AIMS AND OBJECTIVES OF THE STUDY
2. AIMS AND OBJECTIVES OF THE STUDY

The aim of this doctoral thesis was to develop and make use of siRNA-based gene silencing techniques to investigate specific Bcl-xL gene target which is associated with chemotherapeutic resistance.

1. To investigate the molecular mode of action of Mitomycin-C as well as Cisplatin on parental lung cancer cell lines as well as on Bcl-xL specific shRNA transfected clone cell lines.

2. To analyze the antiproliferative effects of these two anti-cancer drugs and also to understand the effects of these anti-cancer drugs on the cell cycle and apoptosis.

3. To investigate the changes in the gene expression pattern of cells after treatment with Mitomycin-C and Cisplatin in order to understand molecular pathways involved in the induction of apoptosis or interference with the cell cycle program.

4. To study the gene expression profiles for the investigation of molecular pathways which confer resistance to Lung cancer cells.

5. To use RNAi-mediated knock-down technology to understand the impact of the regulated genes on apoptosis induced by two anti-cancer drugs.
REVIEW OF LITERATURE
3. REVIEW OF LITERATURE

3.1. LUNG CANCER

Lung cancer is the most commonly diagnosed cancer annually since 1985. Worldwide, there are 1.61 million new cases of lung cancer per year, with 1.38 million deaths, making lung cancer the leading cause of cancer-related mortality (Ferlay et al., 2008). In India, approximately 63,000 new lung cancer cases are reported each year (Ganesh et al., 2011). The major risk factor for developing lung cancer is the use of tobacco and this disease is often viewed solely as a smoker's disease. However, significant numbers of patients with lung cancer have no history of smoking.

Lung cancer is characterized by uncontrolled cell growth in tissues of the lung. If the condition is left untreated, this growth can spread beyond the lung in a process called metastasis into nearby tissue and eventually, into other parts of the body. Most of the cancers that start in lung are known as primary lung cancers which are carcinomas that derive from epithelial cells. Globally, lung cancer in non-smokers demonstrates a marked gender bias, occurring more frequently among women. Up to 15% of the total lung cancer incidences occur among non-smokers. Causes/contributing factors other than smoking have been identified and include genetic predisposition, e.g. by allelic variants of genes important for detoxification, loss of tumor suppressor genes and exposure to environmental factors such as passive smoking, chemicals and exposure to radon (Dasgupta et al., 2006).

In particular, a high proportion of Asian women diagnosed with lung cancer are non-smokers. Smoking related carcinogens act on both proximal and distal airways inducing all major forms of Lung cancer; cancers arising in never-smokers target the distal airways and favour the occurrence of adenocarcinoma (Toh et al., 2006).
However, it is well established that the major cause of lung cancer is tobacco smoking, responsible for approximately 85-90% of bronchogenic carcinoma (Dasgupta et al., 2006). Tobacco smoke contains hundreds of known human carcinogens and smoking is associated with increased cancer rates in virtually every organ of the body including the lung, oral cavity, esophagus, colon, pancreas, bladder, bone marrow, cervix and kidney (Shields, 2002). Prevention is the obvious and only measure to take against lung cancer. More information together with new legislation is needed to deter people from tobacco smoking.

3.1.1. Lung cancer classification

Lung carcinomas are classified into four histological types: small cell lung carcinoma (SCLC), squamous cell lung carcinoma (SCC), adenocarcinoma (AC), and the more undifferentiated large cell lung carcinoma (LC). The histological features and clinical courses make SCLC a separate entity. The other three types are referred to as non-small cell lung cancer (NSCLC, WHO-classification 1977). The characteristics of the different histological types of lung cancer are summarized in Table 3.1.

It is common to find tumors with mixed histology. The cell of origin in lung cancer has not been identified but is thought to be a pluripotent epithelial stem cell that would differentiate into the cell types present in the lung, namely the mucus producing cylindrical cells, neuroendocrine cells and type I and II pneumocytes.

3.1.2. Genetic and molecular changes in lung cancer

The hallmarks of cancer, as described by Hanahan and Weinberg, include growth signal self-sufficiency, insensitivity to growth-inhibitory signals, evasion of
Table 3.1 Histopatological classification of lung carcinoma and some characteristic traits.

<table>
<thead>
<tr>
<th>Squamous cell carcinoma (~30%)</th>
<th>Adenocarcinoma (~30%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Closely correlated with smoking (dose-dependent)</td>
<td>* Most common type of lung cancer in women and non-smokers</td>
</tr>
<tr>
<td>* Tends to spread locally</td>
<td>* Worldwide incidence increasing</td>
</tr>
<tr>
<td>* Highly expressed genes encoding proteins with detoxification/antioxidant properties</td>
<td>* Highly expressed genes encoding small-airway-associated and immunologically related proteins</td>
</tr>
<tr>
<td></td>
<td>* K-RAS mutations frequently reported</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Large cell carcinoma (5-10%)</th>
<th>Small cell lung carcinoma (15-20%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Very primitive, undifferentiated cells</td>
<td>* Occurs almost exclusively in smokers, more prevalent in women than men</td>
</tr>
<tr>
<td>* High tendency to metastasize</td>
<td>* Tendency to disseminate early</td>
</tr>
<tr>
<td></td>
<td>* Initially chemo- and radiosensitive, becoming resistant</td>
</tr>
<tr>
<td></td>
<td>* TP53 is mutated in almost all tumors</td>
</tr>
</tbody>
</table>
programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Genetic and molecular alterations in lung cancer are described below that enable all these features.

3.1.2.1. Growth factor independence

Overexpression of growth-receptors, excretion of growth factors, and dysregulation of the downstream signaling pathways represent ways to obtain independence of growth factor stimulation.

Insulin-like growth factor I (IGF-I) expression is elevated in more than 95% of SCLCs and 80% of NSCLCs (Vincent et al., 2013; Minuto et al., 1986; Reeve et al., 1990). Moreover, a causal role for IGF-II in lung adenocarcinoma was demonstrated in a mouse model (Moorehead et al., 2003).

The IGF-I receptor is present in most lung cancers (Vincent et al., 2013; Kaiser et al., 1993). Up to 80% of SCCs overexpress EGF-R, and 20% of ACs (adenocarcinomas) overexpress Her2/neu (Hirsch et al., 2002). These growth factor receptor tyrosine kinases normally transduce proliferative extracellular signals. In addition, they are now therapeutic targets since the recent development of tyrosine kinase inhibitors.

Virtually all SCLC and up to 70% of NSCLC tumors exhibit neuroendocrine features with production of neuroendocrine peptides, such as gastrin releasing peptide, bradykinin, gastrin, insulin-like growth factor I, and vasopressin, of importance for growth stimulation by para- and autocrine loops (Hirsch et al., 2002).

The small GTPase p21-Ras, encoded by the RAS oncogene, is important for the transduction of receptor tyrosine kinase signaling and subsequent activation of
the mitogen-activated protein kinase (MAPK)-cascade, or the phosphatidylinositol-3-kinase pathway (Macaluso et al., 2002). Mutated Ras is locked in a permanent active state and is thus independent of upstream signals. The main members of the RAS gene family are H-RAS, K-RAS and N-RAS. RAS mutations, especially in K-RAS, were found in ~30% of NSCLC (Macaluso et al., 2002), mainly ACs, and is a marker for poor prognosis (Sekido et al., 1998).

3.1.2.2. Cell cycle dysregulation

Cell cycle progression is tightly controlled via the family of cyclins and their partners, the cyclin-dependent kinases (CDKs). Loss of negative control functions, due to mutation of key regulators, is commonly found in cancer and cause insensitivity to growth-inhibitory signals.

The Rb protein, encoded by the retinoblastoma tumor suppressor gene, is responsible for the timely release of transcription factors of the E2F-family to initiate the entry into the G1-phase of the cell cycle. Inactivating mutations of RB is found in >90% of SCLC but only in 15% of NSCLC (Kelley et al., 1995). However, the p16INK4A gene, encoding an inhibitor of CDK4 and thus negatively regulating the Rb-signaling pathway, is inactivated in >50% of NSCLC (Sekido et al., 1998). Moreover, cyclin D1, the partner of CDK4, is overexpressed in NSCLC (Reissmann et al., 1999). All these changes contribute to the loss of negative cell cycle regulation.

MYC is a proto-oncogene encoding a nuclear transcription factor that is essential for cell proliferation and prevention of differentiation. The function of Myc is regulated through heterodimerization. The Myc/Max heterodimer recognizes the consensus sequence with high affinity, resulting in transcription, while formation of the Max/Mad complex is thought to antagonize Myc function. Abnormal expression of all three members of the Myc family (n-Myc. l-Myc and Myc) is commonly found
in both SCLC and NSCLC (Richardson and Johnson, 1993). In SCLC, Myc and n-Myc overexpression is associated with poor prognosis (Viallet and Minna, 1990).

3.1.2.3. Evasion of apoptosis

Evasion of cell death can be conferred by mutation of the tumor suppressor gene TP53, located at chromosome 17p13.1. This is one of the most conspicuous genetic alterations in cancer and is found in >90% of SCLC and >50% of NSCLC (Greenblatt et al., 1994). TP53 encodes the “Guardian of the genome”, p53. Upon exposure to cellular stress, such as hypoxia or DNA damage, the p53 protein is activated by phosphorylation, resulting in accumulation in the cell nucleus. The induction of p53 can result in either cell cycle arrest or apoptosis through transactivation/transrepression of a number of target genes. An intact p53-response is of paramount importance for the integrity of the genome (Gudkov and Komarova, 2003).

Bcl-xL is a potent anti-apoptotic protein that was found highly expressed in 75% of NSCLC, biopsies (Jiang et al., 1995; Kaiser et al., 1996). The Bcl-2 family consists of both pro- and anti-apoptotic proteins, and the intricate balance between them determined the final influence on apoptotic signaling. The mechanisms of apoptosis inhibition by Bcl-2 family proteins are discussed in detail in section 3.2.1.6.

3.1.2.4. Immortalization

Differentiated cells normally undergo a limited number of possible cell divisions before they become senescent. Telomers are structures of repetitive DNA sequences that cap the ends of chromosomes and protect against loss of chromosomal information, stabilizing chromosomes from degradation and illegitimate recombination. Due to the “end replication problem”, the telomers are shortened at each mitotic cycle and this telomeric shortening is considered to act
like a biological clock, ticking towards cellular senescence. The DNA-polymerase telomerase is capable of restoring telomeric length and is therefore a potential oncogene. Activation of human telomerase and amplification of the telomerase reverse transcriptase gene (hTERT), encoding the rate-limiting component of telomerase activity, was found in both SCLC and NSCLC (Sekido et al., 1998; Zhang et al., 2000).

3.1.2.5. Angiogenesis

Both primary and metastatic tumor growth require blood supply via the recruitment of vessels from the surrounding tissue. Vascular endothelial factor (VEGF) and basic fibroblast factor (bFGF) are the two most important inducers of tumor angiogenesis. VEGF-expression was found to negatively correlate with overall survival, and the receptor for VEGF, Flt-1, is frequently expressed in SCC tumors (Volm et al., 1997). The bFGF expression was detected in ~70% of NSCLCs (Takanami et al., 1996) but the prognostic use of this finding is not so clear.

3.1.2.6. Metastasis

Metastatic potential is determined by several factors; the ability to detach from the basal membrane and survive without adhesion-dependent stimulation, the expression of proteases necessary for invasion of the blood- or lymphatic circulation and other tissues, and finally self sufficiency in growth stimulation and the capability of angiogenesis (Hanahan and Weinberg, 2000). For tissue invasion, the tumor needs to activate proteolytic enzymes to breech the barrier of the basal membrane and to dissolve the extra-cellular matrix. The family of matrix metalloproteinases (MMPs) is important for tumor invasion, metastatic potential and tumor-related angiogenesis. However, not all lung cancers express the MMPs believed to be most
important in promoting the neoplastic process, and there are conflicting reports regarding the prognostic significance of MMPs in lung cancer (Liang et al., 2013; Bonomi, 2002).

Smokers were found to have multiple pre-neoplastic alterations in the mucous membranes outlining the major bronchi. These lesions harbored abnormalities identical to some of those found in invasive carcinoma, including TP53 mutations, upregulation of Myc and Ras proteins, cyclin D1 overexpression, Bcl-2 overexpression, allele loss at several loci (3p, 9p, 8p and 17p) and aneuploidy (Hirsch et al., 2001).

The loss of alleles at 3p is observed in more than 90% of SCLC tumors and approximately 80% of NSCLC tumors (Kok et al., 1987). The FHIT gene, localized at 3p14.2, may represent one of several potential tumor-suppressor genes located on chromosome 3p. Eighty per cent of SCLC tumors show abnormalities of this gene (Sozzi et al., 1996). Loss of the FHIT gene results in the accumulation of diadenosine tetraphosphate that could lead to stimulation of DNA synthesis and cell proliferation (Croce et al., 1999).

3.1.3. Lung cancer therapy - the problem of resistance

The clinical treatment of lung cancer, summarized in Table 3.2, is determined by the histological type and tumor stage. The prognosis for NSCLC correlates closely to the tumor stage. For apparently local disease classified as stage IA (i.e. a tumor less than 3 cm in diameter and no evidence for local lymph node engagement) the 5-year survival after surgery is ~75% thus underscoring the problem of early occult dissemination (Porrello et al., 2002). NSCLC is frequently resistant to both drug- and radiotherapy and complete response to therapy is rare. Therefore, resistance to treatment presents a major problem. The resistance
<table>
<thead>
<tr>
<th>NSCLC</th>
<th>SCLC</th>
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<tbody>
<tr>
<td><strong>Stage IA-B (local disease) and IIA-B (local lymph node engagement)</strong>&lt;br&gt; * Surgery&lt;br&gt;   * Curative radiotherapy if surgery is contraindicated&lt;br&gt;   * Adjuvant or neo-adjuvant chemo- or radiotherapy</td>
<td><strong>Limited disease (i.e within one side of the thorax)</strong>&lt;br&gt; * Concomitant platinum-based chemo-radiotherapy with PCI for responders</td>
</tr>
<tr>
<td><strong>Stage IIIA (local lymph node engagement)</strong>&lt;br&gt; * Surgery in selected cases&lt;br&gt;   * Radiotherapy alone, benefits 5-10% of patients&lt;br&gt;   * Chemotherapy + radiotherapy / neoadjuvant therapy&lt;br&gt;   * Post-operative adjuvant chemotherapy</td>
<td><strong>Extensive disease</strong>&lt;br&gt; * Combined chemotherapy +/- PCI&lt;br&gt; * Palliative radio- or chemotherapy</td>
</tr>
<tr>
<td><strong>Stage IIIB (regional lymph node engagement)</strong>&lt;br&gt; * Chemo- or radiotherapy alone&lt;br&gt; * Chemotherapy + radiotherapy</td>
<td></td>
</tr>
<tr>
<td><strong>Stage IV (distant metastasis)</strong>&lt;br&gt; * Palliative chemotherapy (platinum-based)&lt;br&gt;   * New chemotherapy agents&lt;br&gt;   * Palliative radiotherapy</td>
<td></td>
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</table>
mechanisms are numerous and diverse and depend on the detoxifying capacity of the cells, DNA repair capacity, drug uptake and excretion (Volm and Rittgen, 2000).

SCLC is initially among the most drug and radiosensitive tumors. If the disease is local, i.e. is confined to one side of the thorax and can be covered within an irradiation-field, concomitant chemo- and radiotherapy can be curative. The probability of complete clinical remission is even higher for patients with limited-stage SCLC than for patients with stage III NSCLC after combined radio- and chemotherapy (Erridge and Murray, 2003). However, only approximately 10% of SCLC patients are present with limited disease. SCLC is associated with early metastasis and relapse within 2 years is typical. Relapse is associated with a quick development of resistance to both drug- and radiotherapy, making the overall long-term prognosis for SCLC very poor (less than 10%).

3.1.3.1. Different Therapeutic methods used to treat Lung Cancer

By the time lung cancer is diagnosed, it is often too late to cure. However, if the cancer cannot be cured, there are several types of treatment that are used to slow or stop the disease. These treatments also relieve symptoms.

The right treatment depends on several factors, including:

- Patient's health
- Stage of the cancer at the time of treatment

Possible treatment includes:

- Surgery
- Radiation
- Chemotherapy
- Targeted therapies
3.1.3.1.1. Surgery

In early stages, surgery to remove the cancer may be able to cure the disease. Such surgery may include the removal of part of a lung known as a lobe or it may require the removal of an entire lung. Nearby lymph nodes are also removed to see if the cancer has spread. Surgery may also be used to drain fluid that has built up outside the lungs.

3.1.3.1.2. Radiation

This treatment uses high-energy beams or particles to kill cancer cells or keep them from growing. Current techniques allow doctors to better target the cancer and reduce radiation damage to nearby healthy cells.

Radiation therapy can be done in two ways:

- externally (by machine)
- internally (often via radioactive pellets placed near the cancer cells)

Radiation is often used when surgery is not an option. It is also used to reduce symptoms.

3.1.3.1.3. Chemotherapy

This treatment uses anticancer drugs to shrink tumors or stop their growth and spread. The drugs are either injected or taken by mouth. They are often given in several cycles. Each cycle typically lasts three to four weeks.

For NSCLC, most people get two chemotherapy drugs or combination chemotherapy. There are several chemotherapy drugs available and will be chosen by the doctor based on the specific situation. These are some of the chemotherapy drugs commonly used to treat NSCLC.

- Cisplatin (Platinol)
Carboplatin (Paraplatin)
Paclitaxel (Onxol, Taxol)
Docetaxel (Taxotere)
Vinorelbine (Navelbine)
Gemcitabine (Gemzar)
Etoposide (Toposar, Vepesid)
Irinotecan (Camptosar)
Vinblastine (Alkaban-AQ, Velban, VLB)
Mitomycin (Mutamycin)
Ifosfamide (Ifex)
Pemetrexed (Alimta)
Erlotinib (Tarceva)
Bevacizumab (Avastin)
Cetuximab (Erbitux)
Albumin-bound paclitaxel (Abraxane)

Chemotherapy is given in cycles that last 3 to 4 weeks. The patients will have
time off in between cycles so that their body can recover. They may have 4 to 6
treatment cycles of chemotherapy. Although they can expect to feel tired following
each treatment, many people can continue to work and enjoy their regular activities.

3.1.3.1.4. Targeted therapies
These drugs target specific cell changes caused by cancer. The drugs include:
Avastin (bevacizumab)
Erlotinib (erlotinib)
3.1.3.1.5. Mitomycin

The Mitomycins (MMC) are a family of aziridine-containing natural products isolated from Streptomyces caespitosus or Streptomyces lavendulae (Danshiitsoodol et al., 2006). One of these compounds, mitomycin C, finds use as a chemotherapeutic agent by virtue of its antitumour antibiotic activity. It is given intravenously to treat upper gastro-intestinal (e.g. esophageal carcinoma), anal cancers, and breast cancers, as well as by bladder instillation for superficial bladder tumours.

MMC is an anticancer drug which is typically used as a first or second line regimen to treat NSCLC, and is often combined with other chemotherapeutic agents for advanced NSCLC treatment (Babiak et al., 2007; Booton et al., 2006). MMC causes intrastrand DNA cross-links that lead to collapsing replication forks and generation of DNA double-strand breaks that are typically repaired by homologous recombination repair (HRR) (Dronkert and Kanaar, 2001; Helleday et al., 2008; Warren and Hamilton, 1996). The therapeutic value of MMC depends on the capability of the cells to remove DNA damage (McHugh et al., 2001).

In addition to DNA, mitochondria are postulated as a cellular target for MMC. The membrane permeability transition of mitochondria has been shown to be involved in a variety of toxic and oxidative forms of cell injury as well as apoptosis (Lee et al., 2004).

**Mechanism of action**

Mitomycin C is a potent DNA crosslinker. A single crosslink per genome has shown to be effective in killing bacteria. This is accomplished by reductive activation followed by two N-alkylations. This drug is reductively activated to 2,7-diaminomitosene, which cross-links DNA and subsequently leads to cell death (Sartorelli, 1986; Crooke and Bradner, 1976). Both alkylations are sequence specific.
for a guanine nucleoside in the sequence 5'-CpG-3' (Tomasz, 1995). Potential bis-alkylating heterocyclic quinones were synthetised in order to explore their antitumoral activities by bioreductive alkylation (Renault et al., 1981). Apart from the mechanism of MMC-induced DNA cross-linkage, its mode of action has been also associated with the formation of DNA monoadducts and free radical-induced DNA strand breaks (Sartorelli et al., 1994; Tomasz and Palom, 1997).

### 3.1.3.1.6. Cisplatin

Cisplatin, cisplatinum, or *cis*-diaminedichloroplatinum(II) (CDDP) is a chemotherapy drug. It was the first member of a class of platinum-containing anticancer drugs, which now also includes carboplatin and oxaliplatin. These platinum complexes react *in vivo*, binding to and causing crosslinking of DNA, which ultimately triggers apoptosis.

Cisplatin is administered intravenously as short-term infusion in normal saline for treatment of solid malignancies. It is used to treat various types of cancers, including sarcomas, some carcinomas (e.g. non small cell lung cancer, and ovarian cancer), lymphomas and germ cell tumors. It is used in combinations with bleomycin and vinblastine in testicular cancer (Praveen and Chowdary, 2013).

Cisplatin is a commonly used therapeutic agent in NSCLC, together with a third-generation anticancer drug, such as vinorelbine, gemcitabine, or the taxanes; it is the standard regimen used in the first-line treatment of advanced NSCLC. Of these regimens, CDDP has been evaluated in multiple phase III trials and showed consistent superior efficiency.

Most chemotherapeutic agents including CDDP induce cell apoptosis. Activation of a family of cysteine proteases or caspases is essential for apoptotic cell death (Salvesen and Dixit, 1997). It is believed that DNA damage caused by
chemotherapeutic drugs induces the release of mitochondrial cytochrome c, which facilitates activation of initiator caspase-9, thereby triggering activation of downstream effector caspases, such as caspase-3 (Green and Reed, 1998).

**Mechanism of Action**

Following administration, one of the chloride ligands is slowly displaced by water (an aqua ligand), in a process termed aquation. The aqua ligand in the resulting $[\text{PtCl(H}_2\text{O})(\text{NH}_3)_2]^{+}$ is itself easily displaced, allowing the platinum atom to bind to bases. Of the bases on DNA, guanine is preferred. Subsequent to formation of $[\text{PtCl(guanine-DNA)}(\text{NH}_3)_2]^{+}$, crosslinking can occur via displacement of the other chloride ligand, typically by another guanine (Alderden et al., 2006). Cisplatin crosslinks DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible.

Most notable among the changes in DNA are the 1,2-intrastrand cross-links with purine bases. These include 1,2-intrastrand $d(GpG)$ adducts which form nearly 90% of the adducts and the less common 1,2-intrastrand $d(ApG)$ adducts. 1,3-intrastrand $d(GpXpG)$ adducts occur but are readily excised by the nucleotide excision repair (NER). Other adducts include inter-strand crosslinks and nonfunctional adducts that have been postulated to contribute to cisplatin's activity. Interaction with cellular proteins, particularly HMG domain proteins, has also been advanced as a mechanism of interfering with mitosis, although this is probably not its primary method of action.

Cisplatin is frequently designated as an alkylating agent; it has no alkyl group and so cannot carry out alkylating reactions. It is correctly classified as alkylating-like. A lot of these drugs has been very successful in the treatment of many malignancies; they are, however, therapeutically limiting when faced with problems such as toxicity
and resistance. With CDDP and MMC as the drugs of choice for NSCLC treatment and the emergence of drug resistance as a critical problem in therapy, we examined the influence of Bcl-xL shRNA on drug sensitization in NSCLC cell lines and explored the mechanism of NSCLC cell apoptosis after treatment with CDDP and MMC.

3.2.1. Relationship between cytotoxicity, apoptosis and necrosis

Cytotoxicity is simply the cell-killing property of a chemical compound (such as a food, cosmetic, or pharmaceutical) or a mediator cell (such as a cytotoxic T cell), independent from the mechanisms of death. There are two experimentally distinguishable mechanisms of cell death: necrosis, the “accidental” cell death that occurs when cells are exposed to a serious physical or chemical insult, and apoptosis, the “normal” cell death that removes unwanted or useless cells.

3.2.1.2. Apoptosis

Apoptosis — the regulated death of a cell — is a complicated process. The decision to die cannot be taken easily, and the activities of many genes influence a cell's fate by playing a role in its self-destruction programme. Once the decision is made by the organism, proper execution of the apoptotic programme is essential for the coordinated activation and execution of multiple sub-programmes. Multi-cellular organisms often need to get rid of excess cells, regarding this purpose; they use an organized molecular program. As important as cell division and cell migration, regulated cell death allows the organism to tightly control cell numbers and tissue size, and to establish homeostasis. Programmed cell death acquired a number of names over the past two centuries (Hengartner, 2000). The term apoptosis was finally adopted by Currie and co-workers in 1972 to describe a common type of programmed
cell death that they repeatedly observed in various tissues and cell types (Currie and Wyllie, 1972). They noticed that these dying cells shared many morphological features, which were distinct from the features observed in cells undergoing pathological and necrotic cell death, and they suggested that these shared morphological features should be the result of a common and conserved cell death programme (Wyllie et al., 1980). Apoptosis can now be defined not only by morphology, but also by molecular and biochemical mechanisms. Studies within the past few years have revealed that a complex group of molecules that make up the “death machinery” regulates apoptosis. This pathway can be explained as an active process of cellular self-destruction with distinctive morphological and biochemical features (Yin and Dong, 2003). Two major apoptotic pathways have been defined in mammalian cells: the death-receptor-mediated or the extrinsic apoptotic pathway and the mitochondrial or the intrinsic apoptotic pathway. The extrinsic pathway can be activated through cell surface receptors by external stimuli such as growth factor withdrawal, UV or γ radiation, chemotherapy agents, free oxygen radicals, and heat shock leading to activation of cysteine-aspartic-acid-proteases (Caspases) (Jacobson et al., 1997). The intrinsic pathway is activated through mitochondrion as a result of cellular stress and DNA damage. In this pathway, the BCL-2 family of genes and proteins are perhaps the most important regulators. These proteins can also be responsible for bridging signals from the death-receptor pathway to the mitochondrial pathway.

BCL-2 family consists of both anti-apoptosis and pro-apoptosis members. While the pro-apoptosis members function as sensors for death signals and executors of the death program, the anti-apoptosis members inhibit the initiation of the death program. Also caspase cascade activity is essential in both pathways, thus a short description of caspase proteins as well as two apoptosis pathways will be provided.
The BCL-2 family will be discussed in detailed as the expression of genes of this family is the central part of this study. A flow chart explaining the Apoptosis cascade in simple terms is given as Figure 3.1.

3.2.1.3. The role of caspases in apoptosis

Horvitz’s group for the first time discovered the role of caspases in regulation of programmed cell death during development of the nematode worm *Caenorhabditis elegans* (Horvitz, 1999) and because of his efforts in this field, he was awarded the Nobel Prize in 2002 for studies of the genetic regulation of *C. elegans* development (Wong and Hengartner, 2005). Most of the morphological changes that are observed during apoptosis are caused by a set of cysteine proteases activated specifically in apoptotic cells. These death proteases are very similar to each other in their sequence, and are part of a large protein family known as the caspases. Caspases are highly conserved through evolution, and can be found from nematodes to humans. Over a dozen caspases have been identified in mammals; about two-thirds of them have been suggested to function in apoptosis (Cohen, 1997). The list of effector and initiator caspases are enlisted in Table 3.3.

**Table 3.3 Caspases involving in apoptosis in mammals**

<table>
<thead>
<tr>
<th>Initiator Caspases</th>
<th>Effector Caspases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase 2</td>
<td>Caspase 3</td>
</tr>
<tr>
<td>Caspase 8</td>
<td>Caspase 6</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>Caspase 7</td>
</tr>
<tr>
<td>Caspase 10</td>
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</tbody>
</table>
Note: The apoptotic cascade is comprised of four main phases: initiation, signaling, effector, and degradation. The pathway is initiated by a stimulus, which signals certain receptors on the cell surface. These signals initiate the Bcl-2 family of survival factor proteins, releases mitochondrial proteins, and activate effector or executioner caspases. The cascade ends with the degradation phase followed by either phagocytosis in vivo or secondary necrosis in vitro. Throughout the pathway there are a number of ways the cascade is regulated including survival factors, caspase inhibitors and individual pathway blockers (noted on left). (Figure from Mastrangelo and Betenbaugh, 1998)
All known caspases possess an active-site cysteine, and cleave substrates after aspartic acid residues (Nicholson, 1997). A caspase's distinct substrate specificity is determined by the four residues amino-terminal to the cleavage site (Earnshaw, 1999). Caspases have been divided into subfamilies based on their substrate preference, extent of sequence identity and structural similarities. Caspases can be thought of as the central executioners of the apoptotic pathway because they cause most of the morphological changes that differentiate apoptotic cells from other types of dying cells. Indeed, eliminating caspase activity, either through mutation or applying pharmacological inhibitors, will slow down or even prevent apoptosis (Elmore, 2007). Thus, blocking caspases can increase cell survival.

Activation of caspases does not result in the degradation of all of the cellular proteins. Rather, caspases selectively cleave a restricted set of target proteins, usually at one or more positions in the primary sequence and always after an aspartate residue. In most cases, caspase-mediated protein cleavage results in inactivation of the target protein, which can be either a single polypeptide chain enzymes, such as poly ADP-ribose polymerase, or a complex macromolecular network (Yin and Dong, 2003). Caspase-mediated cleavage of specific substrates explains several of the apoptotic morphological features. For example, cleavage of the nuclear lamins is required for nuclear shrinking and blebbing (Rao et al., 1996). However, caspases can also activate proteins, either directly, by cutting off a negative regulatory domain, or indirectly, by inactivating a regulatory subunit. Several important caspase substrates have been identified in recent years. One of the most exciting discoveries was the elucidation of the mechanism of activation of the nuclease responsible for nucleosomal degradation. Wyllie et al., (1980), first described that nuclease cuts the genomic DNA between nucleosomes to generate DNA fragments of approximately 180 base pairs. The presence of this DNA ladder has been used extensively as a marker for apoptotic cell
death. Nagata showed that the DNA ladder nuclease (caspase-activated DNase, or CAD) is present in living cells prior to apoptosis as an inactive complex with an inhibitory subunit, called ICAD (Nagata, 2000); caspase-3-mediated cleavage of ICAD activates CAD and causes the release and activation of the catalytic subunit (Sakahira et al., 1998).

Since caspases activity plays very important role in apoptotic process, a proper understanding of apoptosis is required to understand how caspases are activated. Similar to other proteases, caspases are synthesized as an inactive pro-enzyme. Most caspases are activated by proteolytic cleavage of the pro-enzyme between the p20 and p10 domains. Interestingly, all these cleavage sites occur at Asp-x sites- candidate caspase substrate sites- suggesting the possibility of autocatalytic activation (Thornberry et al., 1997). Indeed, the simplest way to activate a pro-caspase is to expose it to another, previously activated caspase molecule.

This “caspase cascade” strategy of caspase activation is used extensively by cells to activate three short prodomain caspases, caspase-3, -6 and -7. These three downstream effector caspases are considered the most active members of the caspase family, and are usually more abundant than other ones. The caspase cascade is a useful method to amplify and integrate pro-apoptotic signals inside the cell. The entire caspase activation cascade is summarized in Figure 3.2. The effector caspases are usually activated 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 and, most likely, much more complex that currently understood.
3.2.1.4. Extrinsic apoptotic pathway

The extrinsic or death receptor pathway is activated by extracellular ligands such as CD95L and tumor necrosis factor (TNF-α) that bind to death-receptor superfamily members such as CD95 and tumor necrosis factor receptor I and/or II (TNFRI, TNFRII). The CD95L /CD95 receptor and the TNFR/TNF-α are the most well-known initiators of the extrinsic pathway (Elmore, 2007). Binding of these ligands to their receptors induce receptor clustering and formation of a death inducing signalling complex (Figure 3.3). This complex recruits pro-caspase-8 through the adaptor molecule FADD (Fas-associated death domain protein). Pro-caspase-8 activates caspase-8 which stimulates cleavage and activation of several different effector caspases. Caspase-8 activation can be blocked by recruitment of the degenerate caspase homologue c-FLIP (Meier et al., 2000; Debatin, 2004).

3.2.1.5. Intrinsic apoptotic pathway

The intrinsic or mitochondrial apoptotic pathway is activated in response to either external or internal signals including DNA damage (Meier et al., 2000). In addition, this pathway can be activated when Cytochrome C is released from the intramembrane space of the mitochondria into the cytosol (Figure 3.3). This release is under tight control by the BCL-2 family of proteins.

The BCl-2 protein is usually attached to the intracellular membranes such as endoplasmic reticulum and mitochondria; however, some other members of this family such as Bax, Bad, and Bid proteins, can shuttle between the cytosol and organelles. The cytosolic forms of these proteins are inactive. Pro-apoptotic signals redirect these proteins to the mitochondria, where they are activated and initiate the destruction of the cell.
The Bcl-2/Bax ratio indicates the relative amounts of anti- and pro-apoptotic proteins of the BCL-2 family. This ratio decreases in response to death signals such as DNA damage (through p53) or reactive oxygen species (Debatin, 2004). Members of the BCL-2 family stimulate release of Cytochrome C and other proteins from the mitochondrial intra-membrane space into the cytosol. Cytosolic Cytochrome C binds to apoptotic protease activating factor 1 (Apaf-1) which in turn stimulates binding to procaspase-9 to form a complex known as the apoptosome. When bound to the apoptosome, procaspase-9 is activated. Activated caspase-9 in the apoptosome activates the effector caspases 3, 6, and 7 by cleavage of their respective pro-caspases (Yin and Dong, 2003).

The extrinsic and intrinsic apoptotic pathways meet each other at the level of caspase-3 activation. Caspase-3 activity is inhibited by the inhibitors of apoptosis proteins (IAP), whose activity in turn can be antagonized by the Smac (second mitochondria-derived activator of caspases) or DIABLO (direct IAP binding protein with low pi) proteins released from mitochondria. Downstream of caspase-3, the apoptotic programme results to the cell death. Bid, which is a pro-apoptotic member of the BCL-2 family, provides the integration between two pathways. Activated caspase-8 mediates Bid cleavage to tBid and results in its translocation to mitochondria, where its pro-apoptotic activity promotes cytochrome C release by oligomerization to Bak and Bax proteins, other pro-apoptotic members of this family, and pore formation in mitochondrial membrane (Esposti et al., 2001). Under most conditions, however, the death-receptor and mitochondrial pathways works independently with minimal “cross-talk”.
3.2.1.6. BCL-2 family and its role in apoptosis

The B-cell CLL/lymphoma 2 (BCL-2) family of genes and proteins present a critical intracellular checkpoint of apoptosis. The members of this family are categorized into two main groups. The first group consists of the anti-apoptotic members that share a high degree of structural and functional similarities with Bcl-2 protein, while the second one includes proteins that have fewer similarities to Bcl-2 and show pro-apoptotic activity. The second group is in turn divided into two subgroups, the Bcl-2-associated X protein (Bax)-like death factors and the BH3-only proteins (Table 3.4).

Table 3.4. The BCL-2 family of proteins in different organisms

<table>
<thead>
<tr>
<th>Death function</th>
<th>Organism</th>
<th>Members</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-apoptotic</td>
<td>Mammals</td>
<td>Bcl-2, Bcl-xL, Mcl-1</td>
<td>BH Multi-domain</td>
</tr>
<tr>
<td></td>
<td>C.elegans</td>
<td>CED-9</td>
<td>BH Multi-domain</td>
</tr>
<tr>
<td></td>
<td>Xenopus</td>
<td>XRI, XRII</td>
<td>BH Multi-domain</td>
</tr>
<tr>
<td>Pro-apoptotic</td>
<td>Mammals</td>
<td>Bax, Bak, Bok</td>
<td>BH Multi-domain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C.elegans</td>
<td>EGL-1</td>
<td>BH3-Only</td>
</tr>
</tbody>
</table>

Interestingly, this family of genes and their protein products are evolutionarily conserved and their structures have not been changed from nematodes to human. A number of viruses encode Bcl-2 homologs, including most gamma herpes viruses. Most of these viral homologs are anti-apoptotic, probably because viruses need to keep the infected cells alive for latent and persistent infection (Hardwick, 2001). One of the key features of the BCL-2 family proteins is that members of this family share
sequence similarities in four BH (Bcl-2-homology region) domains—the BH1, 2, 3, and 4 domains—although not all of them poses all four domains (Figure 3.4). These domains correspond to an $\alpha$-helical domain, which mutagenesis studies have indicated are important for the various molecular functions and for protein-protein interactions (homo and hetero dimerization) among this family. Furthermore, most of the members contain a hydrophobic carboxyterminal transmembrane (TM) domain that is probably responsible for their membrane localization (Nguyen et al., 1993).

The BH1 and BH2 domains are necessary for the death-repression function of the anti-apoptosis molecules, whereas the BH3 domain is required for the death-promotion function of the pro-apoptotic ones. In addition, the BH4 domain, which is present mainly in the anti-apoptotic molecules, is also important for death-inhibition functions (Nguyen et al., 1993; Puthalakath, 2002). It seems that the “BH3-only” molecules, such as Bid, Bik, and Bad, are sensors for the peripheral death signals and are able to activate the “multi-domain” executioner molecules, Bax or Bak (Cheng et al., 2001). This process in some ways resembles the caspase cascade, in which the initiator caspases activate the effector caspases.

### 3.2.1.7. Protein interactions among BCL-2 family members

The BCL-2 family of proteins can interact with each other and also with several other proteins. In fact, the first pro-apoptosis BCL-2 family protein, Bax was cloned based on its interaction with Bcl-2. Many other BCL-2 family proteins were also cloned based on this type of interaction. The most common type of interaction is between anti-death and pro-death members, such as Bcl-2 versus Bax. This interaction can result in antagonistic action of the two types of molecules and thus could control the death program (Korsmeyer, 1998). Interestingly, not all anti-death molecules can interact with all pro-death molecules. It seems that some members of one group will
preferentially bind to some members of the other group. For example, the anti-death molecule Bcl-2 binds to Bax, but not to Bak (Ke et al., 2001) and these molecules may only antagonize the function of those molecules to which they bind. This type of selectivity suggests that specific amino acids required for particular interactions may only exist in some but not all of the family members. In addition, it may also suggest that in certain tissues and for certain death stimuli, a specific set of BCL-2 family proteins is critically involved.

The second type of interaction occurs between two pro-death members, usually between a molecule with only one BH3 domain and a multi-domain molecule, such as Bad and Bik with Bax or Bak. Such interactions could be important for the activation of the multi-domain executioner molecules such as Bax or Bak (Ke et al., 2001).

The third type of interaction is “multimerization” of the same molecule. This has been observed in both anti-death molecules, such as Bcl-2 or Bcl-xL, and pro-death molecules, such as Bax and Bak (Cheng et al., 2001). The ability of Bax or Bak to oligomerize has been considered an important factor in their function to produce or open the mitochondrial channels for the purpose of releasing mitochondrial apoptotic factors such as Cytochrome C (Wei et al., 2000). Overall, the relative ratio of pro-survival (Bcl-2-like) and pro-apoptotic (Bax-like and BH3 only) proteins seems to determine the cell sensitivity or resistance to the apoptotic stimuli. Further details about the genes for their relative expression levels are provided in detail as follows.

3.2.1.8. Death-inhibiting and promoting genes of BCL-2 family

3.2.1.8.1. Anti-apoptotic Bcl-2

Bcl-2 is a proto-oncogene that was identified at the chromosomal translocation breakpoint, between chromosomes 14 and 18, t (14; 18), in non-Hodgkin’s follicular B-cell lymphomas. It promotes tumorogenesis by preventing cell death instead of
increasing cell division rate as well as arresting cells in the Go/G1 phase of the cell cycle (Yin and Dong, 2003). Based on this fact, Bcl-2 was classified as a new class of oncogenes. Protein product of indicated gene is 26 kDa consisting of 239 amino acids in human and contains a single highly hydrophobic domain at its C-terminus that helps the protein to localize mainly in the mitochondrial outer membrane.

Bcl-2 gene expression was observed in a wide variety of fetal tissues, whereas it was claimed that this gene shows restricted expression in more rapidly proliferating and differentiating cells in adult tissues (Kirkin and Zornig, 2004). Bcl-2 gene can be up-regulated by tumour suppressor P53 (Honoki et al., 2000).

The main role of Bcl-2 gene product in the process of apoptosis is based on its ability to inhibit ion conductive channels formation in mitochondrial membrane, and in most cases to prevent mitochondrial disruption and the release of Cytochrome C. Therefore, Bcl-2 protein can inhibit the following association of Cytochrome C with Apaf-1 and therefore suppress the activation of caspase-9 (Yin and Dong, 2003). Based on this scenario, two anti-apoptotic roles are suggested for Bcl-2 protein which in one of them its activity is mitochondrial dependent and in the next one Bcl-2 may play a role in the inactivation of initiator caspases, such as caspase-2, that acts upstream or independently of Cytochrome C release and mitochondrial involvement (Cory and Adams, 2002).

3.2.1.8.2. Anti-apoptotic Bcl-X

BCL-2L1 (BCLX, BCL-2L, BCL-X). The BCL-2-like 1 (BCL-2L1) gene maps on chromosome 20q11.21 and consists of 3 exons and 2 introns, encoding a 233 amino acid protein, localised to the outer mitochondrial membrane. This gene undergoes alternative splicing, and, up to now, three different splicing variants have been identified including the Bcl-xL, Bcl-xS, and Bcl-xyγ. The large transcript Bcl-xL
is pro-survival and mainly expresses in long-lived cells and localizes in the mitochondrial membranes (Gonzalez et al., 1994).

Bcl-xL protein shows high similarities with Bcl-2 and contains the BH1 to BH4 conserved domains as well as a hydrophobic region at its C-terminus. According to different crystallography reports, the BH1, BH2 and BH3 motifs of Bcl-xL are very close to each other, thus, forming a “hydrophobic pocket” through that the BH3 region of pro-apoptotic molecules interact with this protein (Yin and Dong, 2003). For example, the presence of an anti-apoptotic molecule, such as Bcl-2 or Bcl-xL, can inhibit the activation of the pro-apoptotic Bax. Therefore, Bcl-xL, like the Bcl-2 protein, regulates programmed cell death by interaction and blocking the death-promoter proteins. Minn et al., (1997), suggested that Bcl-xL may regulate survival by regulating the permeability of the intracellular membranes, preventing the removal of the Cytochrome C in cytosol, and taking care of the membrane integrity. On the other hand, it has been reported that Bcl-xL can bind to Apaf-1, like the Bcl-2 function, and then form a complex that prevents the activation of caspase-9 (Hu et al., 1998). The Bcl-xL gene is transcriptionally reactive (Van Houten et al., 1997). Its expression is up-regulated by irradiation and insulin-like growth factor-1 (IGF-1) (Apte et al., 1995; Miyashita et al., 1994).

3.2.1.8.3 Pro-apoptotic Bax

Apte et al., (1995), while applying somatic cell hybrid and in situ hybridization showed that the human Bax gene is located on chromosome 19q13.3–q13.4. This gene consists of 6 exons and 5 introns that encode a 21 kDa protein contains the three conserved regions BH1, BH2 and BH3, very similar to Bcl-2 structure, which provide the susceptibility to form hetero-dimers with Bcl-2 and imply its death promoting function. The formation of hetero-dimers between Bax and the other members of the
BCL-2 family play important role in the regulation of cell death scenario (Yin and Dong, 2003).

It seems that the tumour suppressor P53 can transcriptionally regulate Bax expression. Actually, in vitro experiments have shown that P53 up-regulates the Bax gene, while in vivo studies using P53 -/- mice have indicated the opposite results (Miyashita et al., 1994).

*Bax* is the first death-promoting member of the BCL-2 family to be identified, and it was co-immunoprecipitated with Bcl-2 (Korsmeyer, 1998). Different studies have demonstrated that in healthy cells Bax protein is localized in cytosol in monomeric conformation, after exposure to a death signal; this protein translocates to the mitochondria and homodimerizes with another Bax or heterodimerizes with other pro-apoptotic members such as Bak protein (Suzuki et al., 2000).

The presence of anti-apoptotic molecules such as Bcl-2 and Bcl-xL, can inhibit the activation of Bax following a death signal (Gross et al., 1999). However, the death promoting function of Bax is not associated with its ability to interact with these molecules. Bax mediates apoptosis through a mitochondrial-mediated pathway that can be either caspase-associated or not, with the caspase-dependent mitochondrial pathway being based on the release of cytochrome C from the mitochondrial membranes (Yin and Dong, 2003).

Bax is an important gene for the control of cell death. Cells that over express this gene show enhanced apoptosis, whereas Bax-null cells are resistant to apoptosis. Bax expression has also been associated with tumour development (Korsmeyer, 1998).
3.2.1.8.4. Pro-apoptotic Bak

Bcl-2 homologous antagonist/killer 1 (Bak1) has been cloned as a Bcl-2-related gene, which consists of 6 exons and maps to chromosome 6p21.3 on the human genome (Ulrich et al., 1997), encoding a 211-amino acid protein with a relative molecular weight of 23400 (Herberg et al., 1998). Bak gene product is very similar to Bax protein structurally and functionally. This protein contains the conserved domains BH1 to BH3 and enhances apoptotic cell death (Korsmeyer, 1998). The widespread tissue distribution of Bak messenger RNA suggests that cell-death-inducing activity is broadly distributed, and tissue-specific modulation of apoptosis is controlled primarily by regulation of molecules that inhibit apoptosis (Thomadaki, 2006). Furthermore, Leu et al., (2004), demonstrated that Bak is regulated at the transcriptional level, with over expression of P53. The anti-apoptotic Bcl-xL interacts with Bak and inhibits its activity (Sattler et al., 1997).

3.2.1.8.5. Pro-apoptotic Bad

Bad or Bcl-xL/Bcl-2-associated death promoter is a death-promoting member of the BCL-2 family that was initially detected because of its ability to interact with the anti-apoptotic proteins Bcl-xL and Bcl-2. This gene maps to chromosome 11q13.1 and its protein product is about 168 amino acids. The interesting fact about Bad protein is that this protein neither hetero-dimerizes with other members of the family, such as pro-apoptotic Bax, nor homodimerizes. Bad forms an inactivating dimer with Bcl-xL, but doesn’t have any effect on the anti-apoptotic activity of Bcl-2 because this protein only has the BH3 domain, which is essential for its heterodimerization with the other family members, and slight similarity with Bcl-2 (Thomadaki, 2006; Petros et al., 2004). Kaipia et al., (1997) indicated that the Bad protein shows a wide tissue distribution with higher expression levels in lung, ovary, uterus and brain. It seems
that the active form of Bad, which bind to Bcl-2 and Bcl-xL in the mitochondria, is the
dephosphorylated form, while some factors such as IGF-1 inactivates this protein
through phosphorylation (Thomadaki, 2006; Korsmeyer, 1998). It has been suggested
that Bad mediates apoptosis through a caspase-dependent pathway, since the cell
death could be inhibited in granulosa cells, which already had shown apoptosis
because of over expression of Bad mRNA, using a caspase inhibitor (Kaipia et al.,
1997). Therefore, the association of Bad/Bcl-2/Bcl-xL results in the release of
Cytochrome C, which in turn, activates the proteolytic caspase-mediated cascade
(Korsmeyer, 1998).

3.2.1.8.6 Pro-apoptotic Bik

The Bik gene maps on chromosome 22q13.3 and translates to a 19kb protein
with 5 exons and 4 introns (Verma et al., 2000). It has been demonstrated by applying
Northern blot analysis that Bik gene expression is elevated in heart and skeletal
muscle (Verma et al., 2000) and restricted in kidney, pancreas, lung, liver, prostate
and testis (Thomadaki, 2006).

Although the protein heterodimerizes with anti-apoptotic molecules, it has been
found that this interaction is not sufficient to cause death. It has been reported that the
heterodimerization with death-inhibiting proteins such as Bcl-2 and Bcl-xL is not
enough for the death-promoting effect of the complex. Mutation analysis by deletion
of the BH3 domain showed that the pro-apoptotic activity is present even in the
absence of the heterodimerizationion (Thomadaki, 2006). This fact shows that Bik
protein mediates apoptosis through a mechanism which is not dependent on its
dimerization. Bik also may be rapidly turned over during apoptosis (Marshansky et al.,
2001).
3.3. Chemotherapy resistance

Resistance to chemotherapy action has long been a problem in the treatment of cancer. This phenomenon is thought to account for treatment failure in over 90% of metastatic disease. Drug resistance may be intrinsic, occurring at the time of first line treatment, or acquired, developing after treatment with chemotherapeutics. There are many mechanisms by which cancer cells can develop this resistance. Many different genes that contribute to various mechanisms of resistance have been identified. Ultimately, the presence of the drug, at its required intracellular concentration, is vital for chemotherapeutic drug efficacy and so, much research has focused on resistance associated with drugs. Tumors can often develop resistance to drugs other than that they were treated with and this is termed multi-drug resistance. The effects of anti-cancer agents and specific gene targeting bio-molecules like RNA interference inducers on these genes which confer chemotherapeutic resistance have been closely looked into as combinational therapeutic options.

3.4. RNA interference

The basic principle underlying RNAi involves the disruption of mRNA by the use of homologous dsRNA. Small interfering RNAs (siRNAs) are generated intracellularly from endogenous and exogenous dsRNA molecules by the cleavage activity of Dicer, a ribonuclease III- type protein (Hamilton and Baulcombe, 1999; Zamore et al., 2000; Bernstein et al., 2001; Elbashir et al., 2001). Dicer contains an RNA helicase domain at the N-terminus, an RNA binding PAZ domain (Piwi/Argonaute/Zwille], two RNase III domains and a dsRBD (double stranded RNA binding domain) (Sawh and Duchaine, 2012; Collins and Cheng, 2005). This Dicer cuts the dsRNA into short 19-21 duplexes having symmetric 2 nucleotide overhangs at the 3’- end and a 5’- phosphate along with a 3’- hydroxyl group which
are referred to as siRNAs. The siRNAs get incorporated into a nuclease containing multi-protein complex referred to as the RNA induced silencing complex (RISC) (Nykanen et al., 2001). The phosphorylation of the siRNA at the 5’ end is required for its entry into the RISC (Khvorova et al., 2003). The helicase domain of the RISC assembly binds to one end of the duplex and unwinds the siRNA in an ATP dependent manner. The RISC gets activated as soon as the anti-sense strand attaches to the complex. The activated RISC containing the anti-sense strand reaches the target mRNA and induces an endonucleolytic cleavage of the mRNA within the target site (Ahlquist, 2002). This cleavage leads to degradation of the entire mRNA molecule while the RISC is recovered for further cleavage cycles. This mechanism clearly highlights the fact that intracellular presence of siRNAs complementary to the target mRNA is important for the induction of RNAi.

3.4.1. Types of RNAi inducers

siRNAs

The dsRNAs are processed by a combination of RNAse III Dicer, TRBP and PACT into small interfering RNAs (siRNAs) about 20-24 mer nucleotides in length with a phosphate group at both the 5’ ends along with hydroxyl groups and two nucleotide overhangs at both the 3’ends (Elbashir et al., 2001; Bernstein et al., 2001; Nykanen et al., 2001). The siRNAs thus processed consist of a sense strand and a complementary anti-sense strand. The siRNAs unwind due to helicase activity of the pre-RISC domain and the help of an AGO protein (Kwak and Tomari, 2012) which later cleaves the sense strand (Matranga et al., 2005; Rand et al., 2005; Czech and Hannon, 2011; Ghildiyal and Zamore, 2009), then the mature RISC containing the anti-sense strand along with the AGO protein attaches and cleaves the complementary mRNA as shown in Figure 3.5. (Zamore et al., 2000).
**shRNAs**

Short hairpin RNAs (shRNAs) are a class of siRNAs which are expressed using U6, H1 or tRNA promoter (Paul et al., 2002; Brummelkamp et al., 2002; Kawasaki and Taira, 2003). In comparison to siRNA, these shRNAs have many advantages like long lasting silencing effects, cost effective as well as easy delivery methods. Generally shRNA is transcribed in cells from a DNA template as a single stranded RNA molecule (about 50-100 bases). The complementary regions are spaced by a hairpin loop thereby getting the name “short hairpin” RNA (Bernstein et al., 2001).

**miRNAs**

The miRNAs are a class of RNAi inducers that bring about post-transcriptional gene silencing and are processed from endogenously expressed transcripts. The Primary miRNAs (pri-miRNAs) are approximately >100 nucleotides long with one or more microRNA stem loops are the initial products of microRNA genes. The pri-miRNAs are transcribed by RNA polymerases [(Borchert et al., 2006; Monteys et al., 2010; Ozsolak et al., 2008) and are processed by the microprocessor complex which consists of an enzyme Drosha and a subunit DGR8 into ~70 nucleotide precursors, called precursor miRNAs (pre-miRNAs) (Zeng et al., 2005; Lee et al., 2003; Gregory et al., 2004). The pre-miRNAs are exported to the cytoplasm with the help of a protein called exportin 5 (Lund et al., 2004; Yi et al., 2003), where a complex that contains the enzyme Dicer, TAR RNA-binding protein (TRBP) and PACT converts the pre-miRNAs into miRNA–miRNA* duplexes (sense and antisense strand) (Provost et al., 2002; Förstemann et al., 2005). The duplex associates with an Argonaute (AGO) protein within the precursor RNAi-induced silencing complex (pre-RISC). One strand of the duplex (the antisense strand) is removed. The mature RISC contains the antisense strand, which directs the complex to the target mRNA and the miRNA anti-
sense strand binds to the 3’ UTR resulting in translational repression. (Hafner et al., 2011; Kiriakidou et al., 2007; Chendrimada et al., 2007).

3.4.2. Delivery of siRNA

The major challenge in the use of siRNA as therapy lies in the delivery of these bio-molecules into the desired cell, tissue or organ. Successful delivery of chemically synthesized siRNAs or in vitro transcribed siRNAs in mice models can be brought about by cationic liposome based system (Sorensen et al., 2003; Sioud and Sorensen, 2003; Hamidreza et al., 2012). A number of innovative delivery methods have been developed by combining siRNAs with other molecules like conjugation of cholesterol to the sense strand of the siRNA (Soutschek et al., 2004), siRNAs in combination with antibody-protamine fusion (Song et al., 2003), siRNAs complexed with cyclodextrin nanoparticles, exosome nanoparticles (Van den Boorn et al., 2011; Kesharwani et al., 2012) and aptamer conjugated siRNAs (Zhou and Rossi, 2010; McNamara et al., 2006). Among the varied methods of delivery the use of scaffolds is in the forefront of current research (Monaghan and Pandit, 2011).

A scaffold is composed of a natural or synthetic biodegradable material or a composite which provides structural support; it must be capable of cellular infiltration and also ensure safe release of encapsulated biomolecules. Scaffolds have been used in the delivery of RNAi molecules and they are more effective than conventional delivery systems (Kulkarni et al., 2010; De Laporte and Shea, 2007). Scaffold mediated delivery has the capacity to maintain bioactivity of siRNAs for longer time duration thereby increasing the chances of transfection. A major advantage in using a scaffold is that it protects the siRNA from immune response as well as degradation by serum nucleases and proteases (O'Rorke et al., 2010).
Most of these approaches resulted in cell or tissue specific targeting, thereby ensuring efficient in vivo siRNA delivery (Figure 3.6).

### 3.4.3. Delivery of shRNA

Apart from chemically synthesized siRNAs, it is also possible to generate DNA based expression cassettes that express shRNA or separate sense and antisense 21 mers from pol III promoters (Brummelkamp et al., 2002; Paddison et al., 2002; Lee et al., 2002; Sui et al., 2002). A conventional shRNA expression cassette includes a pol III promoter along with 19 nucleotides of sense strand of the target sequence, a 4-10 nucleotide base loop followed by the complementary antisense strand of the target sequence and finally a group of four to six uridines as the terminator. It is also possible to incorporate a dual promoter system whereby the separately expressed RNA strands hybridize to produce functional siRNAs (Lee et al., 2002; Miyagishi and Taira, 2002). Once the shRNAs reach the cytoplasm they get processed by Dicer to yield functional siRNA duplexes which will follow the conventional RNAi machinery to degrade the target mRNA.

Expression systems that use pol III promoters (Figure 3.6) produce long term silencing in cell culture systems which are less favorable for therapeutic applications. The expression can be modulated by using inducible systems. Inducible systems are normally controlled by the inducer with dose dependent and reversible regulation of transcription. A number of pol III based systems have been regulated by inducers like tetracycline or ecdysone. The use of doxycycline, a tetracycline analog has successfully been applied for in vivo study in mice (Wang and El-Deiry, 2004). The use of ecdysone has been successful in cell culture system (Gupta et al., 2004).

Viral promoters like CMV promoters are incorporated in pol II based expression systems which produce 5’ capped and 3’ polyadenylated transcripts but
lack in efficient transcription start sites when compared to pol III based promoters (Xia et al., 2002). Many pol II based miRNA systems have been developed and they are being assessed for their in vivo applicability (Chung et al., 2006; Xia et al., 2006; Sun et al., 2006).

Viral vector based shRNAs that use Adenovirus and Adeno-associated virus (AAV) have been found to be efficient delivery vehicle to carry out transient shRNA expression (Hall et al., 2010; Li et al., 2005; Osada et al., 2005; Ragozin et al., 2005).

Retro viral vectors like Murine Leukaemia Virus (MLV) can bring about silencing of target genes by stable transfection as the virus encoded proteins get integrated into the host cell chromosomal DNA (Schaser et al., 2011).

Lentiviral vectors like Human immunodeficiency virus, Feline immunodeficiency virus or Equine infectious anemia virus (HIV-1, FIV or EIAV) have been successfully used in a number of studies and have proven to be quite promising (Matrai et al., 2010; Dittgen et al., 2004; Bahi et al., 2005; Singer et al., 2005). RNAi technology can indeed offer therapeutic approaches to treat a number of chronic infections.

3.4.4. Delivery of miRNA cassettes

In order to bring about stable gene silencing shRNA or miRNA constructs can be developed. It is possible to use miRNA expression cassettes to explore the fundamental pathways involved in the biological system or to develop therapeutic strategies for debilitating diseases.

Viral vectors have been successfully employed in the delivery of miRNAs to target specific genes and many viral vectors have been developed to carry out basic research as well as for gene therapy experiments (Heilbronn and Weger, 2010).
Adenoviral vectors are commonly used in the delivery of miRNA expression cassettes for in vitro studies. In vivo study using adenoviral vectors harbouring miRNAs showed successful targeting of genes in the brain as well as the liver (Xia et al., 2002).

A number of in vivo studies have been carried out using AAV vector borne RNAi bio-molecules to treat muscular dystrophies, cancers, metabolic diseases, cardiac diseases as well as neurodegenerative diseases (Grimm, 2009; McCown, 2005).

Studies by Mclaughlin et al., (2007) showed that lentiviral vectors could be successfully used to deliver miRNAs to inhibit BCR-ABL oncogene, thereby preventing regrowth of leukemic cells both in vitro as well as in vivo. An important issue that needs to be taken into consideration when targeting genes using miRNA expression cassettes is that these miRNA systems must not in any way hinder the endogenous RNAi machinery which can disrupt the function of cellular miRNAs that regulate their natural targets. It is necessary to carry out further studies to elucidate whether these RNAi based techniques can be safely applied as therapeutic tools in medicine.

### 3.5. RNAi in Cancer therapy

The use of chemotherapy has been detrimental to both cancer cells as well as normal cells since it lacks the selectivity to distinguish tumour cells from normal cells. RNAi therapy can be used to specifically target cancer cells. The use of siRNAs to inhibit proliferation of cancer cells has been reported in a number of in vivo and in vitro studies. In vitro studies highlighting chromosomal translocations in lymphomas and leukemias result in oncogenic gene fusions which have been inhibited by RNAi (Radhakrishnan et al., 2004). Allele specific targeting by RNAi based approaches on
single nucleotide mutant forms of tumour suppressor genes such as p53 and ras have been successful in different types of cancer (Brummelkamp et al., 2002; Yang et al., 2003; Cioca et al., 2003; Martinez et al., 2002).

Aptamer-siRNA combinations were successfully used to bring about tumour regression in prostate cancer cells by targeting surface expressed tumour cell marker namely a prostate-specific membrane antigen (PSMA) (Martinez et al., 2006). Another successful study involving siRNAs complexed in atelocollagen was successfully used to limit angiogenesis tumor growth \textit{in vivo} by silencing vascular endothelial growth factor (VEGF) (Takei et al., 2004). The use of cyclodextrin nanoparticles to deliver anti-Ews-Fli1 siRNAs was effective in blocking metastasis in ewing’s tumors (Hu-Lieskovan et al., 2005; Simmons et al., 2012). RNAi has been effectively used to inhibit AKT2 kinase, a class of Protein kinase which is over expressed in malignant gliomas and confers chemotherapeutic resistance to the malignant cells (Cui et al., 2012).

\textbf{ABCC4} (ATP-binding cassette sub-family C member 4) is highly expressed in Pancreatic cancer tissues. RNAi was successfully used to downregulate ABCC4 expression in pancreatic cancer cell lines like Panc-1 and BxPC-3 cells which in turn resulted in inhibition of proliferation and cell cycle arrest (Zhang et al., 2012).

Glycogen synthase kinase-3 beta (GSK-3b), a protein kinase plays an important role in tumor formation in different cancers. Knockdown of the GSK-3b gene using sequence specific siRNAs in pancreatic cancer xenograft mice models resulted in inhibition of tumour and angiogenesis (Zhou et al., 2012).

RNAi has also been used as a combinational gene therapy to target human telomerase reverse transcriptase (TERT) and epidermal growth factor receptor (EGFR) resulting in effective apoptosis in hepatocellular carcinoma cell lines as well as tumour growth inhibition in xenograft mice models (Hu et al., 2011).
3.5.1. RNAi in Tumorigenesis

Many genes have been targeted using RNAi based technology in different tumour cell models and the knock down of these genes have made new inroads in therapy. Some of the genes that have been studied include oncogenes, telomerase, growth factor receptor genes, signalling molecules and other genes.

An important oncogene Bcl-2 is over expressed in many human tumours. A study by Fu et al., (2005), demonstrated that siRNA targeting Bcl-2 induced apoptosis in 50% of the cells in vitro and shRNAs against Bcl-2 suppressed tumour growth by 60% in mice with xenograft tumour.

In vitro studies using synthetic siRNA specific for Bcl-2 when introduced in combination with cationic liposomes inhibited the expression of Bcl-2 protein and inhibited growth of human tumour cell lines. This combination of liposome complexed bcl-2 siRNA also exhibited strong anti-tumour activity in mouse models having liver metastasis as well as in xenograft models of human prostate cancer (Yano et al., 2004). A study by Lima et al., (2004), demonstrated that down regulation of bcl-2 or another antiapoptotic gene, X-IAP (X-linked inhibitor of apoptosis) by using specific siRNAs sensitized breast cancer MCF-7 cells to anticancer drugs like etoposide and doxorubicin.

In vitro studies by Ling and Li, (2004) showed that shRNAs specific against survivin, a gene which is up-regulated in many cancers (Kim et al., 2003), silenced the expression of survivin and resulted in apoptosis of the transfected cells. RNAi induced down regulation of survivin in esophageal squamous cell carcinoma and caused significant inhibition of cancer cells both in vitro and in vivo (Wang et al., 2005). Human rhabdomyosarcoma xenografts when treated with a cocktail of survivin-shRNA encoding plasmids over two weeks resulted in 70% reduction in tumour growth (Caldas et al., 2006).
A member of the signal transduction and activation of transcription (STAT), the STAT3 gene is frequently activated in different types of cancer. RNAi specific to STAT3 resulted in inhibition of DU-145 prostate cancer cell line by inducing apoptotic cell death (Lee et al., 2004). STAT3 siRNA inhibited the growth of Hep2 human laryngeal cancer cell line, resulting in apoptosis and down regulation of Bcl-2 expression (Gao et al., 2005).

The multiple drug resistance (MDR1) gene product P-glycoprotein is over expressed in cancer and poses a major problem in chemotherapeutic treatment of cancer. Retroviral mediated shRNA specific to MDR1 sensitized cancer cells to cytotoxic drugs (Pichler et al., 2005). Similarly a study by Hua et al., (2005) reported that the suppression of MDR1 gene using siRNA expression vector reversed drug resistance to doxorubicin in human uterine sarcoma cell line.

Polo-like kinase 1(PLK1) is a serine/threonine kinase which plays an important role in mitosis as well as in malignant transformation. It has been found that siRNAs specific against PLK1 on Non-small cell lung cancer (NSCLC) cell lines resulted in reduced cell proliferation as well as increased cellular apoptosis and sensitized the cells to chemotherapy.

An important genetic alteration in the protein tyrosine kinase pathway leads to a disease called chronic myeloid leukemia (CML) which occurs as a result of recurrent chromosomal translocation between chromosome 9 and 22 leading to the formation of a hybrid BCR-ABL gene. This gene encodes for a deregulated protein tyrosine kinase which plays an important role in the pathogenesis of chronic myeloid leukemia. Sequence specific siRNAs were used to target BCR-ABL activity (Wilda et al., 2002; Wohlbold et al., 2003; Scherr et al., 2005; Withey et al., 2005). RNAi inhibited BCR-ABL dependent cell growth and induced apoptosis in CML cells.
Further studies revealed that siRNAs against BCR-ABL increased the sensitivity of leukemia cells to a drug called imatinib which targets the deregulated protein tyrosine kinase (Wohlbold et al., 2003).

Another important gene cyclophilin A (CypA) is over expressed in most non-small lung carcinomas. Down regulation of CypA using specific siRNAs in human lung tumour cells resulted in reduced growth of Xenograft tumours along with decreased cancer cell proliferation and increased apoptosis both in vitro and in vivo (Howard et al., 2005). The potential anti-tumour applications of RNAi based techniques offer great hope in designing effective anti-cancer therapy.

3.5.2. RNA interference in Lung Cancer

The use of siRNA technology has increased the chances of combating lung cancer. The use of specific siRNAs targeting survivin encapsulated in PEGylated LPD nanoparticles resulted in down regulation of survivin gene and showed pronounced anti-tumour effect as well as enhanced apoptosis along with inhibition of tumour cell growth in lung cancer cells (Li et al., 2006). Similarly the use of epidermal growth factor receptor specific siRNAs in combination with LPD nanoparticles in Lung cancer xenograft mice model resulted in tumour growth inhibition (Li et al., 2008).

In another study, the use of Akt1 specific siRNAs into urethane induced lung cancer mice model showed downregulation of Akt1 gene thereby resulting in inhibition of tumour growth (Xu et al., 2008).

3.6. Bcl-xL as a Target Gene for anti-cancer therapy

Bcl-X functions as a Bcl-2-independent regulator of apoptosis. It has been demonstrated from earlier studies that alternative splicing results in two distinct Bcl-X mRNA species. The protein product of the larger mRNA, Bcl-xL, encodes a protein
comparable in function to the anti-apoptotic proto-oncogene Bcl-2. The second mRNA species, Bcl-XS, encodes a protein that promotes apoptotic activity Bcl-xL which can form ion channels upon insertion into synthetic lipid vesicles or planar lipid bilayers suggesting that Bcl-xL brings about cell survival by influencing the permeability of the intracellular membranes to which it is distributed. Bcl-xL exists in both soluble and membrane-bound forms. In cells undergoing apoptosis, soluble Bcl-xL shifts to the membrane-bound form, possibly forming ion channels in mitochondria. Bcl-xL prevents apoptosis by limiting the availability of cytochrome c (cyt c) in the cytosol. Bcl-xL acts upstream of caspase-3 and overexpression of Bcl-xL corresponds to an absence of caspase-3 activation in cells treated with various chemotherapeutic agents (Granville et al., 1998). Compounds that inhibit Bcl-2 and Bcl-xL activity sensitize cancer cells to apoptosis and hence these genes are considered as potential therapeutic targets in devising effective anti-cancer agents (Cuddihy et al., 2008). The expressions of anti-apoptotic Bcl-2 proteins are clinically related to multidrug resistance (MDR) in several types of cancer. MDR, the major cause of chemotherapeutic failure in cancer, is a multifactorial phenomenon. Anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL), inhibit cell death by forming complexes with pro-apoptotic ones (Bax, Bad). Similarly, the over expression of Bcl-xL was also related to chemoresistance and tumor progression in several types of tumors. The search for new drugs able to overcome resistance mechanisms is of great interest for cancer therapy (Fernandes et al., 2008).

Based on the above review it is clear that there is scope for developing innovative method to target cancer cells. Is is necessary to develop specialized therapeutic methods using RNAi bio-molecules to specifically target expressed genes in cancer cells without damaging normal cells. Since most of the RNAi inducers target expressed cells in all cells irrespective of whether it is a cancer cell or a normal cell.
There is a need to develop an innovative way of targeting the genes expressed in cancer cells alone by incorporating an miRNA sponge region which attract the miRNAs which are abundantly found in normal cells but absent in cancer cells. These miRNAs degrade the RNAi inducer when it approaches the normal cells.

When specific anti apoptotic genes are down regulated by RNAi bio-molecules, they become sensitized to low doses of anticancer agents. In this study Bcl-xL gene was selected as an effective target for RNA interference based Gene silencing.