CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO CANCER

Cancer (from the Latin word for crab, so called because of the tendency to exhibit spread similar to the legs of the creature) has been recognized as the second most common cause of death in the developed world. A cancer can be defined as a new growth (neoplasm or tumor) resulting from the continuous proliferation of abnormal cells. These tumors have the ability to invade and destroy other tissues and spread to parts of the body remote to the original site of growth (known as metastasis). Tumors exhibiting these characteristics are said to be malignant. In contrast to these malignant cancers are benign (non-cancerous) tumors, which are non-invasive and not prone to invasion or remote spread. Whilst benign tumors do present a risk to patient health, the presence of even a small, untreated malignant tumor is capable of causing a rapidly progressive and potentially fatal disease.

Cancer cells lose the specific functions (known as differentiation) associated with the tissues from which they are derived. As a rule, the less differentiation showed by the cells, the more aggressive the tumor and the worse the prognosis. Any cell or tissue type present in the human body is capable of becoming cancerous - as a disease, cancer refuses to be defined a single entity. Cancer can strike an individual at any time, but the tumor type can vary considerably with age (such as childhood leukemia), sex, and predisposing
factors (such as genetic predisposition and exposure to environmental factors). Some tumor types are rarely encountered outside of certain demographics (such as Kaposi's sarcoma in HIV sufferers).

1.1.1 The Hallmarks of Cancer

After a quarter century of rapid advances, cancer re-difficult search has generated a rich and complex body of knowledge, revealing cancer to be a disease involving dynamic changes in the genome. The foundation has been set in the discovery of mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function; both classes of cancer genes have been identified through their alteration in human and animal cancer cells and by their elicitation of cancer phenotypes in experimental models (Bishop and Weinberg 1996; Lowe and Lin 2000).

1.1.2 Tumorigenesis in humans is a multi-step process

Several lines of evidence indicate that tumorigenesis in humans is a multistep process and that these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives. Many types of cancers are diagnosed in the human population with an age-dependent incidence implicating four to seven rate-limiting, stochastic events (Renan 1993). Pathological analyses of a number of organ sites reveal lesions that appear to represent the intermediate steps in a process through which cells evolve progressively from normalcy via a series of pre-malignant states into invasive cancers (Foulds 1954). These observations have been rendered more concrete by a large body of work indicating that the genomes of tumor cells are invariably altered at multiple sites, having suffered disruption
through lesions as subtle as point mutations and as obvious as changes in chromosome complement (Kinzler and Vogelstein 1996).

Transformation of cultured cells is itself a multi-step process: rodent cells require at least two introduced genetic changes before they acquire tumorigenic competence, while their human counterparts are more re-difficult to transform (Hahn et al., 1999). Transgenic models of tumorigenesis have repeatedly supported the conclusion that tumorigenesis in mice involves multiple rate-limiting steps (Bergers et al., 1998). Taken together, observations of human cancers and animal models argue that tumor development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (Foulds 1954; Nowell 1976).

1.2 **AN ENUMERATION OF THE TRAITS**

Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis. There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs. This complexity provokes a number of questions. How many distinct regulatory circuits within each type of target cell must be disrupted in order for such cell to become cancerous? Does the same set of cellular regulatory circuits suffer disruption in the cells of the disparate neoplasm’s arising in the human body? Which of these circuits operate on a cell-autonomous basis, and which are coupled to the signals that cells receive from their surrounding microenvironment with in tissue? Can the large and diverse collection of cancer
associated genes be tied to the operations of a small group of regulatory circuits?

The vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth (Figure 1.1): 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 capabilities acquired during tumor development - represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues.

1.2.1 Self-Sufficiency in Growth Signals

Normal cells require mitogenic growth signals (GS) before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signalling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. To our knowledge, no type of normal cell can proliferate in the absence of such stimulatory signals. Many of the oncogenes in the cancer catalog act by mimicking normal growth signalling in one way or another. Dependence on growth signalling is apparent when propagating normal cells in culture, which typically proliferate only when supplied with, appropriate diffusible mitogenic factors and a proper substratum for their integrins. Such behavior contrasts strongly with that of tumor cells, which invariably show a greatly reduced dependence on exogenous growth stimulation. The conclusion is that tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from their
Figure 1.1 Novel capabilities acquired during tumor development
(Adopted from The Hall Marks of Cancer, Hanahan and Weinberg, Cell 2000)
normal tissue microenvironment. This liberation from dependence on exogenously derived signals disrupts a critically important homeostatic mechanism that normally operates to ensure a proper behavior of the various cell types within a tissue.

1.2.2 Insensitivity to Antigrowth Signals

Within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis; these signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. These growth-inhibitory signals, like their positively acting counterparts, are received by transmembrane cell surface receptors coupled to intracellular signalling circuits. Antigrowth signals 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 must evade these antiproliferative signals if they are to prosper. Much of the circuitry that enables normal cells to respond to antigrowth signals is associated with the cell cycle clock, specifically the components governing the transit of the cell through the G1 phase of its growth cycle. Cells monitor their external environment during this period and, on the basis of sensed signals, decide whether to proliferate, to be quiescent, or to enter into a post-mitotic state. At the molecular level, many and perhaps all antiproliferative signals are funneled through the retinoblastoma protein (pRb) and its two relatives, p107 and p130. When in a hypo-phosphorylated state, pRb
blocks proliferation by sequestering and altering the function of E2F transcription factors that control the expression of banks of genes essential for progression from G1 into S phase (Weinberg, 1995).

### 1.2.3 Evading apoptosis

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death—apoptosis—represents a major source of this attrition. The evidence is mounting, principally from studies in mouse models and cultured cells, as well as from descriptive analyses of biopsied stages in human carcinogenesis, that acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer. Observations accumulated over the past decade indicate that the apoptotic program is present in latent form in virtually all cell types throughout the body. Once triggered by a variety of physiologic signals, this program unfolds in a precisely choreographed series of steps. Cellular membranes are disrupted, the cytoplasmic and nuclear skeletons are broken down, the cytosol is extruded, the chromosomes are degraded, and the nucleus is fragmented, all in a span of 30–120 min. In the end, the shriveled cell corpse is engulfed by nearby cells in a tissue and disappears, typically within 24 hr (Wyllie et al., 1980).

The apoptotic machinery can be broadly divided into two classes of components, sensors and effectors. The sensors are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die. These signals regulate the second class of components, which function as effectors of apoptotic death.
Further, the life of most cells is in part maintained by cell–matrix and cell–cell adherence-based survival signals whose abrogation elicits apoptosis (Ishizaki et al., 1995; Giancotti and Ruoslahti 1999). Both soluble and immobilized apoptotic regulatory signals likely reflect the needs of tissues to maintain their constituent cells in appropriate architectural configurations. Many of the signals that elicit apoptosis converge on the mitochondria, which respond to proapoptotic signals by releasing cytochrome C, a potent catalyst of apoptosis (Green and Reed, 1998). Members of the Bcl-family of proteins, whose members have either pro- apoptotic (Bax, Bak, Bid, Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W) function, act in part by governing mitochondrial death signalling through cytochrome C release. The p53 tumor suppressor protein can elicit apoptosis by up regulating expression of proapoptotic Bax in response to sensing DNA damage; Bax in turn stimulates mitochondria to release cytochrome C.

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).

1.2.4 Limitless Replicative Potential of cancer cells

The early work of Hayflick demonstrated that cells in culture have a finite replicative potential (Hayflick, 1997). Once such cell populations have progressed through a certain number of doublings, they stop growing; a process
termed senescence. 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 until they enter into a second state termed crisis. Cell that has acquired the ability to multiply without limit, the feature termed immortalisation (Wright et al., 1989).

Provocatively, most types of tumor cells that are propagated in culture appear to be immortalized, suggesting that 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.

The counting device for cell generations has been discovered over the past decade: the ends of chromosomes, telomeres, which are composed of several thousand repeats of a short 6 bp sequence element. Replicative generations are counted by the 50–100 bp loss of telomeric DNA from the ends of every chromosome during each cell cycle. This progressive shortening has been attributed to the inability of DNA polymerases to completely replicate the 39 ends of chromosomal DNA during each S phase. The progressive erosion of telomeres through successive cycles of replication eventually causes them to lose their ability to protect the ends of chromosomal DNA. The unprotected chromosomal ends participate in end-to-end chromosomal fusions, yielding the karyotypic disarray associated with crisis and resulting, almost inevitably, in the death of the affected cell (Counter et al., 1992). Telomere maintenance is evident in virtually all types of malignant cells (Shay and Bacchetti, 1997);
85–90% of them succeed in doing so by upregulating expression of the telomerase enzyme, which adds hexanucleotide repeats onto the ends of telomeric DNA (Bryan and Cech 1999). By one or the other mechanism, telomeres are maintained at a length above a critical threshold, and this in turn permits unlimited multiplication of descendant cells. Both mechanisms seem to be strongly suppressed in most normal human cells in order to deny them unlimited replicative potential.

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. During organogenesis, this closeness is ensured by coordinated growth of vessels and parenchyma. Once a tissue is formed, the growth of new blood vessels—the process of angiogenesis—is transitory and carefully regulated. Because of this dependence on nearby capillaries, it would seem plausible that proliferating cells within a tissue would have an intrinsic ability to encourage blood vessel growth. But the evidence is otherwise. 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 must develop angiogenic ability (Bouck et al., 1996; Hanahan and Folkman 1996).

Counterbalancing positive and negative signals encourage or block angiogenesis. One class of these signals is conveyed by soluble factors and their receptors, the latter displayed on the surface of endothelial cells; integrins and adhesion molecules mediating cell–matrix and cell–cell association also play critical roles. The angiogenesis-initiating signals are exemplified by vascular
endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors (FGF1/2). Each binds to transmembrane tyrosine kinase receptors displayed by endothelial cells (Veikkola and Alitalo 1999). A prototypical angiogenesis inhibitor is thrombospondin-1, which binds to CD36, a transmembrane receptor on endothelial cells coupled to intracellular Src like tyrosine kinases (Bull et al., 1994). There are currently more than two dozen angiogenic inducer factors known and a similar number of endogenous inhibitor proteins.

1.2.6 Tissue Invasion and Metastasis

Invasion and metastasis are exceedingly complex processes, and their genetic and biochemical determinants remain incompletely understood. At the mechanistic level, they are closely allied processes, which justifies their association with one another as one general capability of cancer cells. Both utilize similar operational strategies, involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases.

Several classes of proteins involved in the tethering of cells to their surroundings in a tissue are altered in cells possessing invasive or metastatic capabilities. The affected proteins include cell–cell adhesion molecules (CAMs)—notably members of the immunoglobulin and calcium-dependent cadherin families, both of which mediate cell-to-cell interactions and integrins, which link cells to extracellular matrix substrates. Notably, all of these “adherence” interactions convey regulatory signals to the cell (Aplin et al., 1998).

The second general parameter of the invasive and metastatic capability involves extracellular proteases (Coussens and Werb, 1996;
Chambers and Matrisian, 1997). Protease genes are upregulated, protease inhibitor genes are down regulated, and inactive zymogen forms of proteases are converted into active enzymes. Matrix degrading proteases are characteristically associated with the cell surface, by synthesis with a transmembrane domain, binding to specific protease receptors, or association with integrins (Werb 1997; Stetler-Stevenson 1999).

1.3 DRUG DISCOVERY IN CANCER RESEARCH

1.3.1 Mechanisms based target identification

Cancer as a disease in the human population is becoming a larger health problem, and the medicines used as treatments have clear limitations. In the past 20 years, there has been tremendous increase in our knowledge of the molecular mechanism and pathophysiology of human cancer. Many of these mechanisms have been exploited as a new target for drug development. The fruition of these efforts in the clinic is just now being released with a few encouraging results. The medical treatment of cancer still has many unmet needs. The main curative therapies for cancer; surgery and radiation- are generally only successful if the cancer is found at an early-localised stage. Once the disease has progressed to locally advanced cancer or metastatic cancer, these therapies are less successful.

1.3.2 Medicinal plants: a re-emerging health aid

Interest in medicinal plants as a re-emerging health aid has been fuelled by the rising costs of prescription drugs in the maintenance of personal health and well-being, and the bioprospecting of new plant-derived drugs. The widespread use of herbal remedies and healthcare preparations, as those
described in ancient texts such as the Vedas and the Bible, and obtained from commonly used traditional herbs and medicinal plants, has been traced to the occurrence of natural products with medicinal properties.

The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed (UNESCO, 1996). Furthermore, an increasing reliance on the use of medicinal plants in the industrialised societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies (UNESCO, 1998).

1.3.3 Role of medicinal plants in cancer drug discovery

Throughout medical history, plant products have been shown to be valuable sources of novel anti-cancer drugs. Examples are the *Vinca* alkaloids, the taxanes, and the camptothecins, derived from the Madagscan periwinkle plant *Canharantus roseuhs*, the Pacific yew *Taxus brevifolia*, and the Chinese tree *Camptotheca acuminata*, respectively. Approximately five decades of systemic drug discovery and development have established a respectable armamentarium of useful chemotherapeutic agents (Chabner 1991; Yarbro 1992), as well as a number of important successes in the treatment and management of human cancer. Nevertheless, the need for more effective anti-neoplastic agents remains. The most common tumors of the adult are resistant to available anti-neoplastic drugs (Yarbro 1992; Fact sheet 1996), and the majority of these agents have only limited anti-solid tumor activity (Chabner 1991; Sikora et al., 1999).
Drug development strategies based on naturally derived candidate compounds may present a considerable number of obstacles, which are not posed by those using rational synthesis. There may be problems of procurement due to inaccessibility of collection sites, difficulties with the isolation and production of the pharmacologically active ingredient, and serious legal disputes among governments about intellectual rights properties. Even so, screening of natural products seems more likely to yield a hit when compared with screening of rationally designed compounds.

1.3.4 The impact of naturally derived agents on human therapeutics

Plant-derived substances have traditionally played important roles in the treatment of human diseases (Harvey 1999). Today, about 80% of the world population residing in third world countries still rely almost entirely on plant products for their primary health care. The remaining 20% of individuals living in the first world use, in more than 25% of cases, pharmaceuticals which have been directly derived from plant products (Farnsworth 1984; Cox 1994). These range from common remedies such as aspirin (originally isolated from the Rosaceae Filipendula ulmaria), to prescription drugs such as the analgesic morphine and the cardiac glycoside digoxin (isolated from the Papaveraceae Papaver somniferum, and the Apocynaceae Digitalis purpurea, respectively).

Plant-derived compounds were also of great significance to cancer therapy (Cragg et al., 1997) (Table 1.1). It was, for instance, only upon the addition of the Vinca alkaloid vincristine or oncovin (isolated from Catharanthus roseus, Apocynaceae) (Johnson et al 1963) to mechlorethamine,
prednisone, and procarbazine (the MOPP regimen) that the first cures in a human cancer (Hodgkin’s disease) were achieved (DeVita et al 1970). The combination of the epipodophyllotoxin etoposide (derived from the mandrake plant *Podophyllum peltatum* and the wild chervil *P. emodi*, Berberidaceae (Stahelin 1973)), bleomycin, and cisplatin is currently a highly active and curative regimen in testicular cancer (Williams et al 1987). Etoposide is furthermore one of the most active agents against small cell lung carcinoma (Yarbro, 1992; Chabner, 1991; Harvey, 1999; Williams et al 1987). The more recent development of the structurally and mechanistically novel taxanes (extracted from the bark of the Taxaceae) (Table 1.1).

Table 1.1 Some cytotoxic drugs developed from plant source

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Plant source</th>
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<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></td>
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<tr>
<td>Paclitaxel, docetaxel</td>
<td>Promotion of tubulin stabilization</td>
<td><em>Taxus brevifolia</em> (Taxaceae)</td>
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<tr>
<td>Iriotecan, topotecan,</td>
<td>Inhibition of topoisomerase I</td>
<td><em>Camptotheca acuminata</em></td>
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<tr>
<td>9-aminocampothecin,</td>
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<td>9-nitrocampothecin</td>
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*Taxus brevifolia, T. canadensis, or T. baccata* (Wani et al, 1971 ) and the camptothecins (derived from the bark and wood of the *Nyssaceae Camptotheca acuminata* (Jordan et al., 1996) in the 1990s represented a
landmark in cancer research because of their significant anti-solid tumor efficacy. Paclitaxel is in many countries approved for the treatment of ovarian and breast carcinoma and also has important activity against non-small cell lung cancer (McGuire et al 1996). Irinotecan and topotecan are semi-synthetics from the lead compound camptothecin which are approved for the treatment of advanced colorectal cancer (Bertino, 1997), and as second-line chemotherapy in ovarian carcinoma (Creemers et al 1996), respectively. These agents are also active against several other solid malignancies such as carcinoma of the lung, cervix, and ovary (Bertino 1997; Creemers et al 1996).

Some of the technological advances over recent years would have been unimaginable not too long ago. In the era described as post genomic drug discovery, not only has the technology advanced at an impressive rate but so has the information gained from these technologies. Microarray analysis, genomics and proteomics provide gigantic amounts of data for the cancer researcher. These technologies have now been matched in the drug discovery process by high throughput screening techniques.

Successful drug treatment in human disease requires an adequate therapeutic index reflecting the treatment's specific effects on target cells and its lack of clinically significant effects on the host. In cancer, the therapeutic goal is to trigger tumor-selective cell death. The mechanisms responsible for such death are of obvious importance in determining the efficacy of specific treatments. With the discovery that distinct death pathways exist in biology, and that certain of these are evolutionarily selected and highly efficient, came an explosion of interest in connecting such pathways to the pathophysiology of cancer.
Apoptosis as a therapeutic goal offers advantages over non-apoptotic death mechanisms only if the therapeutic index or the availability of compounds that induce it is greater (Reed 2001). In drug-curable malignancies, such as common pediatric leukemias and certain solid tumors, apoptosis is a prominent (if not the exclusive) mechanism associated with the induction of tumor remission. In addition, the expression of apoptotic modulators within a tumor appears to correlate with its sensitivity to traditional cancer therapies (Figure 1.2).

A drug that activates apoptosis might achieve a suitable therapeutic index in several ways (Lowe and Lin 2000). First, it might activate a death cascade via a drug target that is uniquely expressed in a cancer cell. Alternatively, it might be delivered to the target tissue in a manner that is selective for the cancer cell. These two strategies have proved frustratingly difficult to achieve the core apoptotic machinery. Apoptotic cell death is triggered by intracellular cues such as DNA damage and osmotic stress, and extracellular cues including growth factor withdrawal, matrix detachment, and direct cytokine-mediated killing.

1.4 MICROTUBULE DYNAMICS AND CANCER DRUG DEVELOPMENT

Microtubules are intrinsically dynamic polymers, undergoing two kinds of dynamic behavior: dynamic instability and treadmilling. In dynamic instability, microtubule ends stochastically switch between episodes of prolonged growing and shortening (Mitchison and Kirschner 1984). Microtubule dynamics are important to many functions in cells, the most dramatic of which is mitosis. When cells enter mitosis, the interphase
Figure 1.2 Various apoptotic signaling targets for Anti-cancer drug development
cytoskeletal microtubule array is disassembled and a bipolar spindle is assembled. Spindle microtubules attach to chromosomes at the kinetochore and contribute to chromosome alignment and subsequent segregation at anaphase. Microtubule dynamics are relatively slow in interphase cells, but increase 10 to 100 fold at mitosis (Saxton et al., 1984; Pepperkok et al., 1990; Zhai et al., 1996). Both extensive dynamic instability and treadmillling occur in mitotic spindles, and the rapid dynamics of spindle microtubules play a critical role in the intricate movements of the chromosomes (Mitchison and Kirschner 1989; Hayden et al., 1990; Rieder et al., 1994; Waterman-Storer and Salmon, 1997). In addition, evidence is accumulating that microtubule dynamics may play a crucial role in passage through the metaphase/anaphase checkpoint (Jordan et al., 1992, 1996; Dhamodharan et al., 1995; McEwen et al., 1997; Sorger et al., 1997; Vasquez et al., 1997; Jordan and Wilson 1998).

1.4.1 Natural products that have anti-mitotic activity

A great variety of natural products are used as anticancer agents, their anti-mitotic activity being due to their interaction with microtubular protein. Taxol is an important new cancer chemotherapeutic agent that is effective in the treatment of many types of cancer, including carcinoma of the ovary, lung, head and neck, bladder, and esophagus (Rowinsky 1997). The principal chemotherapeutic target of taxol is microtubules (Schiff et al., 1980; Horwitz, 1992). Plant-derived antimitic drugs that interfere with the normal formation of mitotic spindles and cytoplasmic microtubules include cornigerine (Hamel et al., 1988), alkaloids such as colchicine (Margolis and Wilson 1978), vincristine and vinblastine (Johnson et al., 1963). Interfering with microtubule dynamics or stability can inhibit cell division in several ways. Either stabilizing microtubules or inhibiting polymerization will prevent the cytoskeleton
restructuring that is required at several points in the cell cycle and thus stop progression from one stage in the cell cycle to the next. In addition, microtubules go through a phase of rapid lengthening and shortening during the process of attaching chromosomes to the spindle, and inhibiting this process also prevents completion of cell division (Wilson et al., 1999). The essential role of microtubules in cell division and the ability of drugs that interact with tubulin to interfere with the cell cycle have made tubulin a successful target for applications that include anti-cancer drugs, fungicides, and herbicides.

1.5 TELOMERASE DIRECTED–MOLECULAR THERAPEUTICS

The management of malignant disease remains one of the most challenging areas of modern medicine. The lifetime risk of developing cancer in the western world is estimated to be as high as 1 in 3. Traditionally, surgery, chemotherapy and radiotherapy have been the primary choice of treatment for patients with malignant tumours. Despite advances in the use and development of conventional cytotoxic agents, the cure rate remains disappointing in most patients with advanced disease of the common solid tumours. Consequently, the development of novel anti-cancer therapies is a high priority in cancer medicine. In recent years, a new generation of cancer therapies has emerged, based on a growing understanding of the molecular events that contribute to malignant transformation. A major difference between normal and cancer cells is the ability of cancer cells to multiply in an unrestricted and ungoverned fashion. In this context, there is considerable interest in elucidating the mechanisms that allow this unrestricted proliferation and that ultimately result in immortal cancer cells. It is now clear that the enzyme telomerase confers immortality on cells in most types of cancer. With the cancer cell reliant on telomerase for its survival, telomerase represents an extremely attractive mechanism-based target for the development of new cancer therapeutics.
1.5.1 Telomere structure and function

Telomeres are specialised structures at the end of chromosomes that consist of tandem nucleotide repeats in humans; the repeated sequence is the 6 bp TTAGGG. Telomeres act as protective caps, preventing both degradation of the ends of the chromosomes and their recognition as double strand breaks, which might otherwise result in aberrant recombination. As chromosomes are replicated during cell division, there is incomplete replication of the extreme 3'end of the lagging strand. This is known as the 'end replication problem' and results in the loss of approximately 50 bp of telomeric material with each division. It is postulated that this could act as a molecular counting mechanism, marking the number of cell divisions (Figure 1.3) (Reddel 2000; Bouffler et al 2001). Cell numbers are vigorously controlled within the body and, in human adults, only a few cell types are capable of continued division. Cultured cells in vitro can undergo only a limited number of cell divisions, known as the Hayflick limit, before entering a state of senescence where they remain metabolically active but have lost their replicative capacity (Reddel 2000; Shay and Wright 2000). Reduction in telomere length could provide the signal to cause growth arrest. Cultured cells can be induced to continue to divide beyond the Hayflick limit by expression of transforming oncogenes (Reddel 2000; Hahn et al., 1999).

1.5.2 Telomerase structure and function

Telomerase consists of an RNA component, hTR (also known as hTERC), which includes the template for synthesis of telomere DNA, and a protein catalytic component, hTERT, with reverse transcriptase activity and homology to viral reverse transcriptases. These two components are necessary
Figure 1.3 Regulation of telomere length in normal and cancer cells by telomerase
and sufficient for telomerase activity in vitro, although several additional molecules might play a role in regulating in vivo activity (Bachand et al 2000; Masutomi et al., 2000).

Telomerase adds telomeric repeats on to the ends of chromosomes, thus maintaining their length despite continued cell division. It is postulated that, at crisis, critical telomere shortening results in end-to-end fusions and chromosome shrinkage that leads fusion cycles that cause marked chromosomal abnormalities and apoptosis (Bouffler et al 2001). Expression of telomerase would avoid this catastrophic series of events. This model is supported by the demonstration that forced expression of telomerase allows maintenance of telomere length and increases the replicative lifespan of some cell types without a crisis period.

1.5.3 Telomerase expression in human cancer

In adult humans, most normal tissues have low or no detectable telomerase activity (Holt and Shay 1999; Meyerson 2000; Shay and Bacchetti, 1997). Where telomerase activity has been demonstrated in normal tissue, this has generally been within cells that maintain their replicative capacity into adult life. These include male germ cells, activated lymphocytes, and stem cell populations such as haematopoietic progenitor cells, basal keratinocytes and those in the intestinal crypts (Holt and Shay 1999; Meyerson 2000; Yui et al 1998). By contrast, telomerase activity is found at high frequency across the whole spectrum of human cancers (Holt and Shay 1999; Meyerson 2000). However, the frequency of activation can vary between cancer types, suggesting some tissue-specific regulation (Soder et al 1998, Sarvesvaran et al., 1999). Telomerase activity is assayed using a very sensitive polymerase chain
reaction (PCR)-based assay known as the telomeric repeat amplification protocol (TRAP) (Shay and Gazdar 1997).

1.5.4 Inhibiting telomerase as a therapy for treating cancer

Telomere loss is thought to control entry into senescence. Human telomeres consist of repeats of the sequence TTAGGG/CCCTAA at chromosome ends; these repeats are synthesized by the ribonucleoprotein enzyme telomerase. Telomerase is active in germline cells and, in humans; telomeres in these cells are maintained at about 15 kilobase pairs. In contrast, telomerase is not expressed in most human somatic tissues, and telomere length is significantly shorter. The telomere hypothesis of cellular aging proposes that cells become senescent when progressive telomere shortening during each division produces a threshold telomere length. One important consideration with this proposed treatment regimen is the prolonged time potentially required for a telomerase inhibitor to be effective. Since the mode of action of telomerase inhibitors may require further telomeric shortening of cancer cells before inhibition of cell proliferation, there may be a significant delay in efficacy. The combinatorial use of telomerase inhibitors with other cancer therapeutic agents may more effectively achieve complete cancer remissions.

1.6 SIGNALLING PATHWAYS IN APOPTOSIS AS POTENTIAL TARGETS FOR CANCER THERAPY

The goal of inducing apoptosis in cancer cells is achieved at present primarily via chemotherapy and radiation treatments. As the complex pathways for apoptosis are unraveled, it might become possible to identify biochemical
targets that will enable the design of drugs that induce apoptosis in cancer cells that are resistant to current therapies. Multiple drug-discovery targets for inducing apoptosis in cancer cells have been considered and are currently under study. However, bringing a compound successfully to the clinic will depend not only on choosing the proper target in terms of its clear biological endpoints but also on finding a target that can be readily antagonized by small synthetic organic molecules. Because the desired biological outcome for these drugs is cell death, the particular challenge in developing pro-apoptotic compounds for therapeutic applications will be selectively to promote the death of tumor cells yet to spare normal cells. Targeting the molecular processes that regulate apoptosis might permit us to achieve this goal (Figure 1.2).

1.6.1 Apoptosis and Cancer

In the recent past, molecular oncologists focused their studies primarily on the cellular pathways controlling proliferation. Neoplastic disease was typically envisaged as resulting from defects in these pathways leading to excess cell division. By extension, cancer therapies, when successful, were thought to act by selectively targeting rapidly cycling cells. Apoptosis is now widely accepted to play a role in tumorigenesis. In the same way that programmed cell death by apoptosis may have evolved as a mechanism for regulating cell numbers and their interactions during normal development, apoptosis may also serve to eliminate cells gone "awry" in the adult organism. In fact, many proteins, which are typically known as regulators of cellular proliferation, also are potent inducers of apoptosis. Many oncogenes, which stimulate cellular proliferation also potently, induce apoptosis. Although it still remains unclear whether these genes kill cells by mechanisms related to the manner in which they promote proliferation, tumors, which exhibit deregulated
expression of these oncogenes typically, select genetic aberrations, which inactivate apoptosis. p53 loss or high Bcl-2 levels are common examples of such aberrations, and often correlate with poor prognosis in the clinics. These observations have provided insights on the in vivo process of 'multi-step' tumorigenesis, whereby loss of growth control as well as inactivation of the apoptosis pathway are central for the survival and proliferation of tumors.

1.6.2 Significance of apoptosis in cancer

The study of apoptosis is relevant to many aspects of tumor biology, which include tumorigenesis, tumor homeostasis, angiogenesis, metastasis, and clinical treatment. Malignant cells often harbor mutations in critical components of the apoptotic pathway, which may correlate with poor prognosis. Solid tumors must circumvent the apoptotic pathways, which regulate their anchorage-dependent survival in order to metastasize and establish secondary tumor sites. Although chemotherapy was long thought to kill tumor cells by inactivating critical metabolic pathways, it is now recognized that many effective chemotherapeutics trigger the tumor into killing itself by activation of an apoptotic pathway. It is therefore of prime importance for the discovery of improved treatments to better understand the molecular underpinnings of the apoptosis machinery. It is crucial to remember, however, that much of the knowledge we have gained has been derived from the study of 'developmental apoptosis' - or programmed cell death - in primitive organisms amenable to genetic manipulation. Given the highly conserved nature of the apoptosis machinery (at least parts of it), uncovering its molecular details using a variety of in vitro and genetic systems will likely provide useful information for cancer therapy.
1.7 ONCOPROTEIN INDUCED CELL DEATH

The Myc proto-oncogene has long been implicated in the control of normal cell growth and its deregulation is associated with the development of neoplasia. The myc protein has a well established role as a component of signal transduction pathways promoting both proliferation and apoptosis because signalling pathways that drive cell death and cell proliferation are so tightly coupled, a synergy between genetic lesions leading to suppression of cell death and those promoting cell proliferation is observed during carcinogenesis.

One of the first oncogenes demonstrated to have proapoptotic activity was c-myc. c-myc is one of a family of related mammalian genes that encode the Myc proteins, transcription factors of the bHLH-zip family. Deregulated Myc expression is observed in a variety of tumors, and its enforced expression in animals leads to cellular transformation and tumorigenesis (Marcu et al., 1992). In culture, fibroblasts transformed with Myc display increased growth kinetics when cultured in the presence of adequate serum growth factors. Upon serum withdrawal, however, they undergo rapid apoptosis, the kinetics of which correlate with cellular levels of Myc protein (Evan et al., 1992). In contrast, serum starvation in untransformed fibroblasts typically results in growth arrest. This transformation-selective response is also observed following a number of apoptotic triggers such as radiation and chemotherapy.

1.7.1 Myc-induced apoptosis: dual signal hypothesis

A number of oncoproteins induce apoptosis when overexpressed in cells (Schwartz, 1992). The best characterised examples are the transcription factor c-Myc (Plantefaber and Hynes 1989). Oncogenes activate apoptosis if
their proliferative action is blocked in some way or if the cell’s proliferative machinery is incompletely activated or coordinated. The current view of Myc-induced apoptosis is expressed in the 'dual signal' hypothesis (Re et al., 1994). According to this hypothesis Myc promotes both pathways at the same time proliferation and apoptosis. The apoptotic pathway is suppressed as long as appropriate survival factors deliver anti-apoptotic signals. Such a scenario would act with a general model of survival/cell death regulation according to which the 'default' fate of a cell would be cell death unless suppressed by anti-apoptotic cytokine signalling (Boudreau et al., 1995).

1.7.2 C-Myc and its Target genes

Several candidate Myc-regulated genes have been identified (Frisch et al., 1996; Nikiforov et al., 1996). Two genes suggested as Myc targets, based on the pattern of expression, are ornithine decarboxylase (ODC) and cdc25A. ODC is a rate-limiting enzyme in polyamine biosynthesis, necessary for DNA synthesis, while Cdc25A encodes a tyrosine phosphatase involved in activation of the key inducers of mitosis, the cyclin-dependent kinase (CDK) complexes. Both candidate Myc targets, ODC and cdc25A, when overexpressed, induce apoptosis in cells lacking survival factors (as Myc over-expression does). It therefore seems possible that the cell proliferation and cell death pathways bifurcate 'downstream' of c-Myc. In addition the TNF-R-associated protein TRAP-1 (McGill et al., 1997) and the Bcl-2 family member Bax (Zhang et al., 1995) have been revealed as further Myc targets possibly involved in Myc-induced apoptosis. The emerging model suggests that c-Myc promotes apoptosis by causing the release of cytochrome c, but the ability of cytochrome c to promote apoptosis is critically dependent upon other signals such as CD95 activation. Myc’s cytochrome c-releasing activity might be mediated by upregulation of the pro-apoptotic Bcl-2 family member Bax which
has been suggested as a transcriptional target and mediator of c-Myc-induced apoptosis.

1.8 H-RAS PROMOTES CARCINOGENESIS AND MAINTAINS THE SURVIVAL BALANCE

There is considerable evidence that activated Ras proteins inhibit apoptosis and promote cell proliferation, under many circumstances. There are also many reports stating the opposite: that Ras pathways can promote apoptosis and can inhibit proliferation (Downward 1998). It is clear that effects of ras on these cellular responses depends strongly on the cell type that is being studied and the context of other regulatory influences that the cell is receiving (Downward 1998).

1.8.1 The role of ras in cancer

Ras gene products function as binary switches by cycling between the active state, GTP bound, and the inactive state, GDP bound and are positioned at a critical check point in the cell signalling cascade. That ras plays a direct role in the control of normal and transformed cell growth is supported by several lines of evidence. First, ras activating mutations have been identified in a variety of animal tumors as well as 10-25% of all human tumors (Barbacid 1987; Bos 1989; Willumsen 1992). Second, expression of activated ras or high levels of wild type ras results in mitogenic stimulation of a variety of cell lines (Stacey and Kung 1984). Third, cellular proliferation is inhibited following inactivation of ras. For example, inactivation of c-K-ras by homologous recombination (Shirasawa et al., 1993), inhibition of activated c-H-ras (Mukhopadhyay et al., 1991; Saison-Behmoaras et al., 1991) and c-K-ras by
antisense strategies (Zhang et al., 1993; Aoki et al., 1995), inhibition of post-translational modification by farnesyl protein transferase inhibitors (FPTase) (Kohl et al., 1994).

1.8.2 Regulation of ras activity

The half-life of ras protein, p21, is greater than 24 h (Ulsh and Shih, 1984), suggesting that much of its regulation is at the level of protein activation, either through proper subcellular localization, for example via farnesylation, or increasing the amount of time GTP is bound. It has now been shown that activation of ras, as well as many other members the small GTPase super family, is tightly regulated by families of proteins that either positively or negatively potentiate ras activity (Boguski and McCormick 1993).

As understanding of signalling pathways expands, it seems quite likely that increasingly complex pathways that affect the activation state of ras, either directly or indirectly, will be revealed. It then becomes easy to postulate that alternative mechanisms of ras activation exist, and to conclude that ras can be activated without point mutation or elevated levels of expression. This suggest that a higher proportion of tumors may be ras dependent, via alternative activation mechanisms, than is currently predicted by the incidence of point mutation.

1.9 Bcl-2 FAMILY PROTEINS AND ITS ROLE IN APOPTOSIS

1.9.1 Bcl-2 an anti-apoptotic protein

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). It has been the focus of intense study ever since its demonstrated ability to rescue pre-B cells from apoptosis in response to IL-3 withdrawal. Originally identified as a result of the (Miyashita et al., 1994) translocation in B-cell follicular lymphoma in which its juxtaposition with the IgH enhancer leads to deregulated over expression, Bcl-2's causal role in tumorigenesis was directly confirmed in studies where mice expressing a Bcl-2 transgene in lymphoid cells developed B-cell malignancies (Miyashita et al., 1994). Bcl-2 is now the prototype of a rapidly growing family of interacting proteins, which share its ability to modulate apoptosis. A prevalent model has emerged by which a cell's threshold to apoptosis is determined by the levels of pro- and anti-apoptotic members, which through dimerization, act as a survival "rheostat switch" (Oltvai and Korsmeyer 1994). The biochemistry through which Bcl-2 and its 'partners-in-crime' mediate their effects has only recently started to unfold. A simple 'rheostat switch' model whereby both pro- and anti-death molecules require co-interaction to mediate their effects on apoptosis is not sufficient to explain the above observations. Alternatively, death-promoting and death-suppressing Bcl-2 family proteins may operate in mechanistically different ways.

1.9.2 Bcl-2 family proteins

Bcl-2 family proteins share several homology regions (Bcl-2-homology/BH domains) crucial for both their dimerisation and apoptosis-modulatory functions (Oltvai and Korsmeyer 1994). At least 15 Bcl-2 family members have been identified in mammalian cells and several others in viruses. All members possessed at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4) and can be classified into two main groups based on their influence on apoptosis, as pro-survival and pro-apoptotic. Most pro-survival members, which can inhibit apoptosis in the face of a variety
of cytotoxic insults, contain at least BH1 and BH2, and those most similar to Bcl-2 also bear BH4 group. The BH domains along with the use of the yeast two-hybrid and other methods have been instrumental in the identification of novel interacting proteins such as Bax (Oltvai et al., 1993), Bcl-xL (Boise et al., 1993), Bad (Yang et al., 1995), Bag-1 (Takayama et al., 1995), Bak (Chittenden et al., 1995; Kiefer et al., 1995), Bik (Boyd et al., 1995), Hrk/harakiri (Inohara et al., 1997) and others. The pro-apoptotic Bax protein, cloned through its ability to co-immunoprecipitate with Bcl-2, was originally found to form homodimers as well as heterodimers with Bcl-2 via the BH1 and BH2 domains (Oltvai et al., 1993). Site-directed mutagenesis of these domains in Bcl-2 prevent heterodimer formation with Bax (or Bak) and abrogate its death-repressor activity (Yin et al., 1994). Interestingly, Bcl-2 mutations permissive for homodimerization, but not heterodimerization with Bax, also abolished Bcl-2 activity suggesting that Bcl-2 requires Bax to exert its death-repressor activity.

1.9.3 Bcl-2 family proteins and Mitochondrial integrity

Mitochondria are central to the apoptosis activation pathway in many physiological and pathological conditions. Members of the Bcl-2 family of proteins are known to affect mitochondrial function and regulate the release of apoptosis-activating factors (Susin et al., 1996; Reed et al., 1998; Yang et al., 1997). Anti-apoptotic members of Bcl-2 family (e.g. Bcl-2 and Bcl-xL) act primarily to preserve mitochondrial integrity by suppressing the release of cytochrome c (Yang et al., 1997). In contrast, pro-apoptotic members (Bax, Bid, etc.) induce the release of cytochrome c and cause mitochondrial dysfunction (Eskes et al., 1998; Li et al., 1998). The pro-apoptotic protein, Bax, which normally resides in the cytosol, translocates to mitochondria when triggered by certain stimuli (Eskes et al., 1998). Translocated Bax has been
shown to induce cytochrome \( c \) release both in vivo (Yang et al., 1997) and in vitro (Jurgensmeier et al., 1998) and this is followed by caspase activation (Cregan et al., 1999). The mitochondrial permeability transition, an event that results in disruption of the mitochondrial potential gradient, has been reported to induce cytochrome \( c \) release and apoptosis (Marchetti et al., 1996). However, several other reports suggest that the effects of Bax are targeted at the outer mitochondrial membrane and that the mitochondrial inner membrane remains intact even after Bax-induced release of cytochrome \( c \) (Saikumar et al., 1998).

1.10 p53: THE TERMINATOR

Tumor suppressor gene p53 is a critical regulator of cellular response to DNA damage. Normal cells express very low levels of p53 but they rise rapidly after irradiation or exposure to DNA-damaging drugs (Strasser et al., 1994). Enforced expression of p53 triggers either G1 cell cycle arrest without loss of viability or growth arrest followed by apoptosis. A major arm of the mammalian DNA damage response involves a suite of protein kinases, distantly related to the intracellular signalling molecule phosphatidylinositol 3-kinase (PI 3-kinase), of which the prototypes are ATM (Ataxia Telangiectasia Mutated), mutated in the human autosomal recessive disorder ataxia telangiectasia, and the DNA dependent protein kinase (DNA-PK). ATM and DNA-PK are the mammalian homologs to the yeast Rad-3/MEC1 kinase that mediate DNA damage responses.

1.10.1 Role of Mdm-2 in p53

One pivotal target of ATM (Xu and Baltimore, 1996), and possibly DNA-PK, is the tumor suppressor p53, a transcription factor normally
maintained in abeyance at low levels through interaction with the Mdm-2 protein that signals its degradation. Mdm-2 is itself a target for DNA-PK (Xu and Baltimore, 1996). DNA damage–induced phosphorylation of either p53 or Mdm-2 prevents the two proteins from interacting, thus stabilizing and activating p53. The high frequency with which p53 is functionally inactivated in human cancers attests to its pivotal role as a bulwark against expansion of mutated somatic cells. In many cases, p53 itself is mutated or deleted. However, lesions leading to elevated Mdm-2 also lead to p53 inactivation. Mdm-2 is amplified in certain tumors, and the Mdm-2 protein is a target for p19 ARF, the product encoded by the alternative reading frame within the Ink4a tumor suppressor gene locus—a site frequently deleted in human malignancies (Haber 1997).

1.10.2 p53 activation leads to growth arrest and apoptosis

Two cellular responses to p53 activation are well described—growth arrest (in cell cycle stages G 1 and G 2) and apoptosis. Which of these two responses prevails seems to depend on cell type, cell environment, and factors such as oncogene expression. However, the end points of each of these two processes probably amount to the same thing. Evidence indicates that p53- induced growth arrest after certain types of DNA damage is irreversible (Leonardo et al., 1994) although alive, such cells are genetically dead and thus constitute no further neoplastic risk.

Substantial evidence suggests that a major part of p53-mediated growth arrest proceeds through induction of the cyclin-dependent kinase (Cdk) inhibitor p21 (Hansen and Oren 1997). In contrast, the mechanism by which p53 promotes apoptosis is more obscure, although many studies indicate that it
involves induction of specific target genes (Attardi et al., 1996) that differ from those implementing growth arrest (Hansen and Braithwaite, 1996). Examples of p53 targets implicated in apoptosis are the Bcl-2 antagonist Bax (Yin et al., 1997), the insulin-like growth factor-I (IGF-I) receptor (Ohlsson et al., 1989) and the binding protein IGF-BP3 (Buckbinder et al., 1995) components of the renin-angiotensin system (Harvey 1999), and proteins regulating angiogenesis (Bian and Sun 1997; Dameron et al., 1994). Moreover, in certain circumstances, trans repression of anti-apoptotic genes has been implicated (Sabbatini et al., 1995; Miyashita et al., 1994), and even non transcriptional mechanisms may be involved (Bian and Sun 1997; Caelles et al., 1994).

1.11 DEATH RECEPTOR SIGNALLING IN APOPTOSIS

Although cell death can be triggered by a vast array of stimuli, the manner by which all apoptotic signals engage the cell death machinery. One of the pathway involves triggering cell surface ‘death receptors’, a specialized subset of the tumor necrosis factor receptor (TNF-R) super family, which includes Fas/CD95, TNF-receptor (TNF-R1), and death receptor 3 (DR3). Upon receptor aggregation by ligand, intracellular adapter proteins such as FADD/MORT1, TRADD and RAIDD are recruited to the cytoplasmic regions of the receptors through homotypic death-domain (DD) interactions to form a death-inducing signalling complex (DISC) (Hansen and Braithwaite 1996). In turn, FADD recruits procaspase-8 via interactions between death-effector domains (DEDs) present in both proteins, thereby stimulating caspase-8 autoproteolytic activation and initiating a caspase cascade leading to cell death (Hansen and Braithwaite 1996).
1.11.1 Death Receptors Have Direct Access to the Apoptotic Machinery

Death receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily, which is defined by similar, cysteine-rich extracellular domains (Smith et al., 1994). The death receptors contain in addition a homologous cytoplasmic sequence termed the "death domain" (Tartaglia and Goeddel 1992). Death domains typically enable death receptors to engage the cell's apoptotic machinery, but in some instances they mediate functions that are distinct from or even counteract apoptosis. Some molecules that transmit signals from death receptors contain death domains themselves.

The best-characterised death receptors are CD95 (also called Fas or Apo1) and TNFR1 (also called p55 or CD120a) (Smith et al., 1994; Nagata 1999). Additional death receptors are avian CAR1; death receptor 3 (DR3; also called Apo3, WSL-1, TRAMP, or LARD) (Chinnaiyan et al., 1995; Marsters et al., 1998; Kitson et al., 1996); DR4 (Pan et al., 1997); and DR5 (also called Apo2, TRAIL-R2, TRICK 2, or KILLER) (Pan et al., 1997; Walczak et al., 1997). CD95 ligand (CD95L) binds to CD95; TNF and lymphotoxin a bind to TNFR1; Apo3 ligand (Apo3L, also called TWEAK) (Wu et al., 1997; Chaudhary et al., 1997) binds to DR3 (Liepinsh et al., 1997) and Apo2 ligand (Apo2L, also called TRAIL) binds to DR4 and DR5 (Marsters et al., 1998; Schneider et al., 1997).

1.11.2 Signalling by CD95

Like other TNF family members, CD95L is a homotrimeric molecule. The crystal structure of lymphotoxin in complex with TNFR1 suggests by analogy that each CD95L trimer binds three CD95 molecules (Smith et al.,
Because death domains have a propensity to associate with one another, CD95 ligation leads to clustering of the receptors’ death domains; this is supported by nuclear a genetic resonance structure analysis and mutagenesis studies (Huang et al., 1996). An adapter protein called FADD (Fas-associated death domain; also called Mort 1) (Chinnaiyan et al., 1995; Boldin et al., 1996) then binds through its own death domain to the clustered receptor death domains. FADD also contains a “death effector domain” that binds to an analogous domain repeated in tandem within the zymogen form of caspase-8 (also called FLICE, or MACH) (Boldin et al., 1996). The death effector domain is a specific example of a more global homophilic interaction domain termed CARD (Caspase recruitment domain), which is found in several caspases with large prodomains, including caspases-2, 8, -9, and -10 (Hofmann et al., 1997). Upon recruitment by FADD, caspase-8 oligomerization drives its activation through self-cleavage (Marta Muzio et al., 1998). Caspase-8 then activates downstream effector caspases such as caspase-9 — the mammalian functional homolog of CED-3—committing the cell to apoptosis. Studies with FADD gene knockout mice (Yeh et al., 1998; Zhang et al., 1998) and with transgenic mice expressing a dominant negative mutant of FADD (FADD-DN) in T cells (Newton et al., 1998) establish that FADD is essential for apoptosis induction by CD95. Surprisingly, these mice display reduced proliferation of mature T cells in response to antigenic stimulation; moreover, FADD deletion causes embryonic lethality (Yeh et al., 1998; Zhang et al., 1998; Newton et al., 1998). These results are consistent with FADD having other critical signalling functions besides coupling CD95 to caspase-8.

1.11.3 Signalling by TNFR1

TNF is produced mainly by activated macrophages and T cells in response to infection (Tartaglia and Goeddel 1992). By engaging TNFR1, TNF
activates the transcription factors NF-kB and AP-1, leading to induction of pro-inflammatory and immunomodulatory genes (Tartaglia and Goeddel 1992). In some cell types, TNF also induces apoptosis through TNFR1. Unlike CD95L, however, TNF rarely triggers apoptosis unless protein synthesis is blocked, which suggests the preexistence of cellular factors that can suppress the apoptotic stimulus generated by TNF. Expression of these suppressive proteins probably is controlled through NF-kB and JNK/AP-1, as inhibition of either pathway sensitises cells to apoptosis induction by TNF (Beg and Baltimore, 1996; Roulston et al., 1998). TNF trimerizes TNFR1 upon binding, inducing association of the receptors' death domains. Subsequently, an adapter termed TRADD (TNFR-associated death domain) (Hsu et al., 1995) binds through its own death domain to the clustered receptor death domains. TRADD functions as a platform adapter that recruits several signalling molecules to the activated receptor: TNFR-associated factor-2 (TRAF2) (Rothe et al., 1995; Hsu et al., 1996) and receptor-interacting protein (RIP) (Hsu et al., 1996; Ting et al., 1996) stimulate pathways leading to activation of NF-kB and of JNK/AP-1, whereas FADD mediates activation of apoptosis (Hsu et al., 1996; Chinnaiyan 1996; Varfolomeev et al., 1996).

1.11.4 Activation of TNFR1 by TNF-α leads to apoptosis

Activation of the TNF receptor I (TNFRI) by TNF leads to the recruitment of the death domain-containing protein TNFR-I associated death domain protein and receptor-interacting protein to the receptor complex (Hsu et al., 1996; Ting et al., 1996). Along with the receptor-interacting protein and other signalling molecules, TNFRI-associated death domain protein can activate the NF-κB signalling pathway or trigger the caspase cascade by interacting with FADD (Fas-associated death domain protein) in the absence of
NF-κB activation (Hsu et al., 1996; Chinnaiyan 1996; Varfolomeev et al., 1996). FADD recruits and activates caspase-8. Both caspase-8 and FADD contain death effector domains (DED) that were found to play a critical role for the protein-protein interaction and caspase activation (Chinnaiyan 1996; Varfolomeev et al., 1996). The idea of targeting specific death receptors to induce apoptosis in tumors is attractive, because death receptors have direct access to the caspase machinery. Moreover, unlike many chemotherapeutic agents or radiation therapy, death receptors initiate apoptosis independently of the p53 tumor suppressor gene, which is inactivated by mutation in more than half of human cancers (Figure 1.4).

1.12 THERAPEUTIC POTENTIAL OF NF-KB IN CANCER

NF-κB exists as a dimeric complex, comprising different members of the Rel family: p50, p52, p65, c-Rel, and RelB; all of which contain a well conserved amino-terminal 300 amino acid region. This part of the sequence, known as the “Rel homology region,” is responsible for DNA binding, dimerization, and nuclear localization (Ghosh et al., 1998). In unstimulated cells, NF-κB is located in the cytoplasm as an inactive complex through interactions with inhibitory proteins, IκBa and IκBb. Upon cellular stimulation processes, such as cytokine induction or ionizing radiation, IκBs are rapidly phosphorylated and degraded. This in turn releases NF-κB, allowing translocation into the nucleus, binding to specific kB sites, and subsequently inducing the expression of target genes responsible for cell survival (Chen and Manning 1995). Previous studies have shown that activation of NF-κB is inhibited by a variety of mechanisms. The prevention of degradation or induction of IκB synthesis leads to the inhibition of NF-κB activation (Auphan et al., 1995; Kopp and Ghosh, 1994; Gilmore et al., 1996). Inhibition of p65 phosphorylation, which is
Figure 1.4  TNF-α induced apoptosis

(Adopted from Ashkenzai and Dixit, Death receptors : Signalling and Modulation, Science, 1998)
important for the activation of p65, has also been known to suppress transcriptional activity of NF-kB. Moreover, the cleavage of NF-kB by caspases inhibits its activity during apoptosis (Beg and Baltimore 1996). These reports indicate that inactivation of NF-kB activity plays a crucial role in the apoptotic pathway.

1.12.1 NF-kB regulation of cellular apoptosis and proliferation

In addition to activating the expression of genes involved in the control of the immune and inflammatory response, the NF-kB pathway is also a key mediator of genes involved in the control of the cellular proliferation and apoptosis. Anti-apoptotic genes that are directly activated by NF-kB include the cellular inhibitors of apoptosis (c-IAP1, c-IAP2, and IXAP), the TNF receptor–associated factors (TRAF1 and TRAF2), the Bcl-2 homologue A1/Bfl-1, and IEX-IL (Wang et al., 1998; Wu et al., 1997). One of the best-studied pathways that activates apoptosis is induced following treatment of cells with TNF-α. TNF-α treatment increases the expression of TRAF1, TRAF2, c-IAP1, and c-IAP2 (Wang et al., 1998). The overexpression of these proteins can protect RelA-deficient cells, which are highly sensitive to TNF-α induced apoptosis, from cell death. These antiapoptotic proteins block the activation of caspase-8, an initiator protease, involved at an early step in stimulating the apoptotic pathway (Wang et al., 1998).

1.12.2 Natural products that inhibit the NF-kB pathway

Flavonoids are naturally occurring phenolic compounds, found in plants, that exhibit a variety of biological activities, including suppression of inflammation, cancer chemoprevention, and protection from vascular disease.
Several reports suggest that the properties of the flavonoids quercetin, resveratrol, and myricetin may be mediated through downregulation of the NF-κB pathway (Baeuerle 1998; Beg and Baltimore 1996). For example, resveratrol, which is found in red wine, can inhibit NF-κB activity and induce apoptosis in transformed cells, which may contribute to the ability of red wine to reduce mortality from coronary heart diseases and certain cancers (Baeuerle 1998).

Although the maintenance of appropriate levels of NF-κB activity is a critical factor in achieving normal cellular proliferation, constitutive NF-κB activation is likely involved in the enhanced growth properties seen in a variety of cancers. The potential applications of inhibition of the NF-κB pathway in cancer chemotherapy are in their early stages (Baeuerle 1998). However, such approaches offer the promise of enhancing the efficacy of cancer chemotherapy and reducing abnormal cytokine production, which may contribute to the growth of certain tumors.

1.13 ROLE OF CASPASES IN APOPTOSIS
1.13.1 The executioner and its substrates

Our 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". Our knowledge
of the executioner as well as some of its key modulators stems in large part from genetic studies of the nematode *C. elegans*. 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 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).

1.13.2 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.

1.13.3 Three general mechanisms of caspase activation
1.13.3.1 Processing by an upstream caspase

Most caspases are activated by proteolytic cleavage of the zymogens between the p20 and the p10 domains, and usually also between the prodomain and the p20 domain at Asp-X sites-candidate caspase substrate sites-suggesting the possibility of autocatalytic activation. 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 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).

1.13.3.2 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 procaspase-8, resulting in a high local concentration of the zymogen. Under
these crowded conditions, the low intrinsic protease activity of procaspase-8 is sufficient to allow the various proenzyme molecules to mutually cleave and activate each other. This is however a rather crude way to control the fate of a cell and therefore additional levels of regulation must exist \textit{in vivo} to modulate the process (Liang and Fesik 1997).

\subsection{1.13.3.3 Association with a regulatory subunit}

Activation of procaspase-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. Little is known about the regulation of the interaction between procaspases and their cofactors. Proteins like FLIPs (FADD-like ICE Inhibitory proteins) that are similar in sequence to procaspase-8, except that they lack essential catalytic residues, may compete with procaspase-8 for binding to its cofactor, FADD, thus preventing caspase activation. It appears that procaspase is not the only protein with a decoy, as suggested by the recent discovery of the CARD domain containing protein ARC (apoptosis repressor with caspase recruitment domain). Compartmentalization of caspases and their cofactors are another way of regulating caspase activation (Nicholson 2000).