equipment

Equipment that is used for general laboratory purposes are listed in Appendix D.

3.1.4 Buffers and solutions

Standard buffers and solutions used in cloning and molecular manipulations were prepared according to the protocols in *Molecular Cloning: A Laboratory Manual* [104].

3.1.4.1 Buffers for nucleic acid isolation

Buffer A for agarose gel electrophoresis

1X Tris EDTA acetate (TAE) buffer was used for preparation of 1.5 % agarose gels. Gels were run at 100 mV for 30 minutes. DNA was visualized by including 0.005 % ethidium bromide.

Buffer B for agarose gel electrophoresis

1X Tris Borate EDTA (TBE) buffer was used for 2 % agarose gel electrophoresis. Gels were run at 80 mV for 60 minutes. RNA was visualized by including 0.005 % ethidium bromide.

3.1.4.2 NP-40 lysis buffer for M30 Apoptosense ELISA assay

150 mM NaCl, 1 % NP-40, 50 mM Tris-HCl were dissolved in ddH_2O. pH was adjusted at 8.0, and buffer was stored at -20^oC.

3.1.4.3 Buffers for immunoblotting

10 X PBS (Phosphate Buffered Saline)

80 g NaCl, 2.25 g KCl, 23.27 g $Na_2HPO_4.12H_2O$ and 2.05 g KH_2PO_4 were dissolved in 1000 ml ddH_2O of and pH was adjusted to 7.4.

10 X TBS (Tris Buffered Saline)

87.7 g NaCl, 12,1 g

Tris, 4 ml HCl were dissolved in 1000 ml ddH_2O of and pH was adjusted to 7.4.

1 X TBS-Tween-20

10 X TBS was diluted, 0,2 % Tween-20 was added and pH was adjusted to 7.4.

1 X PBS-Tween-20

10 X PBS was diluted, 0.2 % Tween-20 was added and pH was adjusted to 7.4

10 X Running Buffer

250 mM Tris base, 1.92 M glycine and 1 % (w/v) SDS were dissolved in 500 ml ddH_2O and pH was adjusted to 8.5.

Buffer for SDS polyacrylamide gel electrophoresis

1 X running buffer was prepared from 10 X stock solution and was used for polyacrylamide gel electrophoresis. Gels were run at constant voltage 75 mV, for about 1.5 h.

4 X Tris-Cl/SDS pH 6.8

0.25 M Tris and 0.2 % SDS (w/v) were dissolved in 50 ml ddH_2O. pH was adjusted to 6.8

4 X Tris-Cl/SDS pH 8.8

0.75 M Tris and 0.2 % SDS (w/v) were dissolved in 50 ml ddH_2O. pH was adjusted to 8.8.

10 X Transfer Buffer

0.25 M Tris Base and 1.92 M glycine was dissolved in 500 ml of ddH_2O.

3.1.4.4 Buffers for transfer of proteins into PVDF membrane

1 X transfer buffer was prepared by dilution from 10 X stock solution and 20 % methanol addition prior to the blotting of the proteins into PVDF membrane. The membranes were blocked with blocking solution, 5 % milk powder in 1X P(T)BS-Tween-20 and washed with washing buffer 1X P(T)BS and 1X P(T)BS- Tween-20. The antibodies were diluted in 5 % milk diluent in 1X P(T)BS- Tween-20.

3.1.4.5 Stripping Buffer

62.5 mM Tris-HCl and 2 % SDS (w/v) were dissolved in 500 ml ddH_2O and pH was adjusted to 6.7. Prior to use 352.1 µl of β-mercaptoethanol was added for each 50 ml of solution.

3.1.5 Growth media

Mammalian breast cancer cell lines; MCF-7 (HTB-22) and MDA-MB-231 (HTB-26) were cultured in RPMI 1640 supplemented with 10 % heat inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 UI/ml penicillin and streptomycin. Cultures were maintained in 37°C in a humidified 5% CO_2 atmosphere. Bcl-2 plasmid inserted MCF-7 and MDA-MB-231 cell line were maintained in the same medium formula supplemented with antibiotic G418.

3.1.6 Freezing media

The cells were kept in freezing medium which containing FBS and 10 % di methyl sulfooxide (DMSO) at -80°C for 24 h and then kept in liquid nitrogen.

3.1.7 Plasmids and primers

Bcl-2 plasmid was provided by Dr. O. Kutuk (Addgene plasmid 8768) and used for stable transfection (Figure 3.1)

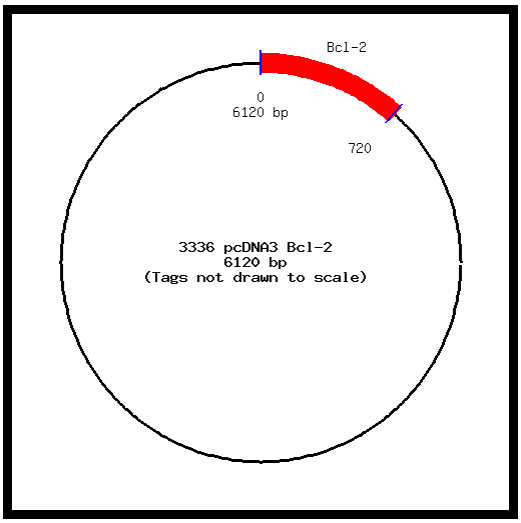

Figure 3.1 Bcl-2 pCI DNA3 plasmid vector map. ORF contains SacI, PstI and BamHI sites for T7 direction [105].

Bcl-2 (Qiagen, Gene globe, QT00025011), Bax (Qiagen, Gene globe, QT00031192), Bcl-x_L (Qiagen, Gene globe, QT00236712), Puma (Qiagen, Gene globe, QT00082859) and Noxa (Qiagen, Gene globe, QT01006138) were primers sets. GAPDH (Qiagen, Gene globe, QT01192646), 18s RNA (Qiagen, Gene globe, QT00199367) and β-actin (Gene globe QT00199367) were used as internal standard gene primer sequences.

3.2 Methods

All methods used in this study are explained below;

3.2.1 Cell culture

MCF-7 (HTB-22) and MDA-MB-231 (HTB-26) breast cancer cells were obtained from American type of cell collections (ATCC, LGCpromochem, UK). The Bcl-2 plasmid transfected stable MCF-7 cell line was obtained from Dr. Ozgur Kutuk.

When cells were grown to confluency, subculturing to a new passage numbers was done with filter-sterilized 1X trypsin-EDTA solution. As soon as cells were

detached in 37°C CO_2 incubator, medium containing serum is added to inhibit further trypsin activity that might be harmful for cells. Cells were counted with a haematocytometer by the following formula:

$$cells/ml = average\ count\ per\ square\ X\ dilution\ factor\ X\ 10^4$$

MCF-7 and MDA-MB-231 breast cancer cell lines were splitted in every 2-3 days and equal volumes of each cell line were passaged to new flask or petri dishes in appropriate seeding densities. Cells were seeded in 96 well plates ($1x10^4$ cells/well), 6 well plates ($4x10^5$ cells/well) and treated as indicated in the experimental protocols. Bcl-2 plasmid inserted MCF-7 and MDA-MB-231 cell lines were splitted in every 5-7 days by the same procedure as indicated above.

For the preparation of frozen stocks, each cell line at log phase growth (50-75 % confluency), was trypsinized, spinned down at 300 ***g*** for 5 min and washed with sterile 1X PBS. Again cells were spinned down at 300 ***g*** for 5 min and were transferred into cryovials. Cryopreservation of cells should be slowly freeze starting from 1 h at -20°C and then frozen at -80°C for 24 h and then kept in liquid nitrogen to remain until thawing. After thawing the cells were immediately washed with growth medium to get rid of DMSO

3.2.2. Cell viability assay

Cell viability was determined using MTT Proliferation kit (Roche, Germany) which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. Each cell line was seeded into 96-well plates ($1x10^4$ cells/well) and treated as indicated experimental scheme. 10 µl MTT labeling reagent was added to each well, after which the plates were incubated for 4 h. The cells were then incubated in 100 µl of the solubilization solution for overnight, and the absorbance was measured with a microtiter plate reader (Bio-Rad, USA) at a test wavelength of 550 nm and a reference wavelength of 650 nm.

$$Percent\ (\%)\ viability = OD\ of\ drug\text{-}treated\ sample/control\ X\ 100$$

3.2.3 M30 Apoptosense ELISA assay

Cytokeratin-18 (CK18) cleavage indicates the activation of caspases and was measured using reagents from Peviva AB. MCF-7 and MDA-MB-231 breast cancer cells ($1x10^4$ cell/well) were seeded on 96-well plates. A solution of 10 % NP-40 was added and mixed for 5 min at room temperature. Two replicates of 25 μl cell lysate/medium were transferred into a specific M30 Apoptosense ELISA assay. This assay was performed according to the manufacturer's instructions. After the washing step, stop solution was added to each well, and samples were incubated in the dark for 5 min. The spectrophotometric measurement was made at 450 nm. Absorbance data was converted to U/L according to standart curve plot analysis.

3.2.4 Mitochondria membrane potential loss

Mitochondria membrane potential loss was measured using rhodamine 123, a green fluorescent dye that accumulates in active mitochondria with high membrane potential. $4x10^5$ cells were seeded into 6 well-plates. Following drug treatment, washed with 1 X PBS twice times. Cells were loaded 5 mM rhodamine 123 in 1X PBS for 30 min at 37^{o}C. Then the cultures were washed three times with 1X PBS. Rhodamine 123 was excited at 488 nm and fluorescence emission at 525 nm was recorded. Samples were also investigated by fluoresence microscopy (Olympus IX70) at green filter [106].

3.2.5 Colorimetric caspase-9 activity determination

Caspase-9 activity was determined using ApoTarget Caspase Colorimetric Protease Assay Sampler Kit (Invitrogen, KHZ1001). $4x10^5$ cells were seeded into 6-well plates. Following drug treatment, cells lysate were mixed with 50 μl cell lysis buffer, supplied in kit. Samples were kept on ice for 10 min. Protein content was determined by Bradford Assay [107]. Each sample diluted with cell lysis buffer to obtain 2 mg/ml protein concentration. Each sample mixed equal volume of reaction buffer 2 X. 4 mM caspase-9 subtrate (final concentration 200 μM) was added into sample and mixed at 37^{o}C for 2 h. Samples were transferred to microtiter plates and

read at 405 nm absorbance. Caspase-9 activity was determined fold comparison of treated samples to untreated sample.

3.2.6 RNA isolation

Total RNA content was isolated using a TRIzol reagent (Invitrogen) following the procedure described by the manufacturer. Total RNA was digested with RNase-free DNase (Boehringer Mannheim, Indianapolis, USA) for 15 min at 37^{o}C.

3.2.7 Reverse transcriptase (RT) reaction

Reverse transcription (RT) was performed using a specific RT kit (Qiagen, Germany). The RT reaction mixture contained 1µl of total RNA, 500 ng of oligo(dT) primer, 5 X RT reaction buffer, 10 mM dNTPs, and 200 U of a reverse transcriptase in a total volume of 20 µl. The all samples were incubated in 37°C for 1.5 h. The quantity of cDNA was calculated using spectrophotmetry by determination of optical density at 260 nm (OD260). Purity was calculated using OD260/280 ratio.

3.2.8 RT-polymerase chain reaction

Real-time PCR was performed in 96-well 0.2 ml thinwall PCR plates (Bio-Rad, USA) using the iCycler Thermal Cycler (Bio-Rad, USA) and carried out with QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany), which contained HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, and SYBR Green I. The real-time PCR reaction mixture contained 1 X QuantiTect SYBR Green PCR Master Mix, 0.3 mM primer pairs, and 500 ng cDNA in a total volume of 25 µl. The mixture was heated initially at 95°C for 15 min in order to activate HotStarTaq DNA Polymerase and then followed by 40 cycles with denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min. Furthermore, the number of amplified products was identified by melt curve analysis. The melt curve protocols designed for increment temperatures of 0.5°C with a starting temperature of 54°C and ending at 90°C were repeated to ensure that primer dimers and other nonspecific products had been minimized or eliminated.

3.2.9 Data expression

Standard curves constructed from serial dilution of a known number of PCR product molecules were analyzed with the iCycler iQ Real-Time PCR Detection System (Bio-Rad, USA). The baseline was set automatically by the software using data collected from cycles 2 to 10, and the threshold cycle (Ct) at which the amplification plot crossed the baseline was calculated. The raw abundance values for each gene was divided by the standard housekeeping genes (18sRNA/GAPDH/β-actin) values obtained from the same samples to derive a normalized value for each sample. In preliminary experiments, standard housekeeping gene mRNA levels within a sample were constant between individual treated study materials

3.2.10 Transfection of siRNA

MCF-7 and MDA-MB-231 cells were placed in a six well plate 24 h prior to transfection. Approximately 4 x 10^5 cells/well were transfected with 20 nM Hs_Bcl-2_9_siRNA (Gene Globe, Qiagen) in 6-well plates. According to manufacturer instructions 1:9 and 1:6 siRNA-transfection reagent complex were used in MCF-7 and MDA-MB-231 cells, respectively. After 48 h incubation, the silencing effect was checked with qRT-PCR and western blot analysis [108].

3.2.11 Plasmid isolation

Plasmid DNA was transformed to competent cells (*E. coli*, DHα) and plasmid DNA was prepared with MidiPrep kit. Starting culture with 2 ml LB broth containing antibiotic ampicillin (100 mg/ml) was inoculated with a single transformant colony. After growth at 37ºC for 8 h with vigorous shaking (250-270 rpm), 1/500 of this culture was inoculated into a larger culture (50 ml) and continued grow for 12-16 h at 37ºC with vigorous shaking. According to manufacturer instructions, plasmid DNA was prepared (MidiPrep, Qiagen). The quality and quantity / concentration of the plasmid DNA were checked by agarose gel electrophoresis (1 % agarose gel 1 X TAE) and spectrophotometry (absorbance at 260 nm with $A_{260/280}$ ratio close to 1.8) [104]

3.2.12 Stable transfection of Bcl-2

For stable transfection of MDA-MB-231, cells were transfected with pcDNA3 Bcl-2 plasmid (Addgene plasmid 8768) using Fugene HD (Roche) and clonal selection was carried out using G418 (Sigma). Antibiotic concentration was gradually increased and resistant each single clone was seeded in 96-well plates. Selected clones were verified by qRT-PCR and immunoblotting analysis of Bcl-2 and maintained in growth medium with 700 μg/ml G418 [105].

3.2.13 Total Protein Isolation

Each cell line was treated with appropriate drugs. First, all samples were washed with ice-cold 1 X PBS and lysed on ice in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, NP-40 0.5 %, (v/v), 1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, protease inhibitor cocktail (Complete from Roche, Germany). After cell lysis cell debris was removed by centrifugation 15 min at 13200 rpm [104].

3.2.14 Determination of Protein Concentration

Protein concentrations in nuclear and total protein extracts were determined by Bradford reagent (Biorad). Bovine serum albumin (BSA) was as the standart protein. 0, 2, 4, 6, 8, 10 μl's of 1 mg/ml BSA were taken which were corresponded to 0, 2, 4, 6, 8, 10 μg/ml proteins, respectively. 1 μl of each unknown protein extract was completed to 10 μl with distilled H_2O. After then 290 μl (1:5) diluted Bradford reagent according to manufacturer instructions was added. All samples were kept at dark for 5 min. Protein concentrations were determined by spectrophotometrically at 595 nm absorbance. The standard curve was generated from A_{595} values of BSA protein standards (*y-axis*) versus protein amounts of BSA (*x-axis*). It is essential the curve is linear with correlation coefficient (R^2 value). Protein concentrations of unknown samples were generated from standard curve graph. For every new assay, standard curve is repeated [107].

3.2.15 Immunoblotting

Total protein lysates (30 μg) were separated on a 12 % SDS-PAGE and transferred onto PVDF membranes. The membranes were then blocked with 5 % milk blocking solution in TBS-Tween-20 and incubated with appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology) in antibody buffer containing 5 % (w/v) milk blocking solution. After washes with TBS-Tween-20, proteins were analyzed using an enhanced chemiluminescence detection system (ECL or ECL-Advance, Amersham Pharmacia Biotech) and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech).

3.2.16 Detection of reactive oxygen species

For assessment of ROS produced by different drugs in the cell cytotoxicity experiment, $2x10^5$ cells were grown on 12 well plates and the 2'-7' dichlorofluorescein (DCF) assay was used. Following drug treatment, MCF-7, MDA-MB-231, MCF-7 $^{Bcl\text{-}2+}$ and MDA-MB-231$^{Bcl\text{-}2+}$ cells were washed with 1 X PBS and DCF-DA (5 μg/ml) in PBS was added into each well. After 30 min, PBS buffer containing excess DCF was removed from the cells and replaced with fresh PBS. Fluorescent intensity was measured at 485 nm excitation and 520 nm emission on a Spectramax Gemini Fluorometer plate reader [109]. Samples were also analyzed by flurosent microscopy (Olympus IX70) [109].

3.2.17 Detection of lipid peroxidation

The extent of lipid peroxidation in control and drug exposed samples was determined by measuring the thiobarbituric acid reactive substance (TBARS). TBARS were determined according to the procedures of Okhawa et al. with minor modifications [110]. At the end of the experimental procedure, $2x10^5$ cells were washed with 1 X PBS and scraped. After then, 10 % SDS was added for solubilization. This was followed by 650 μl of 0.5 % thiobarbituric acid in 20 % (v/v) glacial acetic acid (pH 3.5) were added. All samples were incubated at 80°C for 30 min and cooled to room temperature before absorbance read at 532 nm spectophotometrically on microplate titer (Biorad).

3.2.18 Statistical analysis

All samples were evaluated statistically using an Excel calculation file. MTT cell viability, M30 Apoptosense ELISA assay, caspase-9 activity and relative expression of mRNA was shown as Mean ± Standard Deviation, and the student's t-test was applied to understand the probability efficiency. Differences were regarded as statistically significant at values of $p < 0.05$.

CHAPTER 4

4 RESULTS

4.1 Cisplatin and paclitaxel induces apoptosis

MCF-7 (p53 wt) and MDA-MB-231 (p53 mt) breast cancer cells which are highly metastatic were studied to investigate whether cisplatin or paclitaxel decreased cell viability through the induction of apoptosis.

To assess the cytotoxic effect of cisplatin or paclitaxel, MCF-7 and MDA-MB-231 cells were treated with each agent at various concentrations for 24 h. Cell viability was determined by MTT Cell Proliferation Assay. Since moderate cytotoxic effects were observed at 30 μM cisplatin and 20 nM paclitaxel (30 % and 15 % decrease in cell viability in MCF-7 cells and 29 % and 23 % decrease in cell viability in MDA-MB-231 cells, respectively) (Figure 4.1-2), these concentrations were determined to be used in further experiments. The higher concentrations of cisplatin and paclitaxel were found more than 50 % cytotoxic for both cell lines.

During apoptosis, caspases cleave various cellular proteins. In epithelial cells, one of those substrates is the intermediate filament protein CK18. Cleavage of CK18 results in collapse of the cytokeratin filaments into large aggregates. The M30 antibody detects a neo-epitope region which is exposed after cleavage of CK18 by caspases after

aspartic acid residue 396 (CK18Asp396). To confirm that the decrease in cell viability was indeed due to apoptosis, we performed the M30 Apoptosense ELISA Assay.

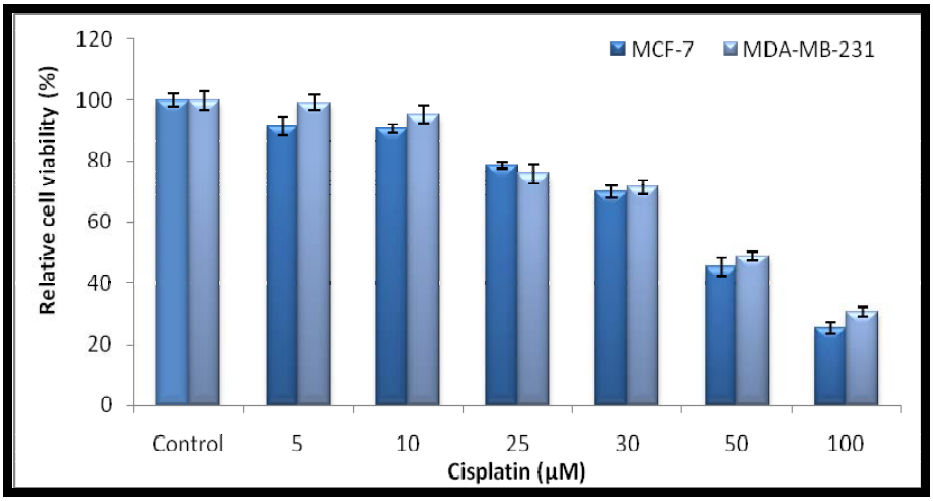

Figure 4.1 The cytotoxic effect of cisplatin was determined by MTT cell viability assay following exposure of MCF-7 and MDA-MB-231 cells to cisplatin for 24 h. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=3), each comprising six replicates. *$P < 0.05$.

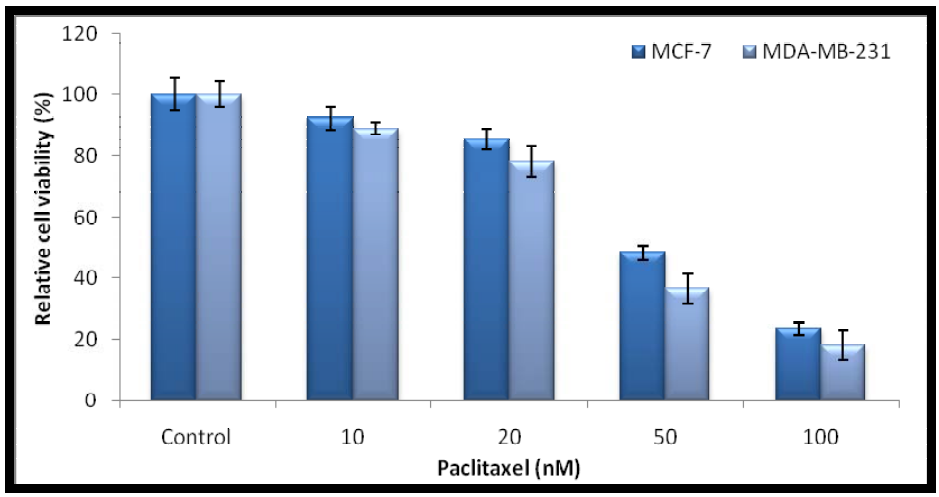

Figure 4.2 The cytotoxic effect of paclitaxel was determined by MTT cell viability assay following exposure of MCF-7 and MDA-MB-231 cells to paclitaxel for 24 h. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=3), each comprising six replicates. *$P < 0.05$.

30 μM cisplatin increased M30 antigen levels by 2.5 fold and 1.9 fold in MCF-7 and MDA-MB-231 cells, respectively (Figure 4.3). In addition, 20 nM paclitaxel increased M30 antigen levels by 1.6 and 2.0 fold compared to control sample in both cell line (drug *vs* control ** P <0.01).

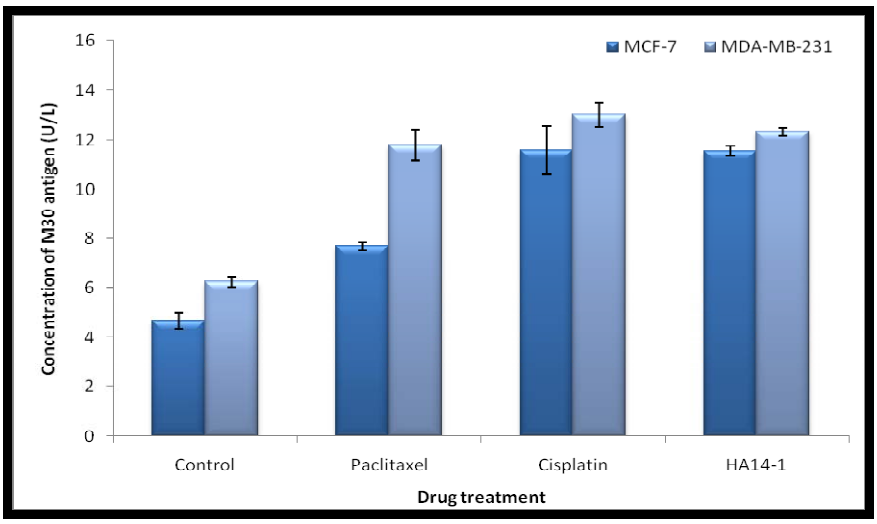

Figure 4.3 Apoptotic cell death was determined by M30 Apoptosense ELISA assay. MCF-7 and MDA-MB-231 breast cancer cells were grown up on 96 well plates and treated with 20 nM paclitaxel,30 μM cisplatin and 10 μM HA14-1 for 24 h. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=2), each comprising four replicates. **P < 0.01.

4.2 Bcl-2 siRNA enhances drug-induced apoptosis in breast cancer cells

The efficiency of Bcl-2 siRNA on Bcl-2 protein levels was assessed at 24 h and 48 h following transfection. Transfection of Bcl-2 siRNA markedly down-regulated Bcl-2 within 48 h. We did not detect any effect of scramble siRNA on Bcl-2 protein levels in both cell lines. As shown Figure 4.4 and 4.5, Bcl-2 siRNA transfection decreased Bcl-2 mRNA or protein levels. Transfection ratio was determined as 1:9 and 1:6 for siRNA:transfection reagent complex in MCF-7 and MDA-MB-231 cells, respectively. The lower transfection ratios were not sufficient to down-regulate Bcl-2 levels in both cell lines.

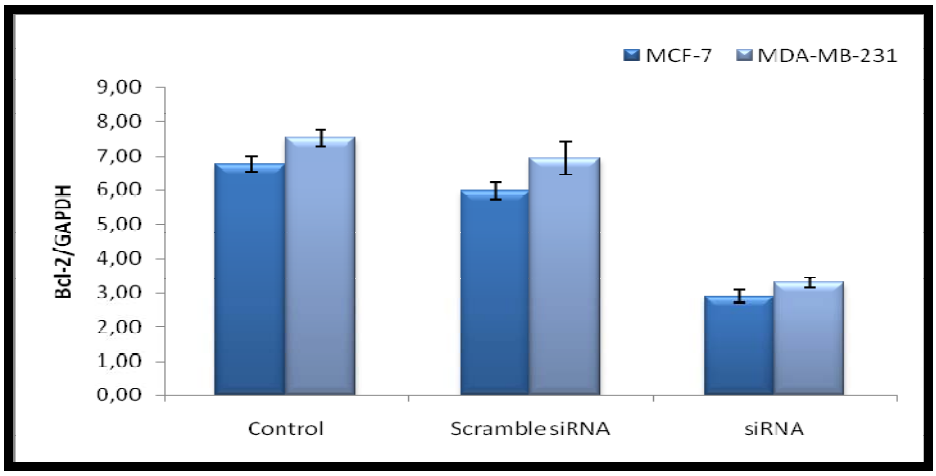

Figure 4.4 Relative mRNA copy numbers were determined by quantitative real time PCR assay following transfection of MCF-7 and MDA-MB-231 cells with Bcl-2 siRNA and scramble siRNA for 48 h. Bcl-2 mRNA copy number was calculated using standard curve of GAPDH. Represented values are normalized by dividing with GAPDH. Columns represent mean (±SEM) of independent two experiments (N=3), each comprising six replicates.

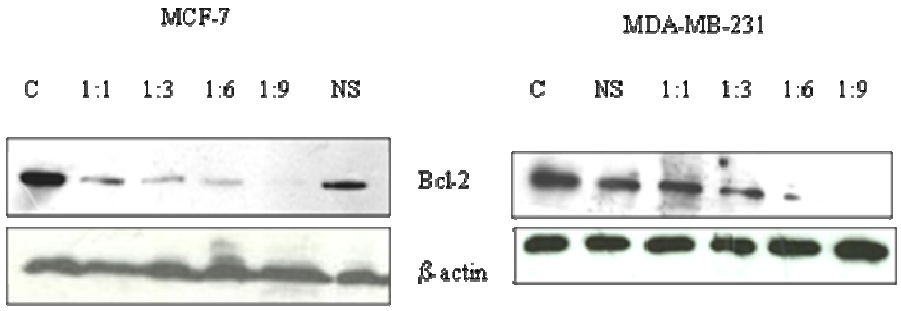

Figure 4.5 Silencing of Bcl-2 by siRNA enhanced drug induced apoptosis. Bcl-2 siRNA silencing effect and efficient transfection reagent complex ratio was determined by western blotting. NS is scramble siRNA.

To investigate if treatment with Bcl-2 siRNA itself had any effect on cell viability, we performed the MTT cell viability assay after 48 h siRNA transfection. The results here (Figure 4.6) showed that Bcl-2 siRNA did not decrease cell viability. When transfection of Bcl-2 siRNA was followed by treatment with cisplatin, cell viability further reduced by 52 % and 57 % in MCF-7 and MDA-MB-231 cells, compared to treatment with scramble siRNA and cisplatin, respectively. Similarly, transfection of

Bcl-2 siRNA followed by treatment with paclitaxel, reduced cell viability by 43 % and 65 % in both cell lines, compared to treatment with scramble siRNA and paclitaxel.

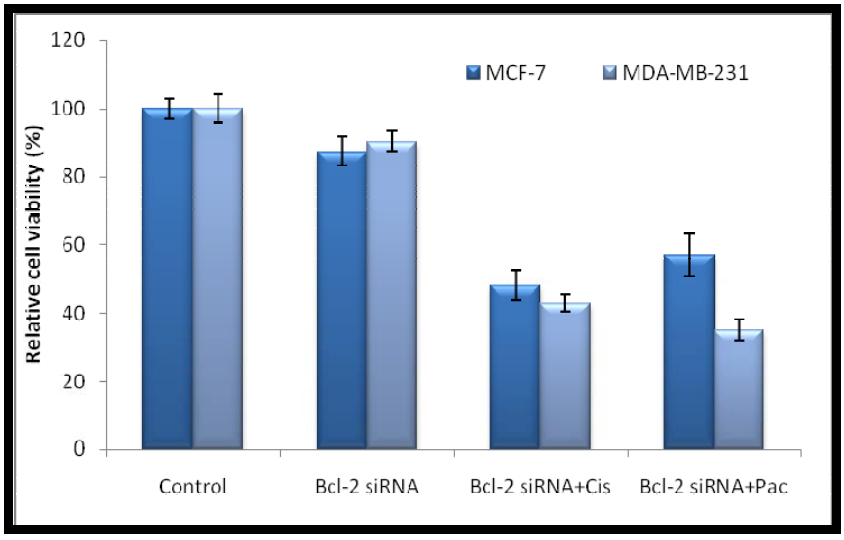

Figure 4.6 Silencing of Bcl-2 by siRNA enhances cytotoxic effects of drugs. Cell viability was determined by MTT assay. After silencing procedure, MCF-7 and MDA-MB-231 breast cancer cells were treated with 30 μM cisplatin and 20 nM paclitaxel for 24 h. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=2), each comprising four replicates. *$P < 0.05$

These findings were consistent with a possible increase in apoptosis and we performed M30 Apoptosense ELISA Assay. Down-regulation of Bcl-2 by siRNA resulted in increased apoptotic response by chemotherapeutic drugs. As demonstrated in Figure 4.7, each chemotherapeutic drug induced M30 antigen levels due to apoptotic cell death more than parental cells in the transiently Bcl-2 silenced cells (P <0.05). This finding indicates that transient silencing of Bcl-2 in both cells enhanced apoptotic effects of cisplatin and paclitaxel.

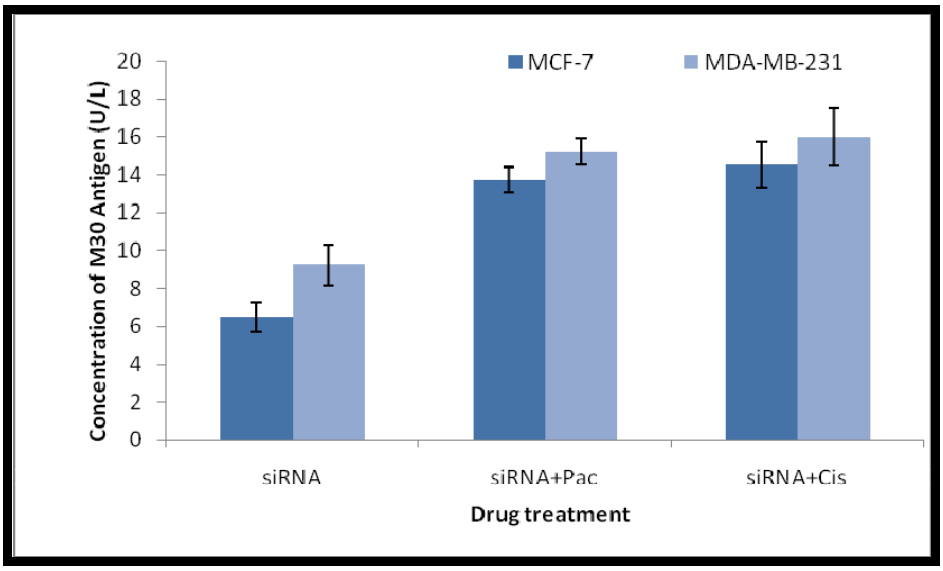

Figure 4.7 Silencing of Bcl-2 by transient siRNA treatment enhanced drug-induced apoptosis. Apoptotic cell death was determined by M30 Apoptosense ELISA assay in MCF-7 and MDA-MB-231 cells. *Columns* represent the mean (±SEM) values fold increased compare to control obtained from at least two different assays (N=2), each comprising four replicates. *$P < 0.05$.

4.3 The Bcl-2 antagonist, HA14-1, sensitizes breast cancer cells to drugs

Since anti-apoptotic Bcl-2 family members likely contribute to resistance in cancer chemotherapy by raising the threshold for apoptosis, we investigated whether the addition of Bcl-2 inhibitor (HA14-1) would potentiate the apoptotic induction by chemotherapeutics. Initially we determined dose dependent effect of HA14-1 on MCF-7 and MDA-MB-231 cells. Cells were exposed to various concentrations of HA14-1 for 24 h (Figure 4.8) and cell viability was determined by MTT assay.

HA14-1 between 1 to 5 μM concentrations for MCF-7 cells and between 1 to 8 μM concentrations for MDA-MB-231 cells did not significantly decrease cell viability in both cell lines as compared to control ($P > 0.05$). Whereas HA14-1 (10 μM) reduced cell viability by 22 % and 15 % in MCF-7 and MDA-MB-231 cells, respectively ($P < 0.05$ Figure 4.9). We found out that pre-treatment of HA14-1 (10 μM) markedly enhanced the cytotoxic effect of cisplatin or paclitaxel as compared to drug alone treatment in MCF-7 cells (Figure 4.9).

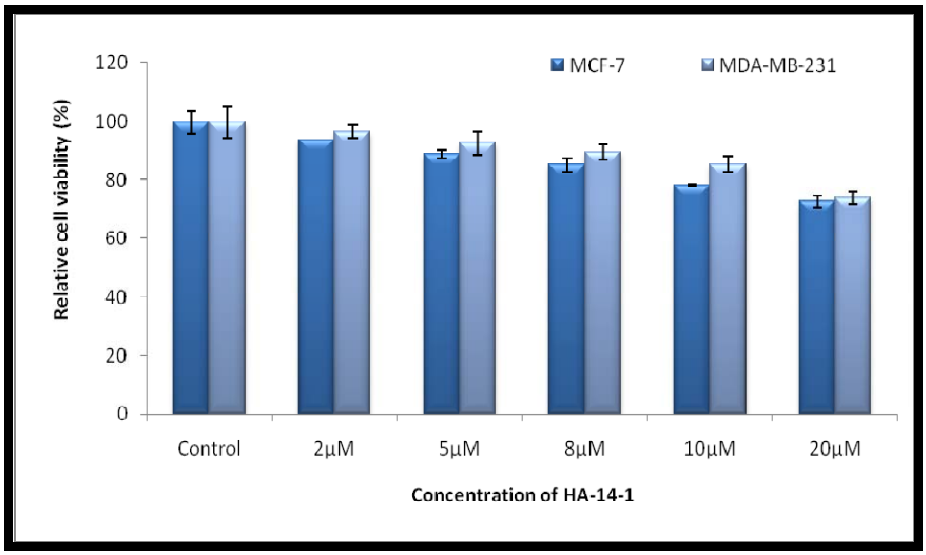

Figure 4.8 Determination of HA14-1-induced cytotoxic response. MCF-7 and MDA-MB-231 breast cancer cells were treated with HA14-1 for 24 h. The number of viable cells was determined by MTT assay. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=3), each comprising six replicates. * $P < 0.05$.

However, HA14-1 (10 μM) pre-treatment followed by cisplatin significantly increased the cytotoxic effect of cisplatin by 41 % (*P <0.05, cisplatin *vs* HA14-1 plus cisplatin), 10 μM HA14-1 pre-treatment was less potent for the cytotoxic effect of 20 nM paclitaxel in MDA-MB-231 cells (paclitaxel *vs* HA14-1 plus paclitaxel, *p>0.05) (Figure 4.10).

These findings were also confirmed with M30 Apoptosense ELISA Assay which determines apoptotic cell populations. Pre-treatment of 10 μM HA14-1 followed by 30 μM cisplatin increased M30 antigen concentration by 3.7 and 2.9 fold in MCF-7 and MDA-MB-231 cells as compare to cisplatin alone, respectively (P <0.05). However, pre-treatment of 10 μM HA14-1 followed by 20 nM paclitaxel treatment significantly increased apoptosis by 3.1 fold in MCF-7 cells (P <0.05), by contrast pre-treatment of 10 μM HA14-1 followed by 20 nM paclitaxel treatment did not significantly increased M30 antigen levels as compared to paclitaxel alone in MDA-MB-231 cells (1.9 vs 2.0 fold; P >0.05) (Figure 4.11).

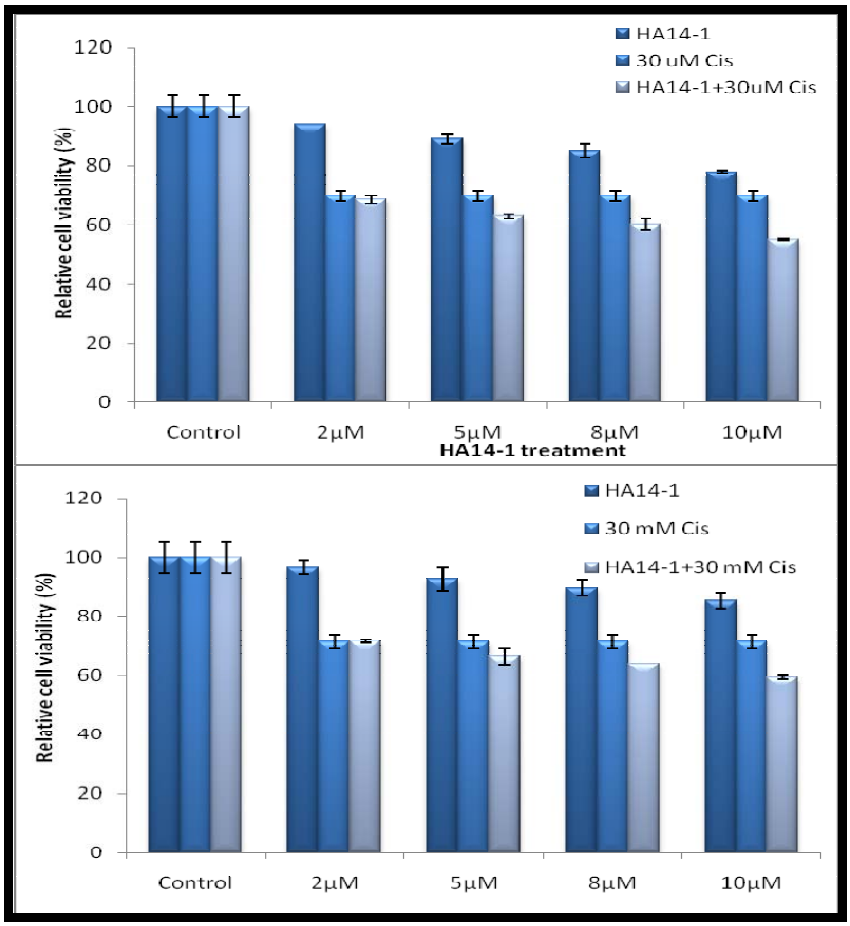

Figure 4.9 Pre-treatment with HA14-1 enhances cisplatin-induced cytotoxic responses in MCF-7 (up) and MDA-MB-231 (down) cells. The number of viable cells was determined by MTT assay. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=3), each comprising six replicates.*$P < 0.05$.

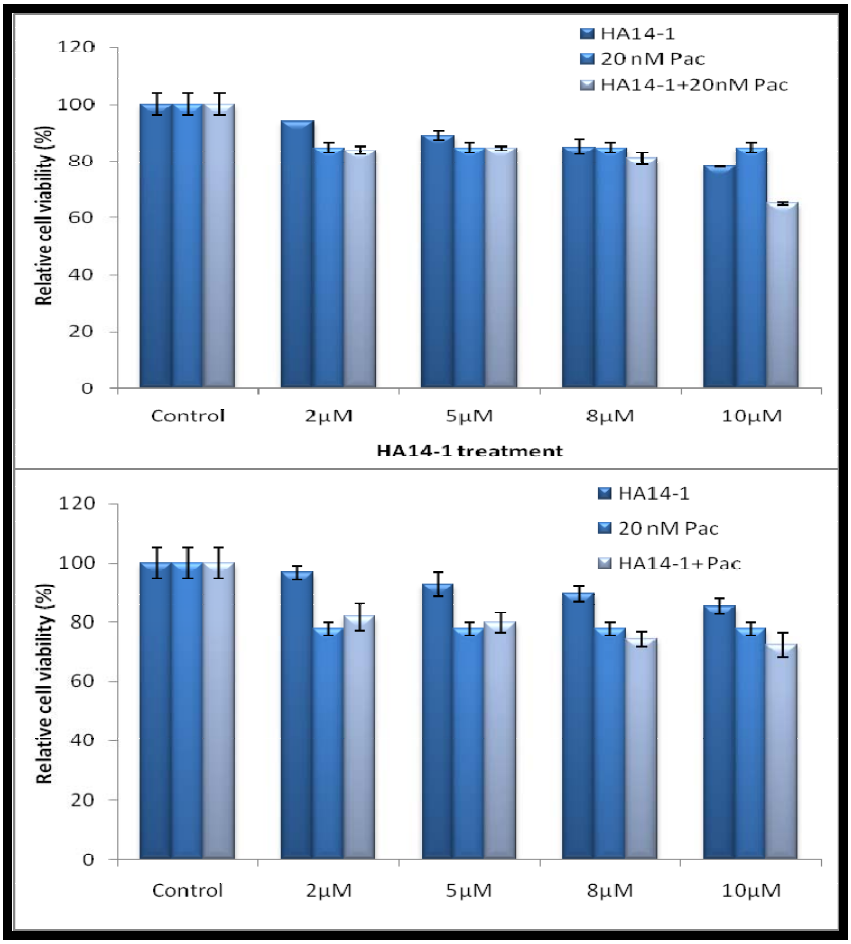

Figure 4.10 Pre-treatment with HA14-1 enhances paclitaxel-induced cytotoxic responses in MCF-7 (up) and MDA-MB-231 (down) cells. The number of viable cells was determined by MTT assay. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=3), each comprising six replicates.*$P < 0.05$.

In order to understand the effect of HA14-1 on Bcl-2 expression, we determined Bcl-2 protein levels following HA14-1 treatment. As shown in Figure 4.12 10 μM HA14-1 did not alter total amount of Bcl-2 at different time points within 24 h of treatment in both cell lines.

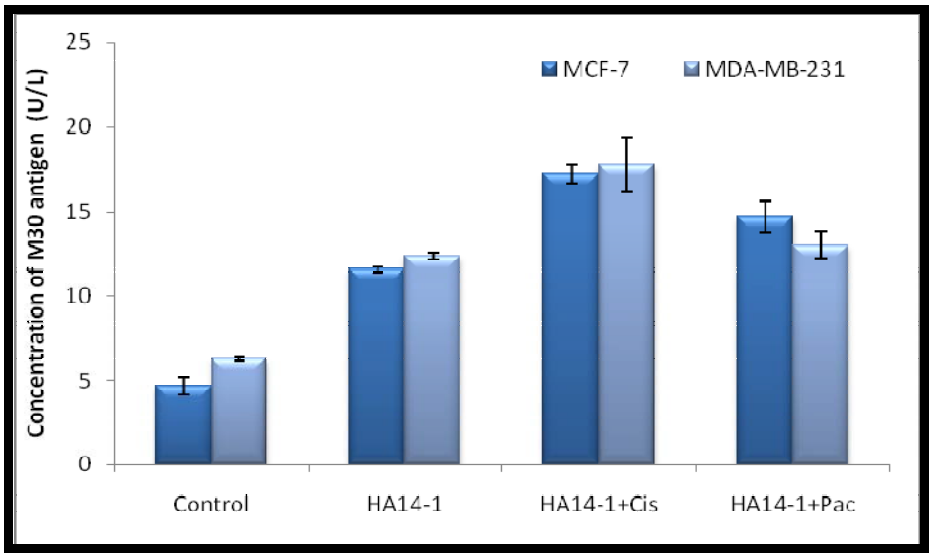

Figure 4.11 Pre-treatment of MCF-7 and MDA-MB-231 cells with HA14-1 potentiates the drug-induced apoptosis. Apoptotic responses were determined by M30 Apoptosense ELISA assay. The concentration of M30 antigen is presented as Units per Liter (U/L). *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=2), each comprising four replicates. *$P < 0.05$.

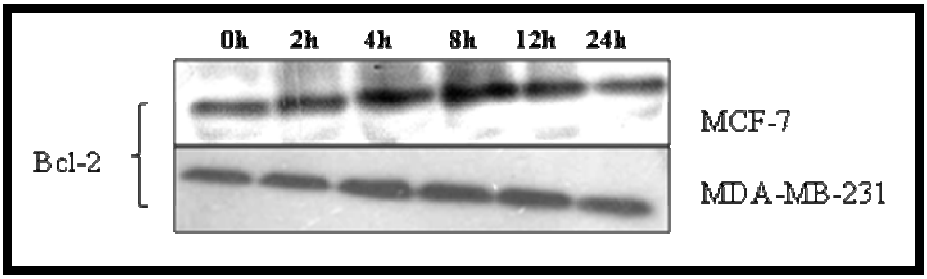

Figure 4.12 MCF-7 and MDA-MB-231 cells were treated with 10 μM HA14-1 for 24 h. Bcl-2 expression was determined by immunoblotting. β-actin was used as loading control.

4.4 Bcl-2 inhibitor potentiates drug-induced apoptosis

To investigate whether drug-induced apoptosis is mediated by caspases, the proteolytic activation of caspase-9 was examined in both cell lines. As shown in Figure 4.13 following drug treatment, caspase-9 activity was increased compare to control in MCF-7 and MDA-MB-231 cells, respectively. Pre-treatment of HA14-1 significantly increased drug-induced caspase-9 activity.

Colorimetric caspase-9 activity results were also confirmed by immunoblotting in drug-insensitive MDA-MB-231 cells. Pre-treatment with HA14-1 followed by cisplatin or paclitaxel treatment induced cleavage of pro-caspase-9 in MDA-MB-231 cells (Figure 4.14).

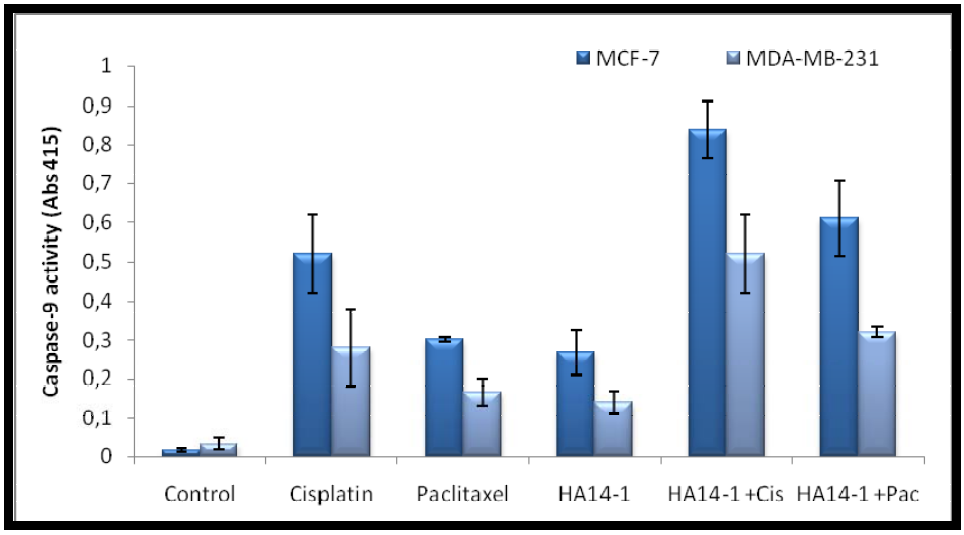

Figure 4.13 Determination of Caspase-9 activity. Colorimetric caspase-9 activity was determined by spectrophotometrically (Abs 415). *Columns* represent the mean (±SEM) values obtained from at least two different assays. Cis= Cisplatin, Pac= Paclitaxel.

As shown in Figure 4.14, caspase-3 was cleaved after cisplatin treatment but paclitaxel and HA14-1 did not induce caspase-3 cleavage in MDA-MB-231 cells. In addition, pre-treatment of cells with HA14-1 followed by paclitaxel induced cleavage of

pro-caspase-3. Thus Bcl-2 inhibitor enhanced paclitaxel induced cleavage of pro-caspase 3 to active form (p19/17) in MDA-MB-231 cells.

Engagement of apoptosis is further confirmed by the detection of PARP degradation. HA14-1 increased the paclitaxel- and cisplatin-induced PARP cleavage as opposed to drug treatment-only cases in drug-insensitive MDA-MB-231 cells (Figure 4.14).

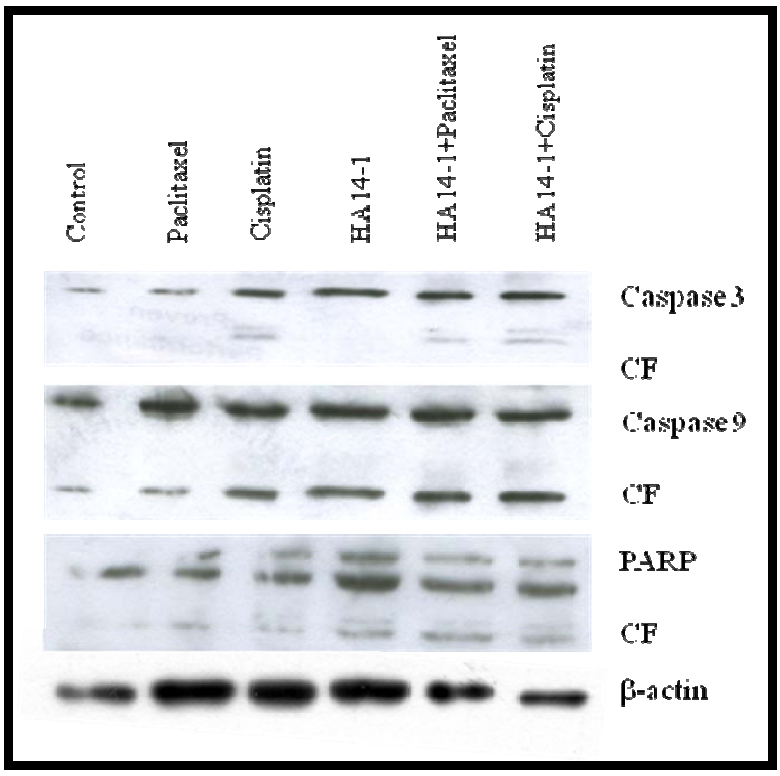

Figure 4.14 The cleavage of caspase-3, caspase-9 and PARP were determined in MDA-MB-231 cells. Thirty micrograms total protein lysate subjected to 12 % SDS-PAGE for western blot analysis with specific antibodies (anti-caspase-3, caspase-9 and PARP). β-actin was used as an internal loading control. CF= Cleaved fragment.

In this study, pan-caspase inhibitor, (z-VAD-fmk), caspase-3 inhibitor (z-DEVD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) were used to address the significance of caspase activation. Drug-insensitive MDA-MB-231 cells were exposed to these inhibitors for 1 h. After this, cells were pre-treated with HA14-1 followed by cisplatin or treated with only cisplatin 24 h. Cytotoxicity was evaluated by MTT assay.

Treatment with caspase inhibitors, each at 20 μM concentration, prevented cytotoxic effects of cisplatin and HA14-1 plus cisplatin. These findings suggest that the synergistic effect of HA14-1 with cisplatin was caspase-dependent (Figure 4.15).

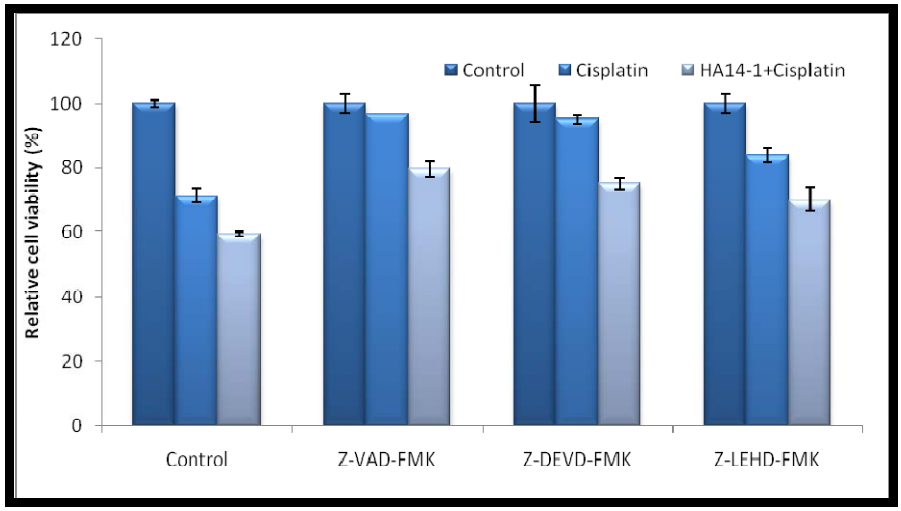

Figure 4.15 Cisplatin induced apoptosis was caspase dependent. MDA-MB-231 cells were pre-treated with HA14-1 followed by cisplatin or cisplatin alone after 1h prior treatment pan-caspase general inhibitor, caspase-3 inhibitor and caspase-9 inhibitor (each 20 μM) for 24 h. Cell viability was analyzed by MTT assay. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=2), each comprising four replicates.

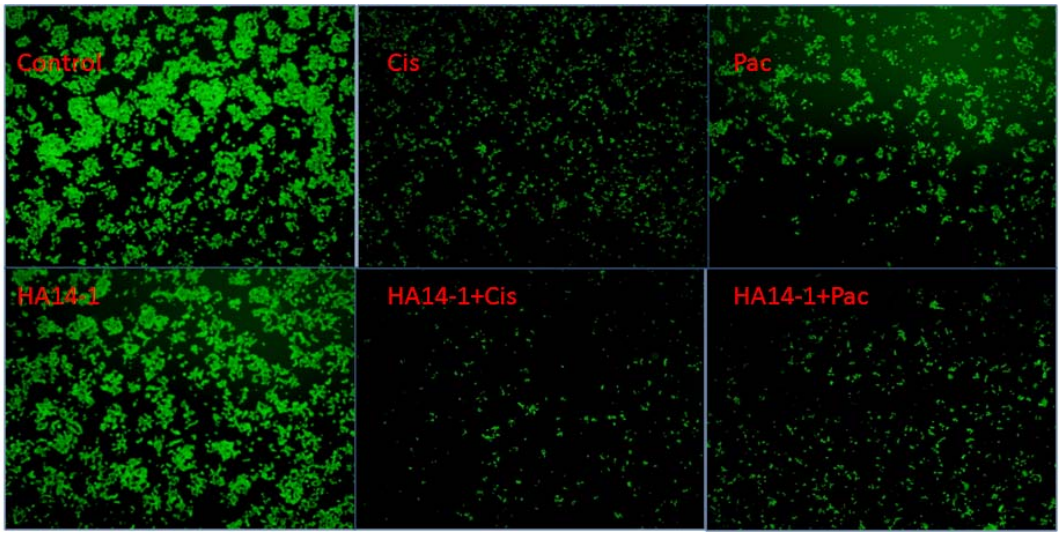

Figure 4.16 Determination of mitochondrial membrane potential (MMP) following drug treatment in MCF-7 cells. The loss of MMP was measured by rhodamine 123 staining and analyzed by fluoresence microscopy. Following treatment of MCF-7 cells with appropriate drugs for 24 h, rhodamine 123 (5 mM) was loaded for 30 min. Results were repeated twice and magnification was 100 X. Green fluorescence filter was used in fluorescence invert microscopy.

In order to determine drug-induced apoptotic events, MMP loss was examined following drug treatment in MCF-7 and MDA-MB-231 cells, respectively. To assess the MMP loss, cells were stained with rhodamine 123 dye which accumulates in living cells and binds to mitochondria membrane. As shown in Figure 4.16 and 4.17, cisplatin and paclitaxel treatment for 24 h increased loss of MMP in both cell lines. Pre-treatment of HA14-1 further increased the MMP loss in cisplatin treated cells in MCF-7 and MDA-MB-231 cells, respectively. Although, prior treatment of HA14-1 followed by cisplatin further increased MMP loss in MDA-MB-231 cells, paclitaxel treatment did not show remarkable increase in loss of MMP. Of note, alone HA14-1 treatment did not decrease MMP in both cell lines compare to control.

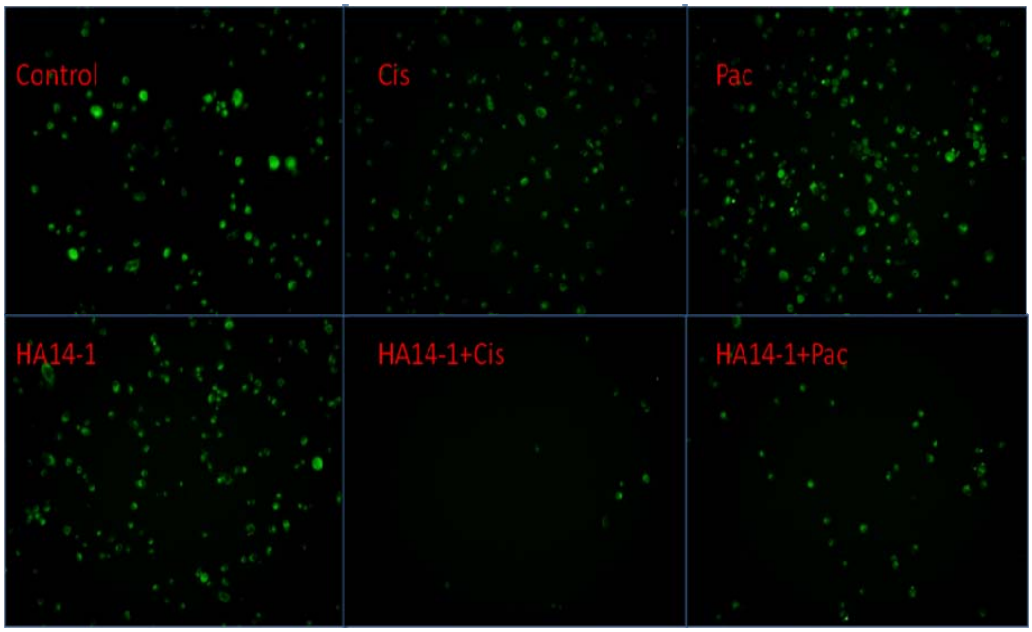

Figure 4.17 Determination of mitochondrial membrane potential (MMP) following drug treatment in MDA-MB-231 cells. The loss of MMP was measured by rhodamine 123 staining and analyzed by fluoresence microscopy. Following treatment of MDA-MB-231 cells with appropriate drugs for 24 h, rhodamine 123 (5 mM) was loaded for 30 min. Results were repeated twice and magnification was 200 X. Green fluorescence filter was used in fluorescence invert microscopy.

4.5 Modulation of Bcl-2 family members in drug-induced apoptosis

Bcl-2 exerts a significant part of its survival function by physically interacting with pro-apoptotic members of the Bcl-2 family. In this study, to identify the role of Bcl-2 family members in drug-induced apoptosis, we investigated mRNA levels and protein levels of various Bcl-2 family members following drug treatment.

Here we showed that cisplatin or paclitaxel did not significantly down-regulate Bcl-2 mRNA ($P > 0.05$ *vs* untreated control sample) and protein level in MCF-7 cells (Figure 4.18). Although cisplatin did not decrease Bcl-2 mRNA levels in MCF-7 cells, pre-treatment of this cells with HA14-1 slightly increased down-regulation of Bcl-2 following cisplatin treatment ($P > 0.05$ *vs* drug treatment).

According to immunoblotting results, pre-treatment of MDA-MB-231 cells with HA14-1 followed by cisplatin down-regulated Bcl-2 expression whereas this ratio was not significant following paclitaxel, HA14-1 and HA14-1 plus cisplatin treatment in MDA-MB-231 cells (Figure 4.20). Interestingly, pre-treatment of HA14-1 followed by cisplatin led to sharp decrease in Bcl-2 protein levels in MDA-MB-231 cells (Figure 4.20). Pre-treatment of MCF-7 cells with HA14-1 followed by cisplatin treatment did not significantly alter Bcl-2 expression.

We determined Bcl-x_L mRNA and protein levels in MCF-7 and MDA-MB-231 cells following drug treatment. Although pre-treatment of MCF-7 cells with HA14-1 followed by cisplatin or paclitaxel treatment did not alter Bcl-2 expression, prior treatment of MCF-7 cells with Bcl-2 inhibitor followed by cisplatin or paclitaxel treatment significantly decreased Bcl-x_L mRNA levels ($P < 0.05$ *vs* alone drug treatment and untreated control sample), Figure 4.18). Results were also confirmed by immunoblotting.

Cisplatin and paclitaxel treatment decreased Bcl-x_L mRNA levels ($P< 0.05$ *vs* untreated control sample) and down-regulated Bcl-x_L expression in MDA-MB-231 cells. Pre-treatment of MDA-MB-231 with HA14-1 also down-regulated Bcl-x_L protein levels, even more drastically than cisplatin or paclitaxel treated MDA-MB-231 cells. Of note, treatment with HA14-1 led to a decrease in Bcl-x_L mRNA levels similar to treatment with cisplatin and paclitaxel in both cell lines in MDA-MB-231 cells.

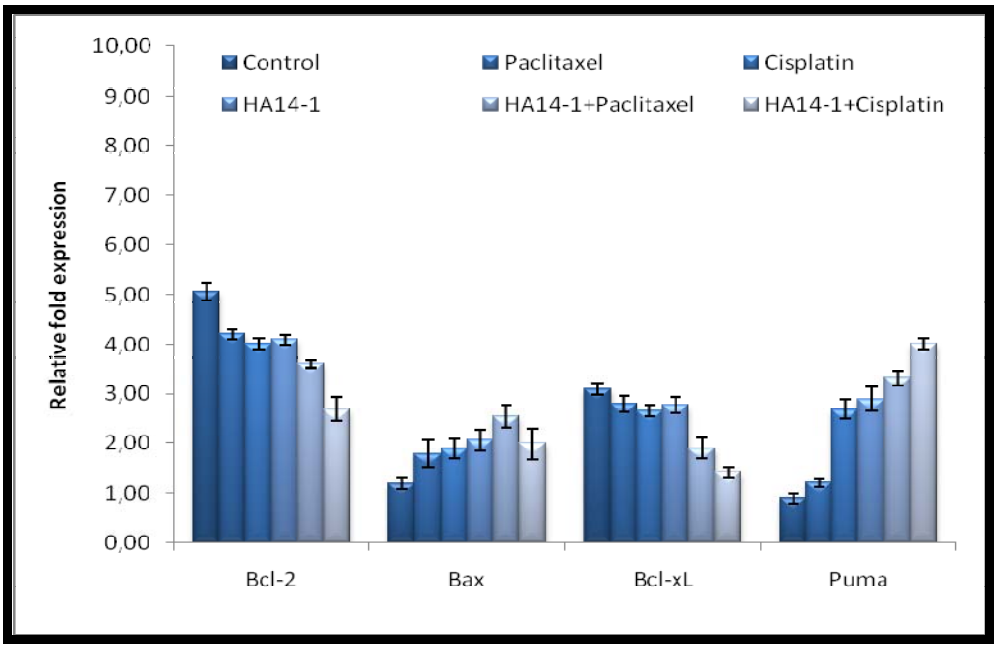

Figure 4.18 Modulation Bcl-2 family members in drug exposed MCF-7 cells. MCF-7 cells were pre-treated with HA14-1 followed by cisplatin or paclitaxel, cisplatin alone, paclitaxel alone and HA14-1 alone for 24 h. Bcl-2, Bax, Bcl-x_L and Puma copy numbers were calculated using standard curve of 18s RNA. These values are normalized by dividing with 18s RNA. *Columns* represented Mean ± S.D and representative of independent two experiments (N=2), and each experiment was repeated three times.

In order to understand the modulation of pro-apoptotic Bcl-2 family members in drug-induced apoptosis, we first determined Bax mRNA and protein levels following drug-treatment in the presence or absence Bcl-2 inhibitor. According to quantitative PCR results, cisplatin, paclitaxel and HA14-1 increased Bax mRNA level in MCF-7 and MDA-MB-231 cells, respectively ($P< 0.05$ *vs* untreated control sample). However, pre-treatment of MCF-7 cells with HA14-1 followed by cisplatin or paclitaxel did not increase Bax mRNA levels ($P> 0.05$ *vs* drug treatment). We established that pre-treatment

of MDA-MB-231 cells with HA14-1 followed by cisplatin increased Bax mRNA levels but paclitaxel did not exert same effect compare to alone paclitaxel treatment in MDA-MB-231 cells. According to immunoblotting results, pre-treatment of MDA-MB-231 cells with Bcl-2 inhibitor increased cisplatin and paclitaxel induced Bax protein levels (Figure 4.20).

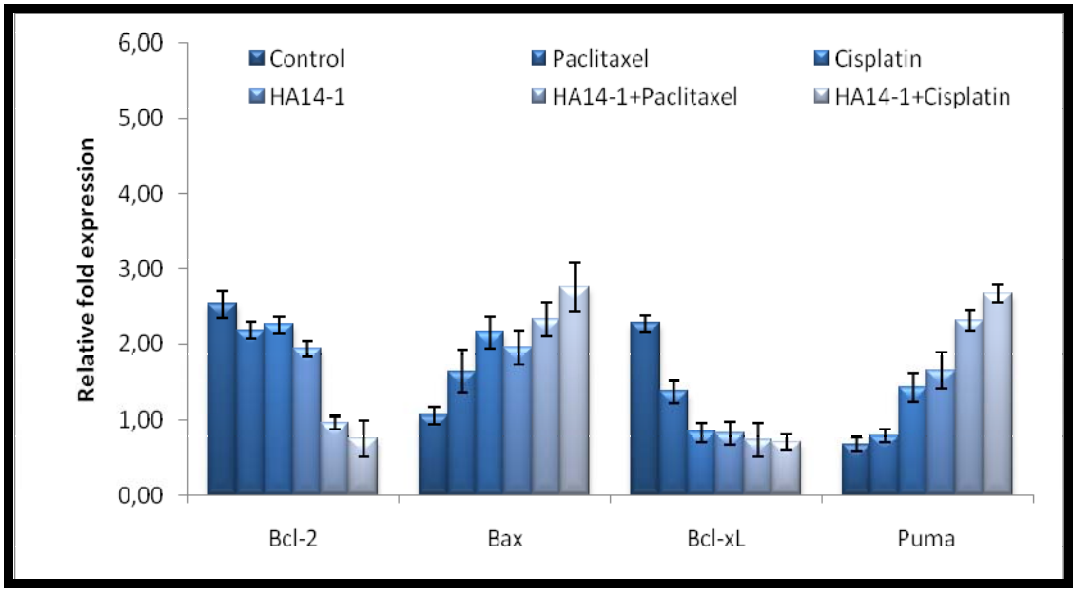

Figure 4.19 Modulation Bcl-2 family members in drug exposed MDA-MB-231 cells. a. MDA-MB-231 breast cancer cells were pre-treated with HA14-1 followed by cisplatin or paclitaxel, cisplatin alone, paclitaxel alone and HA14-1 alone for 24 h. Bcl-2, Bax, Bcl-x_L and Puma copy numbers were calculated using standard curve of β-actin. These values are normalized by dividing with β-actin. The data were represented Mean ± S.D and representative of independent two experiments (N=3), and each experiment was repeated three times.

To assess the role of Puma in drug-induced apoptosis mechanism we determined Puma mRNA and protein levels following drug treatment in MCF-7 and MDA-MB-231 cells, respectively. As shown in Figure 4.18 and 4.19, although cisplatin and HA14-1 treatment up-regulated Puma expression in both cell lines, paclitaxel did not exert same effect in MDA-MB-231 cells. Pre-treatment of MCF-7 and MDA-MB-231 cells with HA14-1 followed by cisplatin and paclitaxel further increased Puma expression ($P<$ 0.05 *vs* drug treatment).

In order to reveal the role of other pro-apoptotic Bcl-2 family protein, Bad in drug-induced apoptosis mechanism, we determined Bad expression following drug treatment in MCF-7 and MDA-MB-231 cells, respectively. Cisplatin and paclitaxel treatment increased Bad expression in MCF-7 and MDA-MB-231 cells. Although HA14-1 treatment increased Bad expression in MCF-7 cells, we did not observe any change in Bad expression profile in MDA-MB-231 cells following HA14-1 treatment. Pre-treatment of MDA-MB-231 cells with HA14-1 followed by cisplatin significantly increased Bad expression but, pre-treatment HA14-1 did not potentiate the paclitaxel induced Bad expression in the same cell line (Figure 4.20).

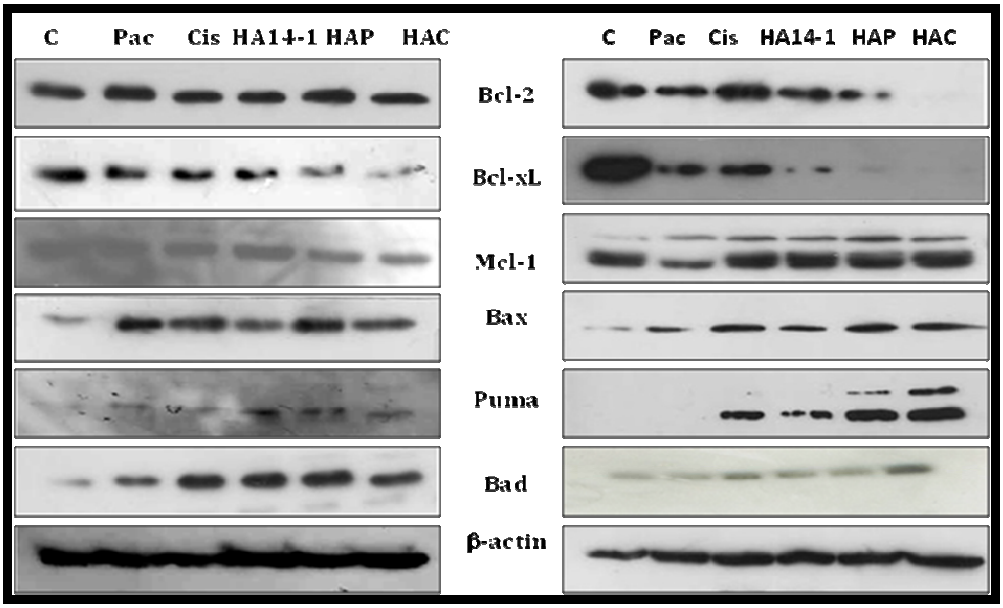

Figure 4.20 Modulation Bcl-2 family members following drug treatment of MCF-7 and MDA-MB-231 cells. Thirty micrograms total protein lysate were subjected to western blots with antibodies directed against the Bcl-2 family members. β-actin was used as loading control. Pac= Paclitaxel, Cis= Cisplatin, HAP= HA14-1+paclitaxel, HAC=HA14-1+cisplatin.

4.6 Prevention of cytotoxic effects of drugs in Bcl-2 overexpressing breast cancer cells

To establish the role of Bcl-2 overexpression in drug induced apoptosis, we set up stable Bcl-2 overexpressing MDA-MB-231 cells following G418 selection. Bcl-2 overexpression was determined by qRT-PCR (Figure 4.21). We also checked relative Bcl-2 mRNA copy number in MCF-7 Bcl-2 overexpressing cells which was provided by Dr. Ozgur Kutuk.

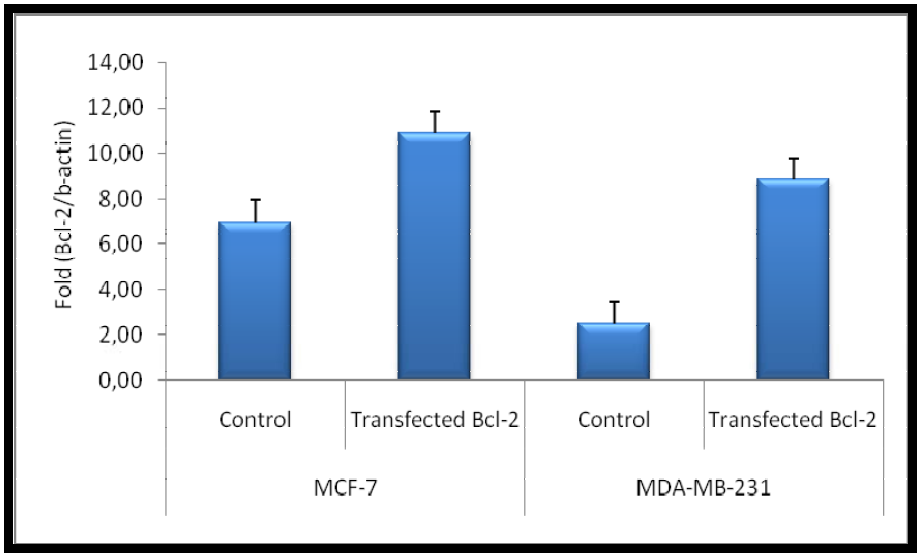

Figure 4.21 Determination of Bcl-2 overexpression in stable transfected MCF-7 and MDA-MB-231 cells. Each sample (700 ng cDNA) was amplified for Bcl-2 with qRT-PCR. *Columns* represents the mean value of two different experiments. β-actin was used as house-keeping gene and fold increase in Bcl-2 mRNA was calculated according to β-actin standart curve. PCR efficiency was determined as at least 85 %.

In order to establish Bcl-2 overexpressing stable cell line, MDA-MB-231 cells were transfected with Bcl-2 plasmid. Following G418 antibiotic treatment for clone selection, resistant clones were picked up under microscopy and seeded in 96 wells. Each clone was isolated for their RNA and investigated for Bcl-2 overexpression ratio (Figure 4.22).

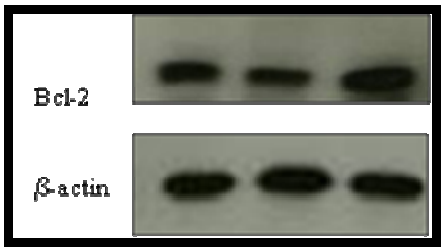

Figure 4.22 MDA-MB-231 cells were transfected with Bcl-2 plasmid and selected with G418 (700 μg/ml). Selected clones were verified by western blotting. From left side to right site clone 1, 2 and 3. Clone 3 was used in further experiments.

Since Bcl-2 is known to protect against various apoptotic insult including chemotherapeutic drugs, we evaluated how Bcl-2 overexpression alters apoptotic response induced by two drugs with different mechanism of action; a microtubule damaging taxane (paclitaxel) and a DNA-damaging platinum agent (cisplatin). First we determined cell viability following each drug treatment. As shown in Figure 4.23, cisplatin decreased cell viability by 50 % after 72 h of treatment in MCF-7 $^{Bcl\text{-}2+}$ (P<0.05 *vs* control), whereas this ratio was 21 % in MDA-MB-231$^{Bcl\text{-}2+}$. While pre-treatment of HA14-1 enhanced the cytotoxic effect of cisplatin in MCF-7 $^{Bcl\text{-}2+}$ cells (cell viability decrease by 75 %), pre-treatment of HA14-1 failed to exert additional cytotoxic effect (cell viability decrease by 27 %) when combined with cisplatin treatment in MDA-MB-231$^{Bcl\text{-}2+}$ (drug *vs* combined treatment p>0.05).

Although paclitaxel, cisplatin or HA14-1 treatment for 24 h significantly induced apoptosis in MCF-7 and MDA-MB-231 cells, overexpression of Bcl-2 protected against apoptosis triggered by paclitaxel, cisplatin or HA14-1 following treatment for 24 h in both MCF-7$^{Bcl\text{-}2+}$ and MDA-MB-231$^{Bcl\text{-}2+}$ cells, respectively. This protective potency of Bcl-2 was still observed following paclitaxel and HA14-1 treatment for 48 h in Bcl-2 overexpressing MCF-7 and MDA-MB-231 cells (Figure 4.23 and 4.24). Although Bcl-2 overexpression abrogated paclitaxel- and HA14-1-induced apoptosis following 48 h treatment in MCF-7$^{Bcl\text{-}2+}$ cells, cisplatin was able to trigger apoptosis.

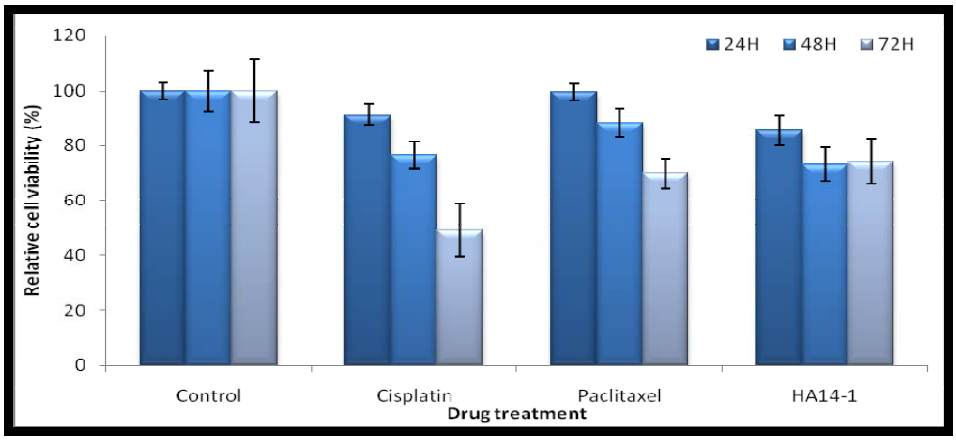

Figure 4.23 The determination of cell viability in MCF-7$^{Bcl\text{-}2+}$ breast cancer cells. MCF-7 $^{Bcl\text{-}2+}$ breast cancer cells were treated with cisplatin, paclitaxel and HA14-1 for 72 h and cell viability was evaluated by MTT assay. *Columns* represents the mean value of two different experiments (N=2).

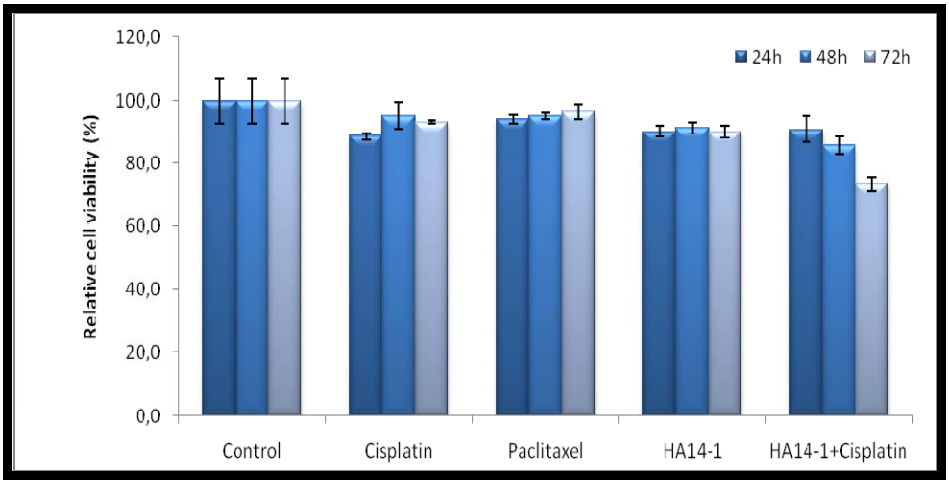

Figure 4.24 The determination of cell viability in MDA-MB-231$^{Bcl\text{-}2+}$ breast cancer cells. MDA-MB-231 $^{Bcl\text{-}2+}$ breast cancer cells were pre-treated with HA14-1 followed by cisplatin for 72 h.

Pre-treatment of Bcl-2 overexpressing MDA-MB-231 cells with HA14-1 did not lead to significant increase in cisplatin induced apoptosis within 72 h ($P > 0.05$, Figure 4.23 and 4.24). According to this result, it can be concluded that MDA-MB-231 cells were more resistant to cisplatin than MCF-7 cells.

4.7 Modulating sensitivity to drug-induced apoptosis: Bcl-2 family members

To evaluate the role of Bcl-2 expression levels in drug-induced apoptosis, we extracted protein samples from MCF-7$^{Bcl\text{-}2+}$ cells following drug treatment (Figure 4.25). In this experiment, MCF-7$^{Bcl\text{-}2+}$ cells were specially chosen because cisplatin could overcome the Bcl-2 protection effect within 48 h. However, MDA-MB-231$^{Bcl\text{-}2+}$ cells were found resistant to cisplatin within 48 h. According to immunoblotting results, cisplatin following 72 h treatment decreased total amount of Bcl-x_L and neither cisplatin nor paclitaxel changed the Bax expression level in Bcl-2 overexpressing MCF-7 cells.

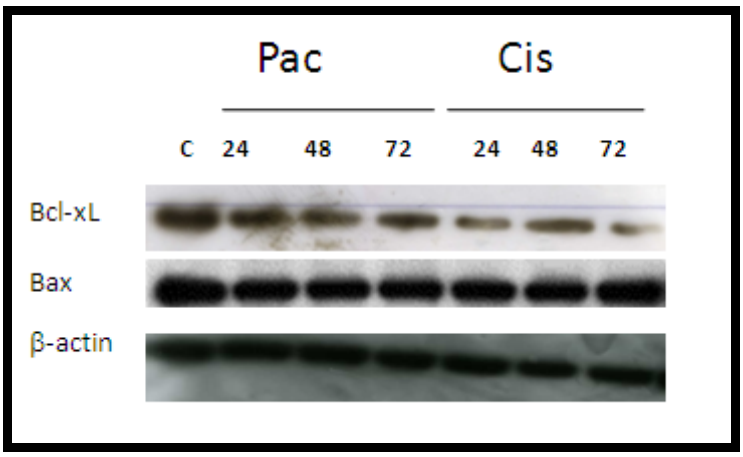

Figure 4.25 Modulation of Bcl-2 family members following drug treatment in MCF-7$^{Bcl\text{-}2+}$ cells. β-actin was used as loading control. M=Mock is control plasmid.

Puma and Noxa expression levels were determined in MCF-7$^{Bcl\text{-}2+}$ cells. MCF-7$^{Bcl\text{-}2+}$ cells were treated with cisplatin for 72 h time interval. Following total RNA isolation, results were analyzed by qRT-PCR. As shown Figure 4.26 and 4.27, qRT-PCR analysis demonstrated that Puma and Noxa transcription was induced following cisplatin treatment in MCF-7$^{Bcl\text{-}2+}$ within 48 h ($P< 0.05$ *vs* control).

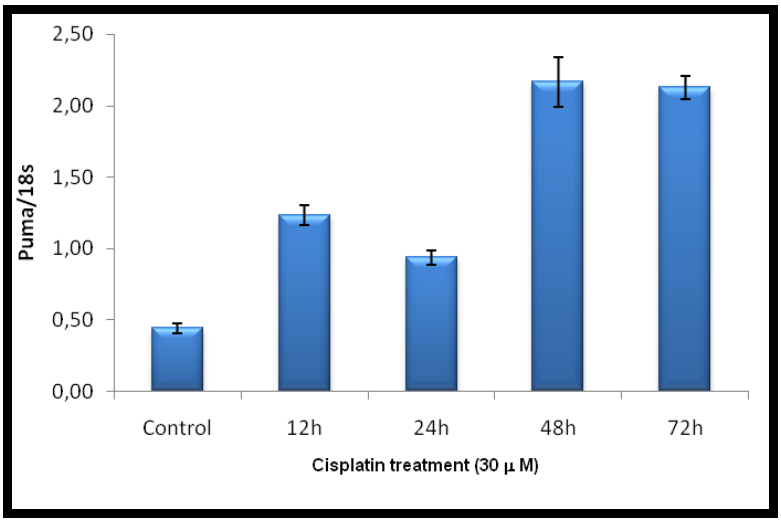

Figure 4.26 MCF-7$^{Bcl\text{-}2+}$ breast cancer cells were treated with 30 µM cisplatin for 0 to 72 h. Puma copy numbers were calculated using standard curve of 18s RNA. These values are normalized by dividing with 18s RNA. The data were represented Mean ± S.D and representative of (N=2) with three replicates.

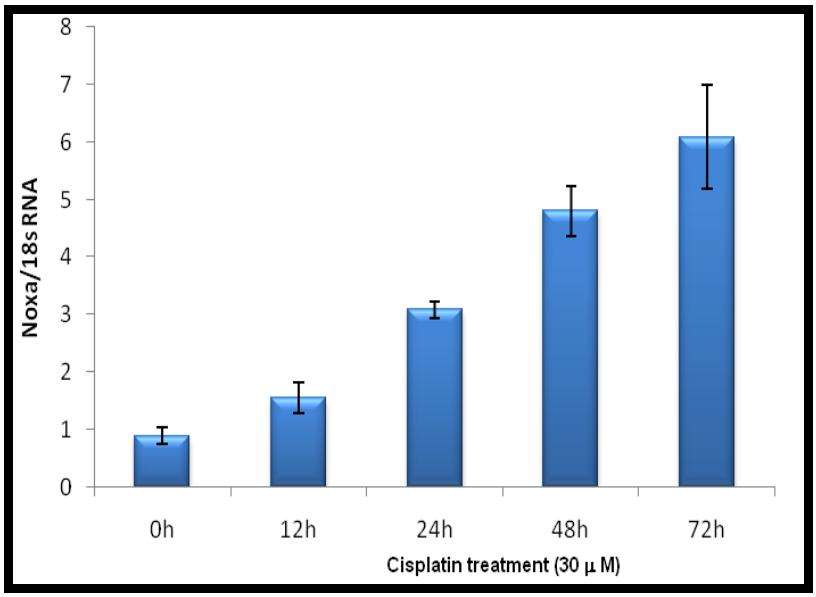

Figure 4.27 MCF-7$^{Bcl\text{-}2+}$ breast cancer cells were treated with 30 µM cisplatin for 0 to 72 h. Noxa copy numbers were calculated using standard curve of 18s RNA. These values are normalized by dividing with 18s RNA. The data were represented Mean ± S.D and representative of independent two experiments (N=2) with three replicates.

4.8 The role of Bcl-2 expression in drug-induced ROS production and lipid peroxidation

In order to understand the role of Bcl-2 in ROS generation and lipid peroxidation in drug-induced apoptosis mechanism, we first examined MCF-7 cells which were more sensitive to cisplatin than MDA-MB-231 cells. While cisplatin alone induced generation of DCF-DA signal, Bcl-2 depletion considerably enhanced this response (Figure 4.28).

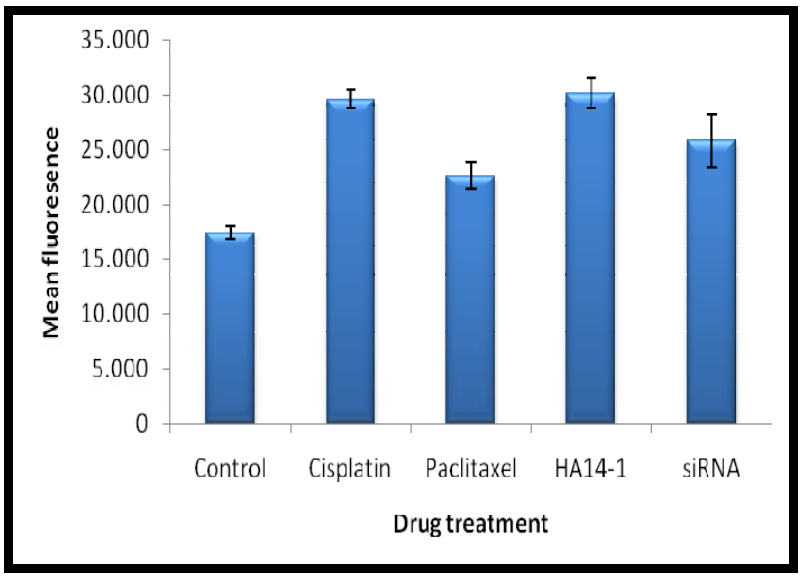

Figure 4.28 The determination of ROS generation in MCF-7 breast cancer cells which were treated with 20 nM paclitaxel, 30 μM cisplatin, 10 μM HA14-1 and Bcl-2 siRNA for 24h. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=3), each comprising six replicates. Excitation 488nm, emission 515nm.

Cisplatin, Paclitaxel, HA14-1 or Bcl-2 siRNA treatment increased ROS generation in MCF-7 cells. Pre-treatment of MCF-7 cells with HA14-1 significantly increased cisplatin induced ROS generation whereas pre-treatment of HA14-1 did not increase paclitaxel-induced ROS generation ($P > 0.05$ combined treatment *vs* paclitaxel treatment, Figure 4.29).

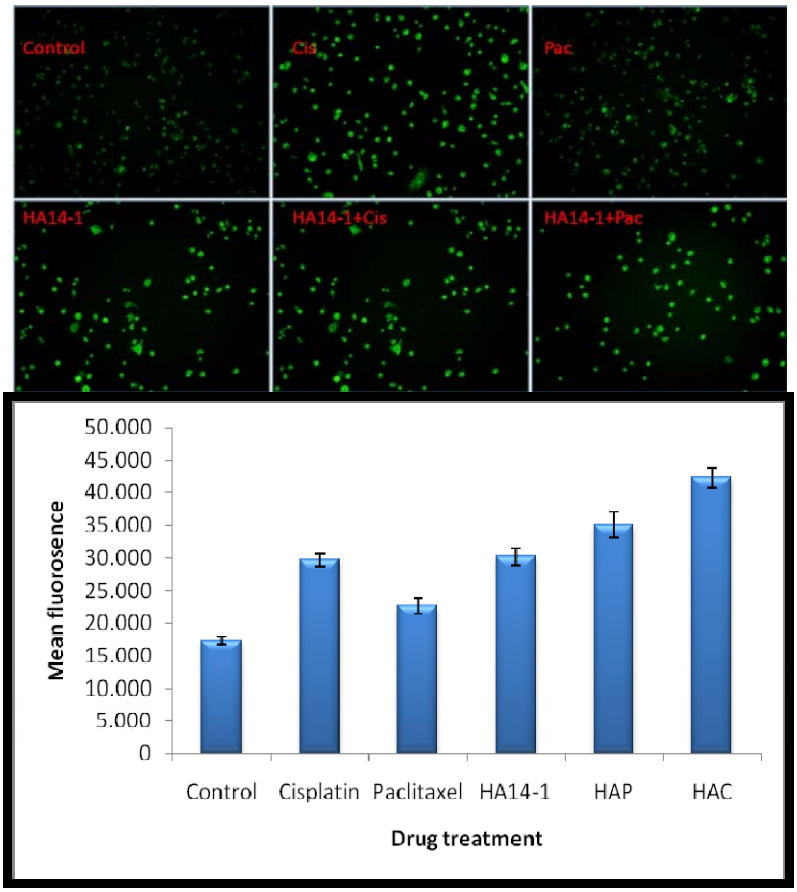

Figure 4.29 The determination of ROS generation in MCF-7 breast cancer cells which were treated with 20 nM paclitaxel, 30 µM cisplatin, 10 µM HA14-1 for 24h. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=3), each comprising six replicates. Excitation 488nm, emission 515nm. HAP= HA14-1 plus paclitaxel, HAC= HA14-1 plus cisplatin.

Bcl-2 silencing in MCF-7 cells increased lipid peroxidation compare to parental MCF-7 cells (Figure 4.30). This finding was confirmed by Bcl-2 inhibitor, HA14-1 in MCF-7 cells (Figure 4.31). Here, we showed that pre-treatment of MCF-7 cells with HA14-1 followed by paclitaxel increased lipid peroxidation more than paclitaxel treatment.

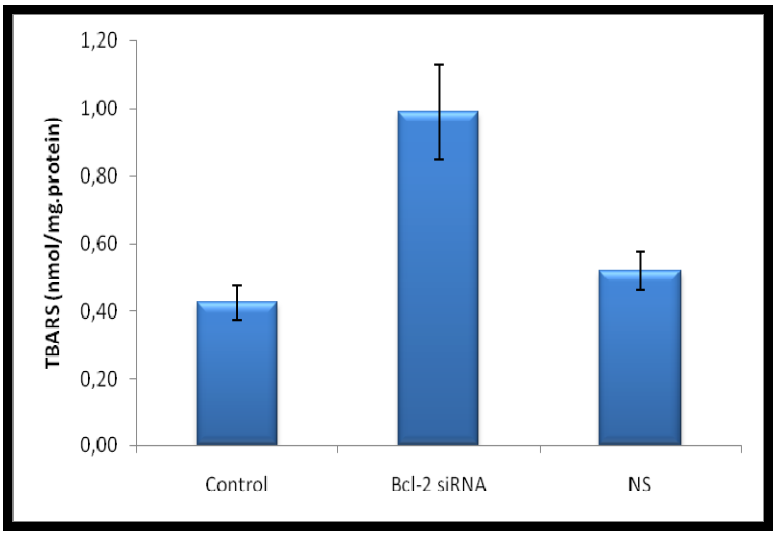

Figure 4.30 The determination of TBARS levels in Bcl-2 silenced MCF-7 cells. NS is non silencing control. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=2), each comprising four replicates.

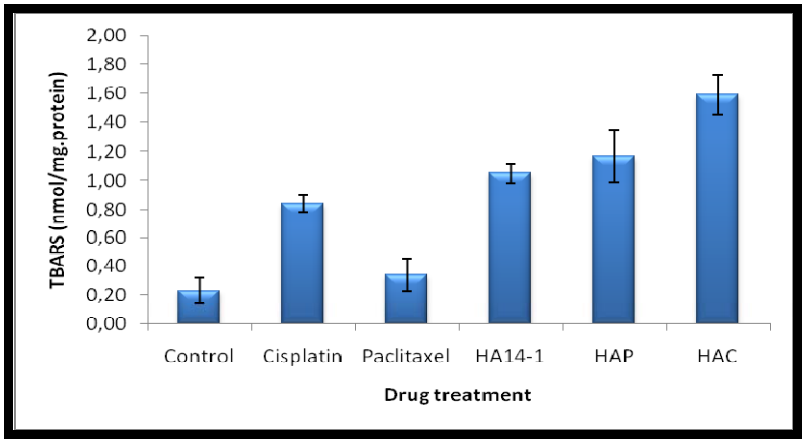

Figure 4.31 TBARS levels were determined for MCF-7 breast cancer cells. The determination of TBARS amount in MCF-7 breast cancer cells which were exposed to 20nM paclitaxel, 30µM cisplatin in presence or absence of 10µM HA14-1 for 24h. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=2), each comprising four replicates. HAP= HA14-1 plus paclitaxel, HAC= HA14-1 plus cisplatin.

To investigate if ROS production was critical for cisplatin-induced apoptosis in MCF-$7^{Bcl\text{-}2+}$ cells, we then similarly monitored the formation of intracellular ROS formation in these cells. Exposure of MCF-$7^{Bcl\text{-}2+}$ to cisplatin for 24 h or 48 h did not trigger any increase in DCF-DA reactive ROS production compare to untreated control cells (Figure 4.32).

As shown in Figure 4.33, cisplatin treatment for 48 h induced lipid peroxidation as determined by elevated levels of TBARS in MCF-$7^{Bcl\text{-}2+}$ cells, but cisplatin treatment for 24 h did not increase lipid peroxidation. Here we conclude that, cisplatin gradually decreased Bcl-2 expression and induced ROS production and lipid peroxidation, albeit with different time-dependent effectiveness.

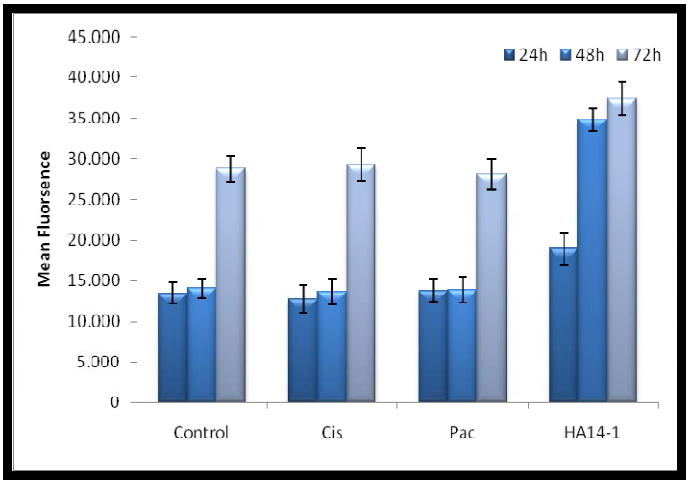

Figure 4.32 The determination of ROS generation in MCF-$7^{Bcl\text{-}2+}$ breast cancer cells which were treated with 20 nM paclitaxel, 30 µM cisplatin, 10 µM HA14-1 for 24h. *Columns* represent the mean (±SEM) values obtained from at least two different assays (N=3), each comprising six replicates. Excitation 488nm, emission 515nm. Cis= Cisplatin, Pac= Paclitaxel.

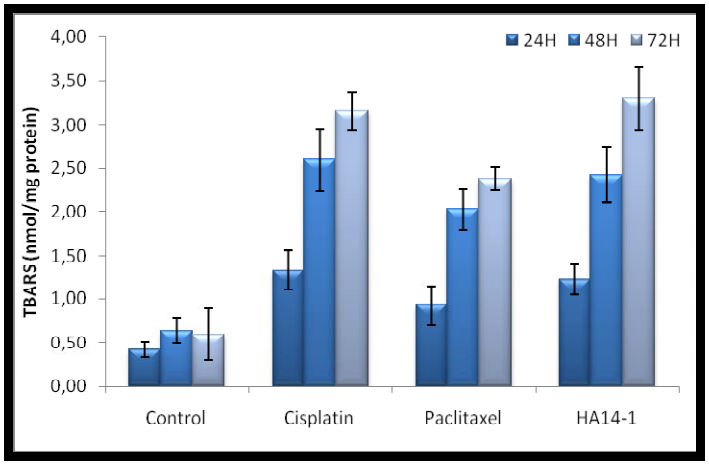

Figure 4.33 The determination of TBARS amount in MCF-$7^{Bcl\text{-}2+}$ breast cancer cells which were exposed to 30µM cisplatin, 20nM paclitaxel and 10µM HA14-1 within72 h time interval. *Columns* represent the mean (±SEM) values obtained from three independent experiments (N=3).

Drug induced ROS production was also examined in drug-insensitive MDA-MB-231 cells (Figure 4.34). Cisplatin treatment significantly increased ROS generation in MDA-MB-231 cells whereas paclitaxel was shown to be less potent to increase ROS generation. HA14-1 treatment did not increase ROS generation but pre-treatment of MDA-MB-231 cells with HA14-1 followed by cisplatin significantly increased ROS generation. Additional N-acetyl cystein (NAC), superoxide scavenger co-treatment with drugs prevented drug-induced ROS generation in MDA-MB-231 cells (Figure 4.34).

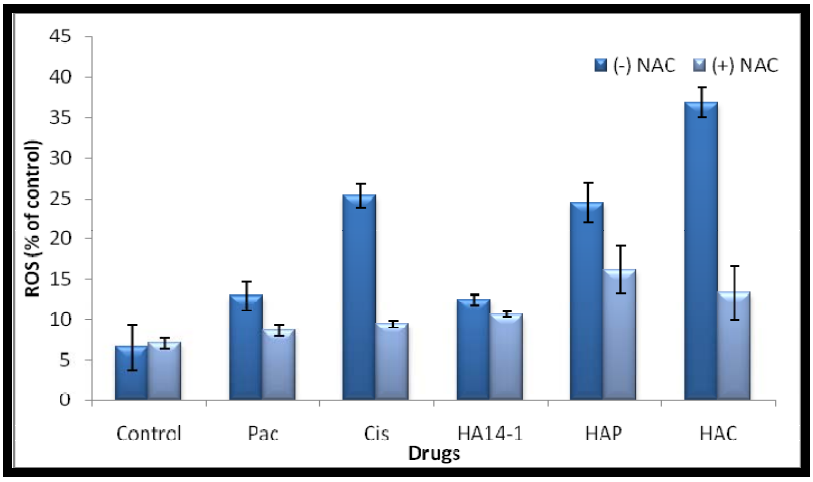

Figure 4.34 The determination of ROS generation in MDA-MB-231 breast cancer cells which were treated with drugs in the presence or absence of N-acetyl cystein (5 μM) for 24 h. *Columns* represent Mean ± S.D and representative of independent two experiments (N=2) with four replicates. Pac= Paclitaxel, Cis= Cisplatin, HAP= HA14-1+paclitaxel, HAC= HA14-1+cisplatin.

CHAPTER 5

5 DISCUSSION

Resistance to anticancer therapy is a major obstacle both in clinical and molecular oncology. Several studies established that the overexpression of anti-apoptotic Bcl-2 family proteins confers resistance to apoptosis and decrease the efficiency of therapeutics in various cancer types [58]. Despite the ongoing controversy on their prognostic role in breast cancer [111] Bcl-2 and Bcl-x_L reported to be overexpressed in majority (70 %) of breast cancer cases. In experimental breast cancer model systems, the overexpression of anti-apoptotic Bcl-2 family members causes increased resistance to drug-induced apoptosis and lead to enhanced angiogenesis and metastatic potential [111]. Accordingly the inhibition of anti-apoptotic Bcl-2 family proteins potently inhibits tumor growth and induces apoptosis or augments the action of conventional cytotoxics both *in vivo* and *in vitro*. Thus Bcl-2 and Bcl-x_L seem to be very promising targets for therapeutic intervention [111].

Solid tumors are difficult to treat, though surgery is performed where it is possible, chemotherapy is mostly the choice of treatment. One of the major limitations of chemotherapy is the inability of anti-cancer agents to induce cytotoxicity due to inaccessibility of certain cells in a tumor. Moreover the histopathological heterogeneity in human tumors is a well-documented phenomenon. It is now widely admitted that breast carcinoma is a genetically and clinically heterogeneous disease as it contains different clones of tumor cells arising from the continual differentiation of transformed progenitor cells [112].

In this study, we determined that cisplatin and paclitaxel display distinct modes of action to induce apoptosis in MCF-7 and MDA-MB-231 cells which represents two different types of breast cancer cells. MCF-7 cells are low-migrating cells which are expressing ERα while MDA-MB-231 cells are lack of ERα with high mobility and invasive potential. Both of them are referred as highly metastatic. According to MTT cell viability assays 30 μM cisplatin and 20 nM paclitaxel were moderately cytotoxic and induced apoptosis in MCF-7 and MDA-MB-231 cells, respectively. The higher concentrations of each drug were found more than 50 % cytotoxic. These concentrations were used for further experiments to evaluate the role of Bcl-2 in drug-induced apoptosis mechanism (Figure 4.1-4.3). It should be highlighted that the optimal dosing of chemotherapeutic agents is critical not only suppression of tumor growth but also to minimize their cytotoxicity in normal cells. Previous reports pointed out that high plasma concentration of several drugs was shown to cause several toxicity to normal tissues, including heart [113]. Thus, optimization of the treatment concentration is very important to build a study model to understand the molecular basis of chemoresistance mechanism.

In recent years gene therapy tools gained importance in order to overcome the non-specific effects of drugs and resistance problem. Antisense compounds are valuable agents to explore the function of gene products as they enable the through specific down-regulation of gene expression, providing opportunity to evaluate the consequences of that down-regulation on physiological events. Among their current applications, antisense molecules also have potential for use as anticancer therapeutics. Single stranded oligonucleotides or siRNAs are in development as agents to specifically modulate processes on which tumor cells depend for viability and growth. They can have reduced non-specific toxicity and improved anti-tumor activity compared with to the traditional cancer drugs. Several of these drugs (including Bcl-2, survivin, XIAP, and clusterin) are under investigation in phase I/II clinical trials [114].

Combination of novel therapeutic models with standard chemotherapeutic agents or radiotherapy was shown to have different effects according to cell characteristics and origin of tissue type. However limitations of gene therapy are still waiting for more progress in this area. A new phenomenon, RNAi is very useful technique to control

gene expression. It provides an additional shut-down mechanism for targeted genes. Therefore, stable or transient transfection of siRNAs which target different kinds of Bcl-2 family members provides a detailed understanding to the overcome chemoresistance problem. Previous reports showed that Bcl-2 silencing approach appears to be a suitable strategy for sensitizing many kinds of cancer cells to various chemotherapeutics whereas their combined action was found drug and cell type specific [114, 115]. In our study, cisplatin and paclitaxel-induced apoptosis was promoted by Bcl-2 silencing approach in MCF-7 and MDA-MB-231 cells more than parental cells (Figure 4.6 and 4.7). Therefore we conclude that Bcl-2 is an attractive target for novel treatment strategies of breast carcinoma. In aggrement with our results, Bcl-2 siRNA augmented cisplatin and paclitaxel induced apoptosis in glioblastoma cells and lung cancer cells, respectively [116].

Since pro-apoptotic Bcl-2 family proteins dock into the BH1-BH2 groove of anti-apoptotic members *via* their BH3-domain, it has been proposed that BH3-mimetics can antagonize the anti-apoptotic members. Currently, a favored strategy for Bcl-2 antagonism is based on small molecules targeting the Bcl-2 anti-apoptotic proteins by mimicking a BH3-domain [61]. Several compounds have been isolated or chemically synthesized, showing different binding specificity and affinity for BH3-domains of Bcl-2 proteins and promoting apoptosis [84, 108, 117]. Among them is HA14-1 which leads to changes in Ca^{2+} homeostasis, inhibition of mitochondrial potential, Bax translocation, ROS generation, Cyt c release and caspase-9/-3 activation, and subsequently PARP cleavage [82, 89, 117-119]. The therapeutic effect of HA14-1 has been described in a variety of tumor cells. HA14-1 cooperates with other drugs such as flavopiridol, bortezomib, dexamethasone, doxorubicin and cytrabine [91, 119, 120]. Additionally, HA14-1 could potentiate non-toxic MAPK inhibitors as lethal agents in the cell culture environment [93]. Similar to HA14-1, ABT-263, AT-101, GX15-070, gossypol or oblimersen sodium (Genta) was also shown as apoptosis inducers as single agent. The success of HA14-1 or other BH3-mimicking drugs in combined or sequential therapy was shown to be cell and drug type dependent [121]. Interestingly gossypol is a natural product which was previously used for male contraception can induce apoptosis in Bax/Bak double deficient cells through changing conformation of Bcl-2 [122]. Similar to gosspol, chelerythrine can induce apoptosis through Bax/Bak independent

mitochondrial mechanism. This finding is very important to manage chemoresistance mechanism in Bax/Bak mutant cells which give less response to chemotherapeutics.

In our study, pre-treatment of MCF-7 cells with HA14-1 (10 μM) significantly potentiated the cytotoxic effect of cisplatin and paclitaxel ($P< 0.05$ *vs* untreated sample). A previous study of hematopoetic cell lines found out that HA14-1 at concentrations higher than 25 μM led to a loss of selectivity for Bcl-2 and marked cytotoxicity in all cell lines tested [119]. In MDA-MB 231 cells, although pre-treatment of HA14-1 potentiated effect of cisplatin significantly, sensitization was much less when cisplatin was replaced by paclitaxel (Figure 4.9, $P <0.05$; Figure 4.10 $P >0.05$). These results were further confirmed by M30 Apoptosense ELISA assay which determines the caspase-induced CK18 cleavage in apoptotic cell populations (Figure 4.11). Additionally, Bcl-2 protein level was checked but not found to be altered by to the treatment of HA14-1 (Figure 4.12).

In order to understand the role of caspases in drug-induced apoptosis, we determined caspase-9 activity following cisplatin and paclitaxel treatment in MCF-7 and MDA-MB-231 cells. Pro-caspase-3 cleavage was only detected in MDA-MB-231 cells. Caspase-9 activity was significantly increased following cisplatin and paclitaxel treatment in MCF-7 and MDA-MB-231 cells, respectively ($P< 0.05$ *vs* untreated control sample, Figure 4.13). Inhibition of Bcl-2 enhanced the caspase-9 activation following cisplatin and paclitaxel treatment in both cell lines. Although cisplatin-induced caspase-3 cleavage, paclitaxel did not exert same effect in MDA-MB-231 cells (Figure 4.14). According to this finding, paclitaxel-induced caspase-3 cleavage might be independent from the caspase-9 activation. It is also possible that paclitaxel acts via activation of another effector caspase, like caspases-6 or -7, or by an independent pathway. Previously, the possible involvement of caspase-7 in paclitaxel-induced apoptosis has been suggested in human esophageal squamous cancer cells and in non-small-cell lung cancer H460 and H520 cell lines [123]. Similar to our results, it was shown that HA14-1 alone itself increased the caspase-3 and caspase-9 activation in cancer cells [89]. In addition, further increased caspase activity was determined following combined treatment of HA14-1 with cytotoxic agents and photo/radiation therapy models [89, 124]. We also determined that cytotoxic effects of cisplatin or pre-treatment of MDA-

MB-231 cells with HA14-1 followed by cisplatin treatment were prevented by pan-caspase, caspase-9 and caspase-3 inhibitors (Figure 4.15). Thus, we conclude that cisplatin-induced apoptosis occurs by caspase dependent pathway. Consistent with our findings, pan-caspase, caspase-9 and caspase-3 inhibitors prevented HA14-1-induced apoptosis in follicular lymphoma B cells [125]. Similar to these findings, it was established that pre-treatment of z-VAD-FMK and α-tocopherol prevented the potentiating effect of HA14-1 with γ-radiation application in prostate cancer cells [89]. Under the light of these findings, we can conclude that inhibition or down-regulation of Bcl-2 was accompanied with drug-induced apoptosis mechanism and increased caspase activation. In order to understand the differential sensitizing effects of HA14-1 pre-treatment followed by paclitaxel in MDA-MB-231 cells, further studies should focus on other effector caspases, or caspase independent cell death mechanisms. Additionally we found out that cisplatin and paclitaxel induced PARP cleavage in MDA-MB-231 cells (Figure 4.14). These findings indicate that cisplatin and paclitaxel induced mitochondrial apoptotic pathway in MCF-7 and MDA-MB-231 cells, respectively. The early loss of the MMP is a hallmark of apoptosis [126]. To assess the MMP loss, MCF-7 and MDA-MB-231 were stained with rhodamine 123 fluorogenic probe following the drug treatment. In this study we determined that pre-treatment of HA14-1 further increased drug-induced MMP loss in MCF-7 and MDA-MB-231 breast cancer cells, respectively (Figure 4.16-4.17). Although caspase-9 activation was observed following HA14-1 treatment, HA14-1 did not significantly trigger MMP loss by itself. Similar to these results, HA14-1 was shown to induce MMP loss in murine leukemia cells and promote rapid loss of MMP following photodynamic therapy [118].

Balance between pro-apoptotic and anti-apoptotic members of Bcl-2 family determines the cell fate. Here the key question is how these proteins are recruited in response to toxic stimuli. As mentioned before, their expression levels have been implicated as major regulators in the control of mitochondrial apoptotic pathway. We presented here, HA14-1 pre-treatment followed by cisplatin or paclitaxel abrogated Bcl-2 and Bcl-x_L expression especially in MDA-MB-231 cells, albeit greater extends with cisplatin (Figure 4.19-4.20). HA14-1 drastically down-regulated Bcl-x_L expression in MDA-MB-231 cells (Figure 4.20) however no significant down-regulation was observed for anti-apoptotic Bcl-2 family protein, Mcl-1. Given that other Bcl-2

inhibitors ABT-763 and GX14-070 (obatoclax) have shown to overcome Mcl-1-mediated resistance, HA14-1 can be a less likely candidate to be used in treatment of Mcl-1 overexpressing cancers [83, 84].

Although the pre-treatment of HA14-1 significantly increased paclitaxel or cisplatin induced cell death in MCF-7 cells, HA14-1 pre-treated MDA-MB-231 cells were more resistant to paclitaxel. Hence, modulation of pro-apoptotic and anti-apoptotic Bcl-2 family members might be an important key answer to understand the sensitizing effect of HA14-1 in various breast cancer cells. Recent reports suggested that phosphorylation of Bcl-2 is an important regulatory mechanism for its function and is a common event in response to anti-mitotic drugs [16]. In addition it was proposed that this post-translational modification may be necessary for Bcl-2's full and potent anti-apoptotic function. Paclitaxel treatment activates members of the JNK/SAPK MAPK family, which then disable Bcl-2 through phosphorylation within its flexible loop region [16, 18]. Although paclitaxel can also bind to the loop of Bcl-2 protein, its significance is unclear. It is possible that mutations in the loop region of Bcl-2 could account for some cases of paclitaxel-resistance in contrast other mitotic blockers does not show this resistance suggesting that their recognition site on Bcl-2 protein is different [19]. However, it was pointed out that while paclitaxel induced Bcl-2 phosphorylation, it could also up-regulate Bax expression [127]. Thereby, other anti-apoptotic genes such as Bcl-x_L should be considered in paclitaxel resistance cases. Further investigation of paclitaxel induced Bcl-2 phosphorylation and related up-stream events might be informative [18].

A number of studies have indicated that cisplatin induces apoptosis via DNA damage responsive pathways which may vary depending upon cell type and molecular context. These pathways include p53 activation lead to transcriptional upregulation of pro-apoptotic members of the Bcl-2 family. Transcriptional regulation of Bcl-2 or Bcl-x_L in response to cisplatin has not generally been examined since suppression of anti-apoptotic molecules has not been considered a transcriptional target of these DNA damage induced pathways [128]. Here, we showed that both HA14-1 or cisplatin treatment did not decrease Bcl-2 expression in MCF-7 and MDA-MB-231 cells, pre-treatment of HA14-1 followed by cisplatin built a synergistic effect and further decrease

Bcl-2 expression (Figure 4.18-20). Identification of up-stream signaling events may clarify how this synergistic effect of both drugs decreases Bcl-2 mRNA and protein levels. Previous reports showed that H_2O_2 mediates Bcl-2 down-regulation by cisplatin through its ability to dephosphorylate the protein, which facilitates its ubiquitination and subsequent degradation by the proteasome [103]. Multiple pro-survival Bcl-2 family members are degraded by the ubiquitin-proteasome system; the best examples are the pro-survival members Bcl-2 and Mcl-1 [129] [130].Here we showed that cisplatin induced generation of ROS and this event may degrade Bcl-2 protein levels by ubiquitin-dependent proteolytic pathway. This event may trigger ubiquitin-dependent proteolytic control of transcriptional regulation of anti-apoptotic Bcl-2 family members. In further studies, proteosomal control of gene transcription and additional translational control of anti-apoptotic genes should be discussed to clarify drug-induced apoptosis and chemoresistance mechanism.

Previous studies pointed out that, lack of Bax might cause a failure in response to HA14-1 [131]. Hence, determination of the Bax expression and related events in HA14-1-exposed cell lines might be significant in the characterization of drug specific sensitizing effect of HA14-1. We herein demonstrated that Bax was markedly up-regulated by the pre-treatment of HA14-1 followed by cisplatin or paclitaxel in MCF-7 and MDA-MB-231 cells, respectively (Figure 4.19-4.20). Previous studies indicated that Bax undergoes conformational changes resulting in Bax homodimerization and mitochondrial membrane insertion in response to death signals from cytotoxic insults, such as survival factor withdrawal [132]. Although pre-treatment of HA14-1 followed by cisplatin induced Bax translocation paclitaxel did not significantly exert same effect in MDA-MB-231 cells [133]. Pre-treatment of HA14-1 did not increase Bax expression levels following cisplatin and paclitaxel treatment in MCF-7 and MDA-MB-231 cells.

Considering the finding that cisplatin induced apoptosis in both parental MCF-7 (p53 wild-type) and MDA-MB-231 (p53 mutated) cell lines, we can conclude that apoptosis induction by cisplatin does not require p53 transcriptional activity in parental breast cancer cells consistent with previous reports. However, the expression of Bcl-2 and Bax appear to be regulated by the wild type form of p53 tumor suppressor gene. The Bcl-2 gene contains a p53-dependent negative response element through which p53

may, either directly or indirectly, transcriptionally downregulate the expression of Bcl-2 [134]. The Bax gene promoter contains binding site sequences for p53 [135]. It was suggested that only wild type p53, not mutant, could transactivate the Bax gene promoter [60]. Similar to Bax, Puma, a BH3-only protein, is also a transcriptional target of p53-mediated apoptosis signaling. However, these genes could also be induced by p53-independent pathways Thus, p53 family members are important mediators of apoptosis and can be induced by a number of chemotherapeutics and radiation [136]. Puma acts by modulating Bax activity through binding to Bcl-x_L and dissociate the interaction of Bax and Bcl-x_L to facilitate Cyt c release from the mitochondria, thereby triggering the apoptotic cascade [137]. It was previously demonstrated that p53-independent Puma up-regulation might be playing role in cisplatin-induced apoptosis in colon cancer cells [138]. Over-expression of Puma induces disorganization/disruption of the cellular microtubule network in apoptotic fibroblasts [138]. Therefore, microtubules displayed de-polymerized and dispersed structures throughout the cytoplasm. In contrast to the mechanistic action of Puma, paclitaxel and docetaxel induce the stabilization of microtubule formation and lead to a subsequent cell death. As shown in Figure 4.19, while cisplatin (30 μM) and HA14-1 (10 μM) were up-regulating Puma mRNA and protein expression, paclitaxel (20 nM) did not change up-regulate Puma expression in MDA-MB-231 cells. Based on these results, we conclude that cisplatin and HA14-1 induce p53-independent Puma up-regulation in MDA-MB-231 cells, whereas paclitaxel failed to show any effect on Puma expression levels.

Bad expression level was increased following cisplatin treatment more than paclitaxel treatment in MCF-7 and MDA-MB-231 cells, respectively. Previous reports showed that p53 transactivates Bad transcription and forms a Bad/p53 complex which induce mitochondrial apoptosis in lung cancer cells [139]. However, Bad plays dual roles in p53 transcriptional-dependent and -independent pathways. Upon treatment with a DNA damaging agent such as etoposide, upregulated p53 directly binds to the upstream promoter region of many target genes, such as Bad, Bax, Puma, Noxa and Bid to transactivate their gene expression. When enough Bad have been translated in the cytoplasm, it in turn associates with p53 to prevent the latter from entering nucleus. Bad expression is thus to be kept at a physiological level; moreover, dephosphorylated Bad is able to direct cytosolic p53 to mitochondria and promotes apoptosis via activating

Bak oligomerization and Cyt c release [140]. In summary, we found that cisplatin and paclitaxel induced Bad expression in wild type p53 expressing breast cancer cells whereas, Bad expression is only induced by cisplatin in mutant p53 expressing MDA-MB-231 cells.

In order to understand the role of Bcl-2 in cisplatin and paclitaxel induced apoptosis, MCF-7 and MDA-MB-231 cells were stably transfected with Bcl-2 plasmid. Results showed that cisplatin did not induce apoptosis in Bcl-2 over-expressing MDA-MB-231 cells (Figure 4.24) whereas apoptosis was induced in MCF-$7^{Bcl\text{-}2+}$ cells following cisplatin treatment (Figure 4.23). These results suggest that cisplatin activates a pro-apoptotic pathway that bypasses Bcl-2-mediated protection against apoptosis only in Bcl-2-overexpressing MCF-7 cells. This protection effect is accompanied with Cyt c release, MMP loss and activation of caspases [133]. Thus, previous reports noted cisplatin induced cell death type is controversial [141], our results clearly indicate that the mode of cell death triggered by cisplatin in MCF-$7^{Bcl\text{-}2+}$ cells is mitochondrial apoptosis (Figure 4.23).

In order to understand the cisplatin induced apoptosis mechanism in MCF-$7^{Bcl\text{-}2+}$ cells, the modulation of Bcl-2 family members were investigated within 72 h. Activation of apoptosis by cisplatin in MCF-$7^{Bcl\text{-}2+}$ cells occurred at a quite late time point compared to parental MCF-7 cells, which suggests a gradual engagement of the pro-apoptotic machinery to overcome Bcl-2-mediated anti-apoptotic potency (Figure 4.23). Paclitaxel treatment for 72 h did not change total amount Bcl-x_L expression level following, whereas cisplatin decreased the Bcl-x_L expression at 72 h. Neither cisplatin nor paclitaxel had any effect on Bax expression (Figure 4.25). These results showed that cisplatin diminished Bcl-2 expression whereas paclitaxel did not exert any effect of Bcl-2 expression. Together, these results suggests that cisplatin induces cell death by decreasing the expression of anti-apoptotic proteins Bcl-x_L, while paclitaxel does not show any effect on the expression levels of these proteins. Therefore, paclitaxel is not effective in inducing apoptosis in cells with increased Bcl-2 anti- apoptotic proteins.

To examine the cell and drug specific apoptotic induction in different kinds of breast cancer cells, we determined the pro-apoptotic Puma and Noxa mRNA levels in

MCF-$7^{Bcl\text{-}2+}$ cells following cisplatin treatment (Figure 4.26-4.27). Results from paralel studies from our laboratory showed that both the cytoplasmic (transcription-independent) pro-apoptotic role of p53 and its transcriptional activity in nucleus seem to be critical for cisplatin-induced apoptosis, particularly in cells with high anti-apoptotic potency due to overexpression of Bcl-2 [133]. Accordingly, the results from this study shown that Noxa, but not Puma, involved in the upstream of MMP loss in cisplatin-induced apoptosis in MCF-$7^{Bcl\text{-}2+}$ cells [133]. Induction of Noxa was shown to be p53-dependent and strictly required for cisplatin-induced apoptosis in Bcl-2-overexpressing MCF-7 cells. In a complementary study, co-immunoprecipitation analysis showed that Noxa binds to Mcl-1 and displaces Bak to promote apoptosis. In contrast, Noxa did not interact with Bcl-x_L or Bcl-2 in untreated or cisplatin-treated cells [133] suggesting that cisplatin induced Noxa expression leads to apoptosis independently from Bcl-2 expression levels.

Previous studies revealed the protective function of Bcl-2 against ROS production and lipid peroxidation [142-144], but the mechanism by which of Bcl-2 exerts these properties is not clearly understood. Here we showed that the gradual development of apoptosis is consistent with a gradual build-up of lipid peroxidation and ROS generation effects. Both ROS and lipid peroxidation have been reported to mediate cisplatin-induced apoptosis in various cancer cell types [145]. Similar to these findings, acute apoptosis induced by cisplatin was shown to be mediated by ROS without the involvement of nuclear DNA-damage response [146]. Bcl-2 itself does not possess any antioxidant activity; rather, it may act indirectly to increase the levels and/or activities of endogenous antioxidants (e.g., glutathione or superoxide dismutase) within cells [147]. Moreover, Bcl-2 over-expression has effect on some antioxidant enzymes and GSH metabolism in different cell lines. In contrary, there are number of studies established that Bcl-2 has no effect on antioxidant enzyme levels and/or activities [142, 148]. Furthermore, mitochondria are a major source of ROS production and are involved in the regulation of Bcl-2 and apoptotic cell death induced by various stimuli. In agreement with an antioxidant role of Bcl-2, silencing or inhibition of Bcl-2 increased ROS generation in MCF-7 cells (Figure 4.28). Thus, pre-treatment of HA14-1 significantly enhanced cisplatin induced ROS generation and lipid peroxidation in MCF-7 cells.

Furthermore, cisplatin, paclitaxel and Bcl-2 inhibitor, HA14-1 increased the ROS levels in MCF-7 parental cells more than Bcl-2 overexpressing cells.

Consistent with these findings, several reports showed that Bcl-2 overexpression protects cellular components such as lipids, proteins and DNA from oxidative stress. Therefore Bcl-2 eases lipid peroxidation induced by various oxidants such as H_2O_2 [144]. Induction of ROS via various chemotherapeutics can lead lipid peroxidation. In order to investigate the effect of Bcl-2 in lipid peroxidation following cisplatin and paclitaxel treatment, MDA-TBA adducts derived from cell membranes were tested using TBARS assay. The high lipid peroxidation products were established following cisplatin treatment in Bcl-2 overexpressing MCF-7 cells for 72 h. In addition, inhibition or silencing of Bcl-2 induced lipid peroxidation in MCF-7 cells. In parallel studies, lipid peroxidation end products were shown to be involved in cisplatin-induced apoptosis downstream of Noxa-induction in MCF-$7^{Bcl\text{-}2+}$ cells [133]. Furthermore, ROS generation was determined following drug treatment in MDA-MB-231 cells. Cisplatin increased ROS generation whereas paclitaxel was less potent to increase ROS. Pre-treatment of MDA-MB-231 cells with HA14-1 increased drug-induced ROS generation in MDA-MB-231 cells. ROS generation upon drug treatment was found to be reversible by additional NAC treatment (Figure. 4.34).

Table 5.1 Summary of results of the study were presented following drug treatment in MCF-7 and MDA-MB-231 cells.

		Bcl-2 ↓		Bcl-2↑
		Bcl-2 siRNA	**HA14-1**	**Plasmid transfection**
MCF-7	**Cisplatin**	Apoptosis ↑	Apoptosis↑↑ Pro-apoptotic Bcl-2 family members: Puma↑ Anti-apoptotic Bcl-2 family members: Bcl-2, Bcl-xL ↑↑ ROS generation↑↑ Lipid peroxidation↑↑	Apoptosis↑↑ Pro-apoptotic Bcl-2 family members: Bax≅, Puma and Noxa↑ Anti-apoptotic Bcl-2 family members: Bcl-2 ↓ ROS generation↑ Lipid peroxidation↑
	Paclitaxel	Apoptosis ↑	Apoptosis ↑ Pro-apoptotic Bcl-2 family members: Bax, Puma, Bad ↑ Anti-apoptotic Bcl-2 family members: Bcl-2, Bcl-xL↓↓ Bcl-2 mRNA↓↓ ROS generation↑ Lipid peroxidation↑↑	Apoptosis ↑ Bcl-2 protein level≅ Bax protein level≅ Bcl-xL protein level≅ ROS generation↑ Lipid peroxidation↑
MDA-MB-231	**Cisplatin**	Apoptosis ↑	Apoptosis↑ Pro-apoptotic Bcl-2 family members: Bax*, Puma, Bad↑ Anti-apoptotic Bcl-2 family members: Bcl-2, Bcl-xL↓ ROS generation↑	Apoptosis≅ ROS generation↑
	Paclitaxel	Apoptosis ↑	Apoptosis ≅* Pro-apoptotic Bcl-2 family members: Bax*, Puma↑ Anti-apoptotic Bcl-2 family members:Bcl-2*↓ ROS generation≅	Apoptosis≅ ROS generation↑

* Not significant

CHAPTER 6

6 CONCLUSION

Since many agents have been identified or designed to target the Bcl-2 family at the mRNA or protein level, chemoresistance mechanism is intensively explored at the molecular level for each agent. Potential effects of Bcl-2 antagonists were similar but heterogeneous cell characteristics of tumors are determinative for their efficiency. The binding affinity anti-apoptotic Bcl-2 family inhibitors or transfection quality of gene therapy elements should be optimized to the clinically achievable concentrations. Agents with high specifity provide ready opportunities to be considered in drug-resistance cases as broader acting agents may introduce unexpected responses to the chemotherapeutics and/or increase their systemic toxicities. Therefore, therapeutic potential of Bcl-2 targeting strategies should be further investigated at molecular level in various types of cancer cells.

In this study, we confirmed that cisplatin and paclitaxel induced apoptosis in MCF-7 and MDA-MB-231 cells through the induction of MMP loss, caspase activation and modulation of Bcl-2 family members. According to our results, utilization of Bcl-2 targeting strategies (siRNA or Bcl-2 inhibitors) has a therapeutic potential to increase the apoptotic efficiency of cisplatin and paclitaxel in both cell lines. However, the potentiating effect of Bcl-2 inhibitor (HA14-1) was found to be drug and cell type specific. In this study, as a first time we demonstrated that paclitaxel was less potential to induce apoptosis in HA14-1 pre-treated ER alpha negative MDA-MB-231 cells. Our results suggest that the efficiency of HA14-1 may not only depend on the inhibition of

Bcl-2 but also other pro-apoptotic and anti-apoptotic Bcl-2 family members. In that, alteration of other pro-apoptotic and anti-apoptotic Bcl-2 family members may dictate the apoptotic response when HA14-1 combined with chemotherapeutic drugs. Therefore further studies should investigate the how HA14-1 increase the drug induced apoptosis at molecular level in different cancer cell lines.

We determined that cisplatin overcame Bcl-2 or Bcl-x_L mediated resistance mechanisms in Bcl-2 overexpressing MCF-7 cells but not in Bcl-2 overexpressing MDA-MB-231 cells. Thus, p53 independent mechanisms which were activated by cisplatin in MDA-MB-231 cells could be successfully abrogated by Bcl-2. We conclude that this differential response in MCF-7 and MDA-MB-231 cells may be related to the selective activation of p53 pro-apoptotic pathway in Bcl-2 overexpressing MCF-7 cells. In addition, previous studies have suggested that cisplatin might induce cell death via mechanisms other than apoptosis but our results clearly indicate that the mode of cell death triggered by cisplatin in Bcl-2 overexpressing MCF-7 cells is mitochondrial apoptosis.

The gradual development of apoptosis was shown to be consistent with a gradual built up of ROS generation which led to lipid peroxidation, suggesting that, Bcl-2 antagonism or silencing approaches prevented the protection role of Bcl-2 against drug-induced ROS generation and lipid peroxidation in breast cancer cells. Here, we confirmed that Bcl-2 possesses an anti-oxidant role and diminishes drug-induced ROS generation. Increased lipid peroxidation in Bcl-2 silenced MCF-7 cells may lead to targeting of lipid peroxidation end products to critical proteins and membranous structures and decreased Bcl-2 levels. However exact mechanism of this observation merits further investigation.

In addition, our findings suggested that cisplatin induced lipid peroxidation was involved in apoptosis downstream induction of Noxa expression in Bcl-2 overexpressing MCF-7 cells. However, further studies should be done to clarify how lipid peroxidation end products modulate Bcl-2 family members in cell death decision placed at mitochondria.

In summary, results of this study indicate two critical important findings. First, administration of Bcl-2 antagonists or silencing tools provided a detailed understanding of drug resistance mechanism in Bcl-2 overexpressing cancer cells. The utilization of Bcl-2 targeting strategies to augment the apoptotic response induced by cisplatin and paclitaxel has therapeutical potential. Here the most critical second observation that cisplatin treatment may overcome Bcl-2 mediated chemoresistance mechanism and this effect requires intact p53 pro-apoptotic pathway at both cytoplasmic/mitochondrial and nuclear. Lipid peroxidation end products are shown to be critical to understand how cisplatin overcome Bcl-2 mediated chemoresistance mechanism.

These insights may be useful for the identification of new therapeutic target sites in Bcl-2 family proteins and elucidate alternative pathways to increase drug-induced apoptosis in drug-resistant cancer cells.

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APPENDIX A

CHEMICALS

Name of material	Suppliers
Acetic acid	Sigma A9967
Acrylamide/bis-acrylamide (30%)	Sigma A6014
Agarose low EEO	Applichem A2114
Ammonium persulphate (APS)	Sigma A3678
Anti β-actin antibody	Cell signal technology 4967
Apoptosis sampler kit	Cell signal technology 9915
Boric acid	Applichem A0768
Bromophenol blue	Applichem A4968
Bradford reagent	Biorad 500-0001
BSA	Promega W3841
Caspase inhibitors	BD Biosciences
Carbondioxide	Karbogaz
Chloroform	Merck 102431
Cisplatin	Bristol Myers Squibb
CM-H_2DCF-DA	Molecular Probes C6827
Complete^{TM} protease inhibitor cocktail tablet	Roche S29130

Coomassie brilliant blue	Merck 1.02082.0005
Developer solution	Agfa
Dimethlysulphoxide (DMSO)	Sigma D2650
Dithiothreitol (DTT)	Sigma D9779
DNA gene ruler 1kb	Fermentas SM1353
DNA loading dye 6X	Fermentas SM1353
Etilendiaminetetraaceticacid (EDTA)	Riedel-de-Haen 32221
Ethanol absolute	Riedel-de-Haen
Ethanol technical grade	Riedel-de-Haen
Ethidium bromide	Merck OCO28942
Foetal bovine serum (FBS)	Biological industries PAN
Fixer solution	Agfa, USA
Fugene HD	Roche 1107.04962095001
Glycerol	Riedel-de-Haen 15523
Glycine	Amresco 0167
Hydrochloric acid (HCl)	Merck 100314
HA14-1	Calbiochem
HEPES	Sigma S7006
IGEPAL CA-630 (NP-40)	Sigma I3021

Isoprapanol (2-propanol)	Riedel-de-Haen 24137
Potassium chloride (KCl)	Fluka 60129
Potassium hydroxide (KOH)	Riedel-de-Haen 06005
Laemmli 2X Sample buffer	Sigma S3401
Liquid nitrogen	Karbogaz
2-Mercapthoethanol	Sigma M370-1
Methanol	Riedel-de-Haen 24229
Magnesiumchloride ($MgCl_2.6H_2O$)	Sigma M9272
Sodium chloride (NaCl)	Riedel-de-Haen 13423
Sodiumbicarbonate ($NaHCO_3$)	Riedel-de-Haen 32320
Sodium hydroxide (NaOH)	Merck 106462
Non-fat dry milk	Sütaş
Paclitaxel	Bristol-Myers Squibb
Phosphate buffer saline 10X (PBS)	PAN chemical
Phosphate buffer saline 1X (PBS)	Biological industries
Penicillin/Streptomycin	Biological Industries
Phenylmethylsulphonyl fluoride (PMSF)	Sigma P7626
Plastic wrap	Cook
Pro-Apoptosis Bcl-2 family antibody sampler kit	Cell signal technology 9942

Pro-survival Bcl-2 family antibody sampler kit	Cell signal technology 9941
Rhodamine 123	Molecular Probes R302
RPMI 1640	Biological Industries
RNA loading dye 2X	Fermentas R0641
Sodium dodecyl sulphate (SDS)	Sigma L4390
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma T7029
N-acetyl cystein	Sigma A7250
Tris base	Fluka 93349
Trizol	Gibco 15596-026
Trypsin-EDTA (1X)	Biological Industries
Tween-20	Merck 822184

APPENDIX B

MOLECULAR BIOLOGY KITS

MTT cell proliferation kit (Roche)

M30 ELISA assay (Peviva AB)

siRNA transfection kit (Qiagen)

One-step RT-PCR kit (Qiagen)

RT reaction kit (Qiagen)

SYBR green assay kit (Qiagen)

ECL western blotting kit (GE Biosciences)

ECL Advance western blotting kit (GE Biosciences)

Qiagen plasmid Midi kit (Qiagen)

Qiagen plasmid Maxi kit (Qiagen)

Caspase activity apoptosis sampler kit (Invitrogen)

APPENDIX C

OTHER MATERIALS

Name of material	Supplier
Hybond P membrane (PVDF)	Amersham Biosciences RPN2020F
Kodak OMATXR film	Kodak
Mass ruler DNA ladder mix	Fermentas SM0403
Protein MW marker	Fermentas SM0441 Amersham RPN303F
RNase A	Roche1119915

APPENDIX D

EQUIPMENT

Autoclave:	Hiclave HV-110, Hirayama
	Masa üstü otoklav, Nüve
Balance:	Sartorious BP211D
	Sartorious BP221S
	Sartorious BP610
Blot module:	Biorad Mini Protean
Centrifuge:	Eppendorf, 5415C
	Eppendorf 5415D
	Eppendorf 5415R
	Heraus multifuge 3L
	Hitachi sorvall RC5C Plus
CO_2 incubator:	Binder
Deepfreeze:	-70 ^{o}C Heraus Hfu486 Basic
	-20^{o}C Bosch
Distilled water:	Milipore, Elix S
	Millipore MilliQ Academic
Electrophoresis:	Labnet Gel System
	Biorad Mini Protean
Gel Documentation:	Gel Doc Biorad
Ice Machine:	Scotsman AF20
Incubator:	Memmert model 300
	Memmert model 600

Laminar flow:	Heraus Hera Safe HS12
Magnetic stirrer:	VELP Scientifica ARE heating magnetic stirrer
	VELP Scientifica microstirrer
Microliter pipette:	Gilson Pipetman
	Finnpipette digital
	Eppendorf pipette
Microwave oven:	Bosch
pH meter:	WTW, pH 540 GLP Multical
Power supply:	Biorad Power pac 300
Refrigerator:	+ 4^{o}C Bosch
Shaker:	Forma Scientific Orbital Shaker 4520
	New Brunswick orbital shaker Innova 4330
	New Brunswick orbital shaker C25HC
Sonicator:	Vibracell 75043, Bioblock
Spectrophotometer:	Schimadzu UV-1208
	Nanodrop
	Amersham 2000 spectrophotometer
	Schimadzu UV-3150
Speed vacuum:	Savant Speed Vac Plus Sc100A
	Savant refrigerated vapor trap RVT 400
Thermocycler:	Eppendorf Mastercycler gradient
	Biorad My Cycler IQ5 quantitative PCR
Vacuum:	Heto, Master Jet Sue 300 Q
Water bath:	Huber, Polystat ccl

Awards

Dr. Gürsel Sönmez Best Thesis Research Grant 2009

Publications arising from this thesis:

Abstracts:

Arisan ED, Kutuk O, Verim A, Basaga H. Molecular mechanism of paclitaxel-induced apoptosis in ERα+/- breast cancer cell lines: MCF-7 and MDA-MB-231. FEBS JOURNAL, 273: 51-52 Suppl. 1, 2006 (Oral presentation in FEBS congress, Istanbul, 2006)

Original articles:

Arisan ED, Kutuk O, Tezil T, Bodur C, Telci D, Basaga H. Small inhibitor of Bcl-2, HA14-1, selectively enhanced the apoptotic effect of cisplatin by modulating Bcl-2 family members in MDA-MB-231 breast cancer cells. Breast Cancer Research and Treatment, 2009 Feb 24.

Kutuk O, Arisan ED, Tezil T, Basaga H. Cisplatin overcomes Bcl-2-mediated resistance to apoptosis via preferential engagement of Bak: critical role of Noxa-mediated lipid peroxidation. Carcinogenesis. 2009 Jul 3.