CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO CANCER

1.1.1 Current Status of Cancer Knowledge

Cancer is a large group of diseases that arises from malfunctioning biological cells and are characterized by an uncontrolled proliferation (abnormal cell cycle regulation), and resistance to death (apoptosis resistance) ultimately disrupting the organization of tissue (Bartek and Lukas 2001). In certain cases the cells additionally gains the potential to migrate and grow at distant sites (metastasis), and have the capacity to induce new blood vessels (attract endothelial cells) (Hanahan and Weinberg 2000). Carcinogenesis, transformation of normal cells into cancerous cells, is a multistep process wherein the genomes of new cancer cells acquire mutant alleles of the proto-oncogenes, tumor-suppressor genes, and other genes that control, cell proliferation (Lowe and Lin 2000). Tumors which are confined to the site of origin are generally called benign, while those having metastatic ability are called malignant tumors. In general, human tumors are classified based on the tissue type and most malignancies arise from epithelial tissue (carcinoma). Other malignancies are indicated by the terms sarcoma (mesenchymal origin), leukemia and lymphoma (neoplasms of hematopoietic system) and melanoma (from melanocytes) (Ruddon 1995).

Once transformed from normal state, cancer cells create their own signals for their sustained growth, further proliferation and progression and
transmit the required signals between proteins through an interconnected complex process commonly referred to as signal transduction (Weinberg 2006).

1.1.2 Causes of Cancer

Cancer is a diverse class of diseases which differ widely in their causes. Tumor initiation begins with the accumulation of multi-gene mutations, which are the result of the interaction between genetic host factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism) and external agents, which may act together or in sequence to initiate or/and then promote carcinogenesis (Weinberg 2006). External carcinogenic agents can be categorized as physical (ultraviolet, heat, hypoxia); chemical (smoking, heavy metals) and biological (viral or bacterial infections) agents. These factors contribute to gene mutations and changes in many genes are required in the process of tumor initiation and progression. Thus it is concluded that cancer is a progressive disease, where the progressive errors slowly accumulate until a cell begins to act contrary to its function in the body.

In a broader view, two set of genes are primarily involved in carcinogenesis (Nebert 2002). Mutations in proto-oncogenes lead to increased amount of the product protein and its activity. Mutated proto-oncogenes now called as oncogenes (e.g. ras) disturb the normal balance of cell cycle regulation in the cell, eventually leading to uncontrolled growth. On the other hand, Tumor suppressor genes (e.g. p53) code for anti-proliferation signals and proteins that controls mitosis and cell growth. The functions of tumor suppressor genes is to arrest the progression of the cell cycle under instructions from cellular stress or DNA damage and allowing the cells to undergo DNA repair, preventing mutations from being passed on to daughter
cells. Mutations in these suppressor genes lead to replication of cancerous cells (Weinberg 1996). In precise terms, oncogenes with dominant gain of function mutations and tumor suppressor genes with recessive loss of function mutations contribute to the initiation of tumorigenesis process (Lowe and Lin 2000).

### 1.1.3 Cancer Incidence

Despite significant investments in capital, manpower, and intellectual innovations for the development of cancer therapies over the past several decades, cancer still remains a powerful threat with high mortality rates across the globe. For the year 2009, in the United States alone, a total of 1,479,350 new cancer cases and 562,340 deaths from cancer are projected to occur as per the American Cancer Society (Ahmedin Jemal et al 2009). It is estimated that there will be 16 million new cancer cases and 10 million cancer deaths per year by the year of 2020 with developing countries accounting for half of the deaths (World Health Organization). In United States, for the year 2009, ACS predicts cancers of the prostate, lung and bronchus, and colon and rectum account for about 50% of all newly diagnosed cancers among men. Prostate cancer alone accounts for 25% of incident cases in men. Breast cancer is expected to account for 27% of all new cancer cases among women followed by lung and bronchus, and colon and rectum among others (Ahmedin Jemal et al 2009). However, in India, the most common cancers found in males were cancers of the lung, pharynx, oesophagus, tongue and stomach with tobacco related cancers accounting for about 52% of all cancers. Further epidemiological data demonstrated that cancers of the cervix, breast, ovary, oesophagus and mouth were common among Indian women. Breast and cervical cancer are the two most important cancer types and account for one-third of all cases diagnosed in women of the developing world and in
India, it is estimated that 59,000 women may die because of breast cancer in the year 2010 (Bobba and Khan 2003).

1.2 BIOLOGICAL PROPERTIES OF CANCER CELLS

Clinically, though cancer is a large group of diseases, in terms of molecular and cell biology it represents a relatively small number of diseases caused by abnormal gene expressions with similar molecular defects in cell functions. Thus, the vast catalogue of cancer cell genotypes is a manifestation of six essential alterations (Figure 1.1) in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these physiologic changes - novel unwanted capabilities acquired during tumor development - represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues (Hanahan and Weinberg 2000).

1.2.1 Self-Sufficiency in Growth Signals

Normal cells proliferate in response to an array of external, mostly locally produced, growth factors produced by one cell type to activate a second. Signalling molecules such as diffusible growth factors, extracellular matrix components bind to transmembrane receptors, which in turn transmit the growth signals into the cell thereby allowing them to replicate. Many of the oncogenes in the cancer catalog act by mimicking normal growth signalling in one way or another. In addition, tumor cells invariably show a greatly reduced dependence on exogenous growth stimulation. These factors led to the conclusion that tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from their normal
tissue microenvironment and disrupting the critically important homeostatic mechanism among various cell types within a tissue (Vats and Emami 1993).

![Figure 1.1](image)

**Figure 1.1** Novel capabilities acquired during tumor development  
(Adapted from The Hall Marks of Cancer, Hanahan and Weinberg, Cell 2000)

1.2.2 **Insensitivity to Antigrowth Signals**

In normal tissues, antiproliferative signals, which maintains cellular quiescence and tissue homeostasis, can block proliferation by two distinct mechanisms. Cells may be forced out of the active proliferative cycle into the quiescent (G0) state from which they may reemerge on some future occasion when extracellular signals permit. Alternatively, cells may be induced to permanently relinquish their proliferative potential by being induced to enter into post-mitotic states, usually associated with acquisition of specific
differentiation-associated traits. Incipient cancer cells evade these antiproliferative signals and dictate their own terms leading to unchecked growth (Weinberg, 1995).

1.2.3 Evading Apoptosis

Studies from mouse models and cell lines have proved that acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer. Apoptosis — programmed cell death — plays a major role in the rate of cell attrition. Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. Surely, the most commonly occurring loss of a proapoptotic regulator through mutation involves the p53 tumor suppressor gene. The resulting functional inactivation of its product, the p53 protein, is seen in greater than 50% of human cancers and results in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade (Harris 1996) (Vaux et al 1988, Weinberg 1995).

1.2.4 Limitless Replicative Potential of Cancer Cells

In general, cultured cells have a finite replicative potential and cell populations after progression through a certain number of doublings, stop growing; a process termed senescence (Hayflick 1997). The senescence of cultured human fibroblasts can be circumvented by disabling their pRb and p53 tumor suppressor proteins, enabling these cells to continue multiplying for additional generations, the feature being termed as immortalisation (Wright et al 1989). Provocatively, most types of tumor cells that are propagated in culture appear to be immortalized, suggesting that their limitless replicative potential is a phenotype that was acquired in vivo during tumor progression and was essential for the development of their malignant growth state (Hayflick 1997). This result suggests that at some point during
the course of multistep tumor progression, evolving premalignant cell populations exhaust their endowment of allowed doublings and can only complete their tumorigenic agenda by breaching the mortality barrier and acquiring unlimited replicative potential (Bryan and Cech 1999).

1.2.5 Sustained Angiogenesis of Cancer Cells

The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100 mm of a capillary blood vessel. Once a tissue is formed, the growth of new blood vessels—the process of angiogenesis is transitory and carefully regulated (Folkman 2002). The angiogenesis-initiating signals are exemplified by vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors (FGF1/2) (Veikkola and Alitalo 1999). However the cells within aberrant proliferative lesions initially lack angiogenic ability, curtailing their capability for expansion. In order to progress to a larger size, incipient neoplasias develop angiogenic ability in an uncontrolled fashion and exploit the positive and negative signals, encouraging sustained angiogenesis and allowing the tumor to grow beyond the limitations of passive nutrient diffusion (Bouck et al 1996; Hanahan and Folkman 1996).

1.2.6 Tissue Invasion and Metastasis

Invasion and metastasis are exceedingly complex and closely allied processes, with both utilizing similar operational strategies, involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases. Metastases is a process where tumor will eventually outgrow its surroundings and release a group of cells to reach out distant sites and grow as new colonies. In order to achieve this cancer cells must acquire
the invasive property, which they ably do. Several classes of proteins such as integrins, extracellular proteases are involved in the tethering of cells to their surroundings in a tissue are altered in cells possessing invasive or metastatic capabilities (Aplin et al 1998).

1.2.7 Current Status of Cancer Treatment

Cancer can be treated by surgery, chemotherapy, radiation therapy, hormone therapy, monoclonal antibody therapy or other methods depending upon the type of the tumor, grade of the tumor, the stage of the disease, the condition of the patient and patient’s response to initial treatment. Ultimately, the purpose of the treatment is complete removal of the entire tumor mass without much damage to the rest of the body.

Surgery is the oldest as well as the best approach to cure non-hematological cancers. The goal of the surgery is the removal of the tumor and can be achieved either by removing only the tumor, or the entire organ. The latter is advisable in certain cases where recurrence is inevitable. Surgery offers complete cure in treating most types of solid tumors when the tumor is confined to the site of origin. However, if the cancer has metastasized to other sites in the body prior to surgery, complete surgical excision is usually impossible and also needs other therapies such as chemotherapy and/or radiation therapy to eliminate the cancer cells in secondary sites (Lenhard et al 2001, King 1996).

Another conventional approach is radiation therapy where ionizing radiation is used to kill cancer cells and can be administered externally (external beam radiotherapy) or internally (brachytherapy). Radiation therapy can be used to treat almost every type of solid tumor. The advantage of radiation therapy over surgery is that radiation therapy can also be used to
treat leukemia and lymphoma. The final radiation dose employed to treat cancer relies on several factors such as the radiosensitivity of each cancer type and the sensitivity of the surrounding tissues and organs. Thus, as with every form of treatment, radiation therapy also has its pitfalls like the effects are localised and confined to the region in most cases and the damage caused to the adjacent normal cells (Kufe et al 2003).

Chemotherapy has become an essential component of treatment of most solid-organ cancers and is the mainstay of the treatment for patients with advanced or recurrent disease. Chemotherapy is used to target presumed micrometastatic disease in an adjuvant setting. Though chemotherapeutic agents include a diverse group of compounds with different mechanisms of action, their ultimate ability to induce apoptosis may signify a unifying concept for the mechanism of chemoprevention. Previously, researchers believed that the mechanism of cell death mediated by cytotoxic chemotherapy and ionizing radiation was through irreversible DNA damage with subsequent mitotic failure. However, it is now proven that the induction of endogenous apoptotic process is responsible for the cancer cell death in response to ionizing radiation as well as most of the chemotherapeutic agents (Martin 1997). Chemotherapy is given before or after surgery, in combination with radiation treatment or alone. One of the side effects of this systemic therapy is that apart from inducing apoptosis in cancer cells, they also damage the rapidly dividing normal cells (DeVita et al 2005).

Complementary and alternative medicine (CAM) is becoming increasingly popular, particularly among patients with advanced stages of cancer, and shares a sizeable percentage in the treatment methods employed in cancer cure which generally given alone or along with conventional therapies (Gerber et al 2006, Pal 2002). Preparations of plants, based on ancient recipes from Chinese or Japanese medical tradition, are mainly
popular in Asia and North America (Henderson and Donatelle 2004) and many mixtures are known to have an anticancerous effect \textit{in vitro} (Jo et al 2004, Campbell et al 2002, Bocca et al 2004). In India, use of Ayurvedic medicines like Valipani, Navjeevan and Kamdudha have shown efficacy in some leukemia patients. CAM encompasses a wide range of treatment modalities, including dietary supplements, and herbal medicines. CAM treatments are considered, to reduce therapy-associated toxicity, to improve cancer-related symptoms, to foster the immune system and even have direct anticancer effects.

1.2.8 Need for Newer Therapies

Clinically, the treatment of cancer still has many unmet needs. Aforementioned conventional strategies for cancer treatment are beneficial only if the cancer is detected at an early stage. In most cases the long term use of chemotherapy has been persistently associated with significant toxicity. Though therapies such as immunotherapy are more target oriented, still there is no breakthrough achieved in the delivery of a magic bullet. These unmet criteria necessitates the urgent need in development of more safe and effective anti cancer agents.

1.3 DRUG DISCOVERY IN CANCER RESEARCH

1.3.1 Natural products in Drug Discovery

Over the past several centuries, humankind has relied on natural products as the primary source for medicines. Natural products which include plants, marine biotics, and micro-organisms have a vast molecular diversity and functionality. There was no considerable change observed in the way the traditional medicine was practiced over much of this time period. However
the last two centuries have witnessed an explosion of knowledge regarding
the production of natural products, its interaction with other organisms and its
chemical nature. The last two centuries have seen the isolation of the first
commercial drug (morphine), use of microbial products as medicines
(penicillin), and even a use for the lowly leech (the anticoagulant, hirudin)
were some of the milestones achieved in the last 200 years.

World Health Organization estimated that 80% of the world’s
inhabitants rely on traditional medicines primarily plant-based for health care
(Mahidol et al 1998). In addition, 60% of all prescribed drugs are derived or
synthesized from natural products. Natural products have the potential to
provide medicine with a source of novel structures that are unobtainable from
sources such as combinatorial synthesis. Nature is capable of producing
complex molecules with multiple chiral centers that are designed to interact
with biological systems (Young 200, Cordell 1999, Cragg and Newman
2005). In developing countries, medicinal plants continue to be the main
source of medication.

1.3.2 Natural Products in Anticancer Drug Discovery

Natural products are the most consistently successful source of drug
leads and they continue to provide structural diversity. So they offer major
opportunities for finding novel lead structures that are active against a wide
range of assay targets (Newman et al 2003). Drug discovery from medicinal
plants has played an important role in the treatment of cancer and, indeed,
most new clinical applications of plant secondary metabolites and their
derivatives over the last half century have applied towards combating cancer
(Balunas and Kinghorn 2005). Analysis of drugs approved by the Food and
Drug Administration in the US in a period of 22 years from 1981-2002 and
found that 62% of anticancer agents were natural products or their derivatives (Newman et al. 2003).

Several phytochemicals have been found to reduce neoplastic transformation and extensive studies reveal the cancer-inhibitory activity of such natural compounds was achieved through decreasing growth, inducing apoptosis, altering cell cycle kinetics, and interfering with intracellular signal transduction events in malignant cells.

Anticancer drugs from plants that are in current clinical use fall into four main classes of compounds namely vinca alkaloids, epipodophyllotoxins, taxanes and camptothecins (Table 1.1). The mechanism of action of these compounds ranges from that of tubulin inhibition to the inhibition of essential DNA enzymes topoisomerase I and topoisomerase II or both topoisomerases I and II.

**Table 1.1 Anticancer drugs and their plant source**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Plant source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinblastine, vincristine</td>
<td>Inhibition of tubulin polymerization</td>
<td><em>Catharanthus roseus</em> (Apocynaceae)</td>
</tr>
<tr>
<td>Etoposide, teniposide</td>
<td>Inhibition of topoisomerase II</td>
<td><em>Podophyllum peltatum</em> P. emodi (Berberidaceae)</td>
</tr>
<tr>
<td>Paclitaxel, docetaxel</td>
<td>Promotion of tubulin stabilization</td>
<td><em>Taxus brevifolia</em> (Taxaceae)</td>
</tr>
<tr>
<td>Iriotecan, topotecan, 9-aminocampothecin, 9-nitrocamptothecin</td>
<td>Inhibition of topoisomerase I</td>
<td><em>Camptotheca acuminate</em></td>
</tr>
</tbody>
</table>
1.4 NATURAL CHEMOPREVENTIVE AGENTS AND THEIR MOLECULAR TARGETS

Conventional therapies cause serious side effects and at best merely extend the patient’s life span by a few years. There is thus the need to utilize alternative concepts or approaches to the prevention of cancer. Many natural product derived compounds have been implicated in cancer prevention and that promote human health without recognizable side effects. These active principles originate from vegetables, fruits, plant extracts, herbs and shown to interfere with several cell signalling pathways. Extensive research during the last half century has identified various molecular targets that can potentially be used for cancer therapy. The active principles identified from natural sources and the molecular targets modulated may be the basis for how these agents function effectively in not only preventing but also treating cancer and other diseases (Table 1.2) (Aggarwal and Shishodia 2006).

Thus natural products consist of a wide variety of biologically active compounds that are ubiquitous in plants, many of which have been used in traditional medicines for thousands of years. Most modern medicines currently available for treating cancers are very expensive, toxic and less effective in treating the disease. Thus one must investigate further in detail the agents derived from natural sources, described traditionally for the prevention and treatment of cancer.
Table 1.2 Phytochemicals and their molecular targets

<table>
<thead>
<tr>
<th>Molecular targets</th>
<th>Dietary agents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nuclear factor-kappa B (NF-κB)</strong></td>
<td>Curcumin, Guggulsterone, Ursolic acid, Betulinic acid, Emodin, Gingerol, Flavopiridol, Zerumbone, Evodiamine, Indole-3-carbinol, Elagic acid, Anethole, Green tea catechins, Lycopene, Diosgenin</td>
</tr>
<tr>
<td><strong>Activator protein-1 (AP-1)</strong></td>
<td>Green tea catechins, Quercetin, Resveratrol, Curcumin, Capsaicin, Oleandrin</td>
</tr>
<tr>
<td><strong>Cell cycle</strong></td>
<td>Curcumin, Resveratrol, Genistein, Dietary Isothiocyanates, Apigenin, Silibinin</td>
</tr>
<tr>
<td>Cyclin-dependent kinases(CDK)</td>
<td>Genistein, Indole-3-carbinol, Diosgenin, Curcuminoids, EGCG</td>
</tr>
<tr>
<td><strong>Cell survival kinase Akt</strong></td>
<td>Curcumin, Green tea polyphenols, Gingerol, Resveratrol, Kaempferol, Apigenin</td>
</tr>
<tr>
<td><strong>Tumor necrosis factor (TNF)</strong></td>
<td>Galangin, Luteolin, Apigenin, 6-hydroxykaempferol, Quercetagenin, Sasanquol, Genistein, Wogonin, Green tea catechins, Curcumin, Resveratrol</td>
</tr>
<tr>
<td><strong>Cyclooxygenase-2 (COX-2)</strong></td>
<td>Curcumin, Resveratrol, Genistein, Luteolin, Catechins</td>
</tr>
<tr>
<td><strong>Angiogenesis</strong></td>
<td>VEGF and bFGF MMP-2 and MMP-9</td>
</tr>
</tbody>
</table>
1.5 MEDICINAL PROPERTIES OF PLANTS UNDER INVESTIGATION

1.5.1 *Piper longum*

Family: Piperaceae  
Common name: Long pepper  
Vernacular Name: Vaal milagu

The genus *Piper*, belonging to the Piperaceae, has been received considerable attention in recent years because of its chemico-biological properties. Various *Piper* species including *Piper longum*, widely distributed in the tropical and subtropical regions of the world, have been used as a spice and also as a folk medicine for various ailments. *Piper longum*, a traditional herb, used in Indian medicine and as spice throughout the world is commonly used as a good remedy for treating a variety of diseases and disorders like gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infection, chronic gut related pain and arthritic conditions (Neelima Pathak 2006).
1.5.2  *Ficus racemosa*

Scientific Name  *Ficus racemosa* L.

Family  Moraceae

English names  Cluster fig, Country fig, Gular fig.

Vernacular names  Aththi.

Traditional use:  Leaves: in small pox, pneumonia and bronchitis

All parts in the tree were used in traditional medicines for curing piles, internal wounds, removes impurities from blood, worms from alimentary canal; useful in skin diseases, including leprosy, sinus, oedema, impurities of blood and in piles; useful in antifertility, pimples and wounds; removes worms, cures thrombophlebitis, syncope, burning sensation and unusual thirst; astringent, sweet and heavy, cures pimples and wounds, diseases caused by deranged phlegm and deranged bile. In addition, *Ficus racemosa* is also used in the recipe’s of *AYURVEDA* and *SIDDHA*. 
1.5.3 *Cissus quadrangularis*

All parts in the plant were used to treat Analgesia, Asthma to remove impurities of blood and in piles. In addition it was also used for healing fractures in bone also used as Anthelmintic digestive.

1.6 APOPTOSIS AS TARGET FOR CANCER THERAPY

1.6.1 Overview of Apoptosis

Apoptosis is a highly intricate, distinct, intrinsic cell death program important for development and maintaining homeostasis of cells. Depending upon the condition, inhibition or induction of apoptosis (programmed cell death) plays a major role in various physiological and pathological situations (Hengartner 2000). In general, apoptosis can be divided into the initiation phase, the effector, and the degradation phase. Any extrinsic or intrinsic stimuli (e.g. radiation, growth factor withdrawal and chemotherapeutic agents) can initiate the apoptosis process. The intracellular proteins which
respond to the stimuli carry out the apoptotic process further and thus leading to the last degrading phase, an irreversible one, which displays unique morphological features. The unifying features of apoptosis, irrespective of cell type or stimuli, are largely morphological and include externalization of phosphatidylserine, chromatin condensation, DNA fragmentation, blebbing of the plasma membrane, and cell shrinkage (Kerr 1972, Rodriguez and Schaper 2005). Ultimately cells are broken down into apoptotic bodies which are phagocytosed to prevent inflammation (Lauber et al 2004).

1.6.2 Cancer and Apoptosis: First Principles

Recent studies have proven that killing of tumor cells by cytotoxic therapies such as chemotherapy, gamma irradiation, and immunotherapy is mediated by triggering apoptosis in cancer cells (Herr and Debatin 2001). Though cancer is a highly heterogenous disease, displaying great genetic diversity, some studies have suggested that the underlying etiology and progression of the disease can be reduced to two factors, (i) mutations that lead to excessive proliferation and (ii) a compensatory disruption of survival signaling pathways that ensures the persistence of these hyperproliferative cells. This theory creates the link between neoplasia and apoptosis, as demonstrated by the ability of oncogenes including Myc and tumor suppressors such as p53 to actively engage apoptosis.

Since apoptosis is a physiological process, induction of this form of cell death in tumor cells is associated with fewer side effects than non-apoptotic modes of cell death. However the most important advantage of targeting apoptosis is that its induction directly leads to tumor regression and reduces risks of selecting more aggressive and/or drug-resistant phenotypes that are often responsible for tumor regrowth and treatment failure. Many studies have revealed that DNA damage by anticancer drugs is commonly recognized as an apoptotic stimulus with varying magnitude.
However with increased understanding of the complexity of cellular responses to drug interactions with a variety of cellular targets that ultimately result in cell death lead to the conclusion that not only the DNA damage but certain drugs belong to the category of agents that bind to cellular DNA but mainly react with cellular proteins causing damage. Protein damage is known to distort the cell redox homeostasis, which facilitates apoptosis execution. (Kaufmann and Earnshaw 2000).

Moreover several reports emphasize that apoptosis is relevant to many aspects of tumor biology, which include tumorigenesis, tumor homeostasis, angiogenesis, metastasis, and clinical treatment. Thus it is empirical that controlling several stages in the cancer solely through targeting apoptosis could be sagacious.

All these factors substantiate the fact that induction of apoptosis could be the ideal mode of targeting tumor cell and understanding the direct or indirect drug interactions with molecular targets that regulates apoptotic machinery may lead to new strategies for cancer chemotherapy and increase the therapeutic efficiency in cancer treatment (Martin 2006).

1.7 MAJOR PATHWAYS IN APOPTOSIS

Two major pathways namely the death receptor pathway (the extrinsic pathway) and the mitochondrial pathway (the intrinsic pathway) (Figure 1.4) (Kim 2005) eventually results in activation of caspases leading to cell death (Thomberry and Lazebnik 1998).
1.7.1 The Death Receptor Pathway (Extrinsic)

Several members of the tumor necrosis factor-a (TNFa) receptor superfamily, a group of type 1 membrane polypeptides that are activated by members of the TNFa family of ligands, signal cell death under certain circumstances (Bouralexis et al 2005, Reed 1999, Wang et al 2005). In particular, Fas, TNFa receptor 1, DR3, TRAIL receptors 1 and 2 (DR4 and DR5) and DR6, which all contain related intracellular motifs called death domains (DDs), can initiate signaling that results in cell death. Upon receptor activation, the DD undergoes homotypic interaction with a DD in the adapter proteins FADD/ MORT1 (Chinnaiyan et al 1995, Perik et al 2005) or TRADD (Hsu et al 1995). FADD/MORT1 binds directly to CD95 and TRAIL receptors DR4 and DR5 and indirectly to p55 TNF-RI via TRADD, and is essential for cell death signaling from all of these receptors (Hsu et al 1996, Newton et al 1998, Zhang et al 1998). Upon receptor-mediated oligomerization, FADD in turn serves as a cofactor for the oligomerization and activation of caspase 8 and/or caspase 10, two members of a cysteine protease family that cleave substrates on the carboxyl terminal side of aspartate. Caspases 8 and/or 10 then cleave a limited number of substrates, including procaspase 3 and Bid, to initiate the cell death process. As a BH3-only polypeptide that is activated by caspase 8-mediated cleavage, Bid represents an important means of crosstalk between this pathway and the mitochondrial pathway in some cells. Anti-cancer strategies undergoing preclinical or clinical testing can affect this pathway in several ways including, ligation of DR4 and/or DR5, up-regulation of DR5 and enhanced DR clustering, dephosphorylation of FADD, enhanced procaspase 8 recruitment to FADD, down-regulation of c-FLIP.

1.7.2 The Mitochondrial Pathway (Intrinsic)

The intrinsic pathway involves the mitochondria and control by the Bcl-2 family proteins. Bcl-2 family proteins comprise of pro-apoptotic and
anti-apoptotic members which alters the mitochondria in a number of ways. This pathway is characterized by alterations in the mitochondrial polarization and release of mitochondrial proteins, including cytochrome c, endonucleases G, secondary mitochondria-derived activator of caspase (SMAC)/direct inhibitor of apoptosis protein (IAP) binding protein with low pI (DIABLO), apoptosis-inducing factor (AIF) and its homolog AIF-homologous mitochondrion associated inducer of death (Sun et al 2004).

In this pathway, cytochrome c is released from the cristae of mitochondria into the cytosol, a key step in apoptosis induction through the mitochondrial pathway. This can be induced by the elevated levels of the pore forming proapoptotic Bcl-2 family protein such as Bax. Once released to the cytoplasm, cytochrome c binds and activates the apoptotic protease activating factors (Apaf-1), enabling binding and activation of procaspase 9, an initiator caspase leading to the assembly of a multiprotein caspase-activating complex called the apoptosome (Cain et al 2002). Thus the release of cytochrome c from mitochondria results in caspase 3 activation through the formation of apoptosome complex. SMAC/DIABLO promote caspase activation by antagonizing the inhibitory effects to IAPs, while AIF and endonucleases G cause large scale DNA fragmentation and chromatin condensation (Guimaraes and Linden 2004).

There are multiple connections between the receptor and the mitochondrial pathway. Activation of caspase 8 may lead to cleavage of Bid, a BH3 domain containing protein of the Bcl-2 family. Bid upon cleavage then translocate to the mitochondria and associate with proapoptotic Bax or Bak resulting in cytochrome c release from mitochondria thereby initiating a mitochondrial loop. Moreover, mitochondria triggered caspase 6 cleavage may feed back to the receptor pathway by cleaving caspase 8 (Rossi and Gaidano 2003).
Figure 1.2 The mitochondrial pathway
1.7.3 p53 and Apoptosis

p53 is a tumor suppressor gene is the most intensely studied apoptosis factor contributing to cancer. It plays a central important role in regulating cell signal transduction, cellular response to DNA damage, cell cycle control and apoptosis. It is inactivated in presumably more than 50% of all human cancers. It is activated as a transcription factor in response to oncogene activation, hypoxia and especially DNA damage resulting in growth arrest or inducing apoptosis by stimulating the expression of various p53 target genes such as p21, Bax, Puma, Noxa, Apaf-1, Fas and DR5 (Vousden and Lu 2002) or by repressing the expression of antiapoptotic proteins namely Bcl-2, Bcl-xL or survivin (Hoffman et al 2002, Wu et al 2001). Due to the importance of p53 in apoptosis induction following DNA damage, different strategies have been undertaken to target the tumor suppressor. A promising approach is the restoration of normal functions of mutant p53.

The importance of p53 in the induction of apoptotic cascade through the intrinsic pathway is associated with the mitochondrial release of factors such as cytochrome c and SMAC/DIABLO (Schuler and Green 2001). p53 can also mediate the extrinsic apoptotic pathway in response to DNA damage. Apoptosis induced by UV and X-ray radiation is mediated through a p53 dependent mechanism. The death receptor CD95/Fas/Apo-1 has been reported as a direct target gene for p53 (Owen-Schaub et al 1995). Several investigations have reported the role of p53 at the mitochondrial membrane following DNA damage wherein a small proportion of p53 directly binds to Bcl-2 and Bcl-xL and neutralize their antiapoptotic action (Marchenko et al 2000, Mihara et al 2003). Some of the natural compounds known to induce p53 activity are curcumin (Han et al 1999), resveratrol (Huang et al 1999), epigallocatechin-3-gallate (Gupta et al 2000), and silibinin (Gu et al 2005).
1.8 THE Bcl-2 FAMILY: REGULATORS OF THE CELLULAR LIFE-OR-DEATH SWITCH

1.8.1 Bcl-2 Family Proteins

Bcl-2 is the mammalian homologue of *ced-9*, which, in *C. elegans*, is required to protect cells that normally survive from undergoing, programmed cell death (Hengartner et al., 1992; Yarbro, 1992). Based on homology within their Bcl-2 homology (BH) 1-4 domain organization, Bcl-2 family of proteins fall under two functional categories, those that inhibit apoptosis, collectively called as anti-apoptotic and those that promote apoptosis, called as pro-apoptotic Bcl-2 family members ((Adams and Cory 1998, Oda et al 2000). The proto-oncogene Bcl-2 was first discovered as the target gene present at the translocation site of the t (14; 18) chromosomal translocation breakpoint in the tumour cells of approximately 80% of patients with human follicular B-cell lymphoma (Strasser et al1997).

Several reports suggest that Bcl-2 family members are involved in p53-mediated apoptosis where Bcl-2 family members such as Bax, Bak, and noxa are transactivationally upregulated by p53 (Ho et al 1999; Miyashita et al 1994; Oda et al 2000). On the contrary, Bcl-2 is transcriptionally suppressed by p53 (Miyashita and Reed, 1995) and overexpression of Bcl-2 blocks apoptosis of mammalian cells triggered by a number of different stimuli, such as growth factor deprivation, irradiation, c-Myc, p53, and certain anti-cancer drugs (Alnemri et al 1992; Rao et al 1992).

1.8.2 Classification of Bcl-2 Family

All members possessing at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4) are antiapoptotic and
include Bcl-2, Bcl-xL, Mcl-1, A1 and Bcl-W. The Bcl-2 family also contains two proapoptotic subgroups. Bax, Bak, Bok, Bcl-x_s and Bcl-G_L are proapoptotic members and are identical to the pro-survival group in their domains containing two or three BH regions. The other proapoptotic members like Bik, Bad, Hrk, Bid, Bim, Noxa, Puma and Bmf share with each other and the other Bcl2 family members only the BH3 domain and therefore often called as BH3-only proteins. Both the proapoptotic subgroups are essential for apoptosis and the BH3-only proteins initiate cell death signalling (Huang and Strasser 2000). The BH4 domain of Bcl-2 has been shown to be necessary for Bcl-2 to heterodimerize with Bax, as part of antiapoptotic function (Hirotani et al 1999, Huang et al 1998). The Bcl-2 proteins both anti- and proapoptotic are located on the membranes, including the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear membrane (Susin et al 1998).

1.8.3 Role of Bcl-2 Family in Apoptosis

Four conserved regions within Bcl-2 related proteins have been identified that have been designated Bcl-2 homology (BH) domains BH1, BH2, BH3, and BH4. The BH1 and BH2 regions are important for the anti-apoptotic function of Bcl-2 and Bcl-xL, and for their interaction with Bax. The BH3 domain is sufficient for proapoptotic Bcl-2 members to interact with the anti-apoptotic Bcl-2 members. Furthermore, BH3 is required for the killing activity of the proapoptotic Bcl-2 members. The BH4 domain of Bcl-2 has been shown to be necessary for Bcl-2 to heterodimerize with Bax, and to inhibit apoptosis (Hirotani et al 1999; Huang et al 1998).

The switching on and off of apoptosis is determined by the ratio of proapoptotic to antiapoptotic proteins (Cory and Adams 2005). Upon induction of apoptosis, the proapoptotic proteins including Bax, Bak and Bad...
are triggered in response to the anticancer drugs. The activation of the bax gene leads to increase in the Bax protein that are translocated from the cytoplasm to mitochondria (Nomura et al 1999). The BH1 and BH2 domains show ion channel activity for regulating the release of cytochrome c from mitochondria (Kluck et al 1997, Yang et al 1997). The homodimerized Bax acts on the voltage-dependent anion channel (VDAC) on the outer membrane of mitochondria resulting in the release of cytochrome c which activates the caspase cascade. The cytochrome c release is also induced by heterodimers with Bax and Bak in the same way (Tsujimoto and Shimizu 2000).

![Figure 1.3 Bcl-2 family members](image)
The transcriptional-independent function of cytoplasmic p53 requires Bax to induce apoptosis leading to cytochrome c release and caspase activation (Chipuk and Green 2004, Chipuk et al 2004). Several studies have reported that the release of cytochrome c is inhibited by the antiapoptotic proteins Bcl-2 and Bcl-xL which like Bax possess ion channel activity (Minn et al 1997, Narita et al 1998, Schendel et al 1997). BH3-only proteins like Bad and Bim bind preferentially to the antiapoptotic proteins Bcl-2 or Bcl-xL for initiating apoptosis (Chittenden 2002). Interaction of Bad with the antiapoptotic members results in the release of Bax and Bak from Bcl-2 and Bcl-xL. The dissociated Bax and Bak are then activated by Bid like activators. Similarly Bid and Bim promote apoptosis by their release from the Bcl-2 and Bcl-xL (Paquet et al 2004).

Within the limits of a cancer scenario, the members of Bcl-2 family can be envisaged as determinants of cell proliferation and cell death; the Yin and Yang of life and death. In other words, the Yin stands the pro-apoptotic Bcl-2 members, upon expression lead to phenotypic changes such as cell death predominantly by inducing apoptosis. In contrast, Yang represents the anti-apoptotic Bcl-2 members, the overexpression of which leads to the cell survival and proliferation by evading apoptosis. Bcl-2 family proteins have emerged as potent targets for cancer chemoprevention, as these proteins dictate the cell to die or not. Several bioactive components like indole-3-carbinol, curcumin, beta carotene, genistein, the garlic compounds diallyl sulfide (DAS) and diallyl disulfide (DADS) are reported to regulate the proapoptotic and antiapoptotic proteins promoting the release of cytochrome c from the mitochondria to induce apoptosis (Martin 2006).

1.8.4 Pro-apoptotic Member Bax in Apoptosis

Bax functions as one of the effectors of p53 to promote apoptosis. Upregulated Bax protein levels were observed in BRK cells after p53
activation (Han et al., 1996). Bax changes conformation in response to a p53 death signal, which causes the exposure of the Bax amino terminus.

However, Bax is not the only player downstream of p53-mediated apoptosis. p53-induced Bax accumulation by itself is not sufficient to mediate apoptosis. Some other proapoptotic Bcl-2 family members, such as Bak and Bad, share certain functionality with Bax and may partially substitute Bax function in Bax-deficient cells. A newly identified BH3-only member of Bcl-2 family, Noxa, has also been implicated in p53-mediated apoptosis by functioning at the mitochondria (Oda et al 2000). These observations suggest that some proapoptotic Bcl-2 family members function downstream of p53 death signaling by pushing cells through the mitochondria death checkpoint.

1.9 ROLE OF MITOCHONDRIA IN MAMMALIAN APOPTOSIS

Cytochrome c normally resides in the space between the outer and inner membranes of mitochondria where it participates in oxidative phosphorylation. Upon exposure of cells to apoptotic stimuli, cytochrome c is released from mitochondria into the cytosol, where it is one of several factors implicated in the proteolytic activation of caspase-3 by caspase-9, thereby making it a potential therapeutic target to modulate cell death in cancer (Alirol and Martinou 2006, Cereghetti and Scorrano 2006, Galluzzi et al 2006, Reed 1999). The CARD domains in Apaf-1 and the prodomain of caspase-9 interact and, in the presence of cytochrome c and either ATP or ADP, this induces autocatalytic activation of the caspase which then activates the downstream caspase effector cascade involving caspases-2, -3, 6 and -7. The Apaf-1 - caspase-9 interaction is clearly reminiscent of the mechanism of Ced-3 activation by Ced-4 in C. elegans. This similarity extends to the involvement of the mammalian anti-apoptotic Bcl-2 and Bcl-xL, proteins, which are homologues of Ced-9. Bcl-2/Bcl-xL resides in the outer
mitochondrial membrane, where they function to suppress apoptosis in both or either of two ways: blocking cytochrome c release and binding to Apaf-1 to prevent its activating caspase-9. The pro-apoptotic Bcl-2 family members such as Bax, Bak and Bik may promote apoptosis by displacing Apaf-1 from Bcl-2/xL.

Mitochondria serve as an immediate target for a few anticancer agents like etoposide and paclitaxel (Grad et al 2001). Because the activation of mitochondria during the apoptotic process results in the point of no return in the cells, the mitochondria is considered to be a potential therapeutic target. Natural compounds such as curcumin, capsaicin, epigallocatechin gallate, beta carotene and lycopene can open the mitochondrial permeability transition pore (MTP) induce cytochrome c release and ultimately resulting in apoptosis (Martin 2006).

1.10 MITOCHONDRIA AS A TARGET IN CANCER THERAPY

Mitochondria have a central role as the major energy factory of living cells, but they can also either trigger or amplify the signals that lead to cell death (Green and Reed 1998). Recent investigations have demonstrated the impact of mitochondria in cancer and its role (Alirol and Martinou 2006, Cereghetti and Scorrano 2006, Galluzzi et al 2006). Dysregulation of several parameters of mitochondrial physiology such as loss of mitochondrial membrane potential, the generation of reactive oxygen species (ROS), the termination of oxygen consumption and the release of apoptogenic proteins, has been described as hallmarks of apoptosis mediated through mitochondria (Green and Reed 1998). Conventional chemotherapies result in induction of apoptosis by triggering the intrinsic death pathways that converge on the mitochondria leading to an activation of caspases.
1.11 ROLE OF CASPASES IN APOPTOSIS

1.11.1 The Executioner and its Substrates

Caspases are synthesized as inactive zymogens called procaspases usually activated by proteolytic cleavage. Procaspases of the apoptosis activator and inflammatory mediator caspases possess long prodomains. These prodomains contain the death effector domain (DED) and the caspase recruitment domain (CARD) that plays a key role in the procaspase activation and caspase cascade regulation through protein-protein interaction (Fan et al 2005). Caspases which are involved in apoptosis are classified as initiator caspases and the effector caspases.

Molecular understanding of apoptosis has advanced profoundly since its original description by Wyllie and colleagues (Bishop and Weinberg 1996). While the first hallmarks of apoptotic cell death membrane blebbing, chromatin condensation and cellular fragmentation into 'apoptotic bodies' - were purely morphological, biochemical hallmarks have superseded these. Apoptosis is now studied as a cascade of proteases and endonucleases, where oligonucleosomal DNA laddering and cleavage of a variety of substrates by cysteine proteases have become the modern "gold-standards". Apoptosis is a developmentally programmed process in C. elegans whereby the death of individual cells is genetically determined and reproducibly observed. Cloning of Interleukin 1-beta Converting Enzyme (ICE) protease as a mammalian homologue for CED-3 provided the first indication that proteases may play a critical role in apoptosis (Cohen 1997, Evan and Littlewood 1998). Following this observation, numerous ICE-family proteases have been identified in mammalian cells and are thought to constitute the core of the apoptosis executioner. The ICE proteases all belong to the cysteine protease subfamily
Figure 1.4 Involvement of mitochondria in apoptotic cell death
**Figure 1.5  Caspase structure**

(A) The caspase family. Three major groups of caspases are presented. Group I: inflammatory caspases; group II: apoptosis initiator caspases; group III: apoptosis effector caspases. (B) Scheme of procaspase activation. (C) The 3D structure of caspase-3 heterotetramer.

Caspases: pharmacological manipulation of cell death

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The Journal of Clinical Investigation Volume 115 Number 10 October 2005
characterised by a cysteine residue at the active site. ICE/ced-3 homologues contain the conserved QACRG sequence surrounding the catalytic cysteine and show a preference for cleaving substrates after aspartate residues. Phylogenetic relationships among the proteases have led to their subdivision into three families (Ashkenzai and Dixit 1998). Typically, these enzymes can auto-catalytically cleave and activate themselves as well as other ICE-family proteases. This may lead to amplification and diversification of available substrates during the execution phase of apoptosis in cells. In the attempt to simplify the nomenclature for this ever-expanding family of enzymes, apoptosis proteases have recently been renamed 'caspases' (or cysteine proteases which cleave after aspartic acid), and numbered in function of the chronology of their discovery (Huang and Oliff 2001).

1.11.2 Caspases Activation Leads Stereotypic Nucleosomal Cleavage

One of the central questions remains how proteolysis leads to the demise of the cell and its stereotypical changes. It is still unclear which substrates known to date (if any) are instrumental in the death pathway and which are simply biochemical markers of the process. Poly ADP-ribose polymerase (PARP) was one of the first proteins reported to be cleaved during apoptosis (Kiess and Gallahur 1998), and is a target of the Yama/CPP32 protease, caspase-3 (Huang and Oliff 2001).

As is true of most proteases, caspases are synthesized as enzymatically inert zymogens (30 to 50 kDa). These zymogens are composed of three domains: an N-terminal pro-domain, and the p20 and the p10 domains, which are found in the mature enzymes. The mature enzyme is a heterotetramer containing two p20/p10 heterodimers and two active sites.
1.11.3 Three general mechanisms of caspase activation

1.11.3.1 Processing by an upstream caspase

Most caspases are activated by proteolytic cleavage of the zymogens and 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 the cells for the activation of three short prodomain caspases, caspase-3, -6 and-7. This cascade is a useful method to amplify and integrate pro-apoptotic signals (Lincz 1998).

1.11.3.2 Induced proximity

Caspase-8 is the key initiator caspase in the death-receptor pathway. Upon ligand binding death receptors such as CD95, TNFR1 form membrane complexes that recruit, through adapter proteins, several molecules of pro-caspase-8, resulting in a high local concentration of the zymogen. Under these crowded conditions, the low intrinsic protease activity of pro-caspase-8 is sufficient to allow the various proenzyme molecules to mutually cleave and activate each other (Wang et al 2005, Liang and Fesik 1997).

1.11.3.3 Association with a regulatory subunit

Activation of pro-caspase-9 occurs via a conformational change and not proteolysis and requires the association with Apaf-1 through the CARD (Caspase recruitment domain) in presence of dATP and cytochrome-c to form a complex- the apoptosome. Thus it is common that compartmentalization of caspases and their cofactors are another way of regulating caspase activation in apoptotic induction (Nicholson 2000).
1.12 CELL CYCLE AND CANCER

The progression of eukaryotic cells through the cell cycle is orchestrated by sequential activation and inactivation of the cyclin-dependent kinases (cdks) associated with their respective cyclin subunits (Sherr 1996, Sherr and Roberts 1995). G1 progression and G1/S transition are regulated by cdk4/cdk6 that assembles with D-type cyclins in mid-G1 and cdk2 that combines later with cyclin E. While cdk2 controls the S-phase when associated with cyclin A, G2/M transition is regulated by cdc2 in combination with cyclins A and B (Girard et al 1991, Guadagno et al 1993, King et al 1994, Krek and Nigg 1991). It has been conclusively thought that cell cycle arrest-inducing compounds are promising candidates for the treatment and prevention of various cancers.

![Cell Cycle Diagram](image)

**Figure 1.6 Stages of cell cycle**

The site of activity of regulatory Cdk/cyclin complex is also indicated. Adapted from The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer, Katrien vermeulen et al cell proliferation 2003
1.13 ROLE OF REACTIVE OXYGEN SPECIES IN APOPTOSIS

ROS are produced in all mammalian cells due to normal cellular metabolism and also when cells are exposed to many stimuli such as UV, ionizing radiation, cancer chemotherapeutics, and TNF-α. Because of their high reactivity, ROS affect various cellular molecules, such as fatty acids, carbohydrates, proteins and nucleic acids. An excess of ROS may lead to cell death when their level overwhelms the cellular antioxidant capacity, which is linked to the antioxidant level. Recent studies have indicated that cancer chemopreventive agents induce apoptosis in part with generation of intracellular ROS and the disruption of redox homeostasis. Generated ROS can directly activate the mitochondrial permeability transition and this leads to loss of mitochondrial membrane potential. Mitochondrial dysfunction such as loss of membrane potential eventually results in release of cytochrome c into cytoplasm from mitochondrion leading to activation of DEVD-specific caspases and to nuclear fragmentation and cell death in vitro.