PREFACE

The development of more effective drugs for treating patients with cancer has been a major human endeavor over the past 50 years, and the 21st century now promises some dramatic new directions. While improvements in surgery and radiotherapy have had a major impact on cancer treatment, the concept of systemic chemotherapy, specific for cancer cells and free of major side effects, remains a critical goal for the future. The issues underlying the achievement of this goal are complex, extending from an understanding of how cancer growth is controlled, through the technology of drug synthesis and testing, to the multifactorial requirements for clinical trial. For anyone working in any single area of anticancer drug development, it is important to have an overview of the whole process.

This book aims to provide such an overview. The opening chapters discuss possible targets for drug design, including the cell division cycle, growth signal transduction, apoptosis induction, and the manifold interactions between tumor cells and host tissues. Succeeding chapters then consider techniques of identifying new potential drugs, including molecular modeling, chemical synthesis, and screening. The concluding chapters detail the required steps that any new potential anticancer agent must go through before it can be considered for routine clinical treatment. In each of these areas, a number of eminently qualified contributors have provided commentaries. Inevitably there are areas of overlap, but these have been retained because they reflect the interdependence of different areas of research.

We hope that this book will provide a useful commentary, including both overviews and specific detail, on this vital but fascinating subject. We also hope that it will stimulate original thought and further encourage those from both scientific and medical backgrounds who are committed to improving the outlook of cancer patients worldwide.

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CHAPTER 1

A BRIEF HISTORY OF CANCER CHEMOTHERAPY

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Summary

Clinical cancer chemotherapy in the 20th century has been dominated by the development of genotoxic drugs, initiated by the discovery of the anticancer properties of nitrogen mustard and the folic acid analogue aminopterin in the 1940s. The development of inbred strains of mice in the early part of the 20th century led to the use of transplantable tumors for the screening of very large numbers of compounds, both natural and synthetic, for experimental antitumor activity. Such screening led to the identification of clinically useful drugs at a rate of approximately one every 2 years. New targets for cytotoxicity were identified in this program, including tubulin and DNA topoisomerases I and II. The huge expansion in our basic knowledge of cancer has facilitated the development of two new anticancer strategies: the inhibition of specific cellular growth pathways and the inhibition of growth of cancer as a tissue. One of the most important principles to emerge is that loss of growth control of cancer cells is mechanistically associated with an increased tendency to undergo programmed cell death, or apoptosis. Thus, cancer growth is a balance between cell birth and cell death. The balance is maintained not only by the genetic status of the cancer cell but by interactions with host cells and extracellular matrix components in the tumor environment. The identification of estrogen as a factor for stimulating the growth led to antiestrogens as therapeutic agents and, more recently, to antagonists of growth factor receptor-mediated pathways. The early use of bacterial toxins in cancer treatment has led to strategies based on host–tumor interactions, such as antiangiogenic and immune approaches. Current research has underlined the enormous complexity not only of growth and death control systems within the tumor cell but of interactions of tumor cells with vascular endothelial, immune, and other cells in cancer tissue. The challenge of future development of low-molecular-weight anticancer drugs is to apply knowledge gained in basic studies to develop new strategies.

1. Introduction

It is difficult to assign a date to the beginning of the treatment of cancer with drugs because herbal and other preparations have been used for cancer treatment since antiquity. However, the 1890s, a decade that represents an extraordinarily creative period in painting, music, literature, and technology, encompassed discoveries that were to set the scene for developments in cancer treatment in the 20th century. The discovery of penetrating radiation, or x-rays, by Roentgen in Germany in 1895 was complemented 3 years later by the discovery of radium by Marie and Pierre Curie. The discovery of ionizing radiation led not only to radiotherapy as form of cancer treatment but eventually to the development of anticancer drugs that mimicked the effect of radiation by

damaging DNA. The discovery by George Beatson, working in Scotland in 1896, that the growth of a breast cancer could be halted by removal of the ovaries indicated that the growth of cancer cells in the body could be influenced by external factors. This provided the basis for cancer treatment strategies that changed the regulation of cancer cell growth. The demonstration by William Coley in 1898 that the administration to cancer patients of a bacterial extract, sterilized by passage through a porcelain filter, caused regressions in lymphoma and sarcoma indicated that activation of the body's defense systems might provide a strategy for cancer treatment. Each of these three advances lent weight to the bold assumption, made by Paul Ehrlich and others in the early part of the 20th century, that low-molecular-weight drugs might be used in the management of cancer as well as infectious diseases. This chapter considers each of these three approaches in turn.

2. Genotoxic (Cytotoxic) Therapy

The first practical anticancer drugs were discovered accidentally. One such discovery was an outcome of war, stemming from the finding that sulfur mustard gas, used as a toxic vesicant in the First World War, caused myelosuppression. Although gas warfare was not employed in the Second World War, a considerable stock of mustard gas canisters was maintained in the Mediterranean area. An accident in the Italian port of Bari, involving leakage of one of these canisters, rekindled interest in the myelosuppressive effect of nitrogen mustard, leading to clinical trials in lymphoma patients (Karnofsky *et al.,* 1948; Kohn, 1996).

The identification of vitamins as small low-molecular-weight enzyme cofactors was an important biochemical achievement in the early part of the 20th century. The structural elucidation and crystallization of folic acid in 1946 led, as with other isolated vitamins, to studies on its effect on the course of a number of diseases. Unexpectedly, administration to leukemia patients of folic acid and its glutamylated derivatives resulted in an increase in tumor growth. While the use of low-folate diets in the management of leukemia was investigated, the development of the folic acid analogue aminopterin provided a significant advance in the management of childhood acute leukemia (Farber *et al.,* 1948; Bertino, 1979).

The link between these two disparate types of drugs and their biological activity was found to be related to their damaging effect on DNA. Although Friedrich Miescher had characterized DNA as a substance in 1862, the informational complexity and significance to life of DNA was not appreciated until the 1940s. The elucidation in 1953 by James Watson and Francis Crick of the double-helical structure of DNA had a singular impact on strategies of anticancer drug development. The cancer chemotherapeutic agent nitrogen mustard was found to react chemically with DNA (Kohn *et al.,* 1966). Studies on aminopterin indicated that it interrupted DNA biosynthesis and in so doing caused DNA damage. The next two decades brought a massive development of new drugs that affected the integrity of the cell's genetic material, with approximately one new drug entering widespread clinical use every 2 years. Many of these drugs, which revolutionized the treatment of many types of cancer, are shown in Figure 1.

A. Development of *in Vivo* Cancer Screening Systems

Developments in chromatography and analytical chemistry in the first half of the 20th century allowed compounds of defined structure to be isolated from a variety of plants, animals, and microorganisms (see Chapter 12). The evolution of synthetic organic chemistry over this time provided anticancer drugs in addition to antimicrobial and other medicinal drugs (see Chapter 11). It was quickly realized that it would be impossible to test such a large number of compounds in cancer patients and that some type of model tumor system was required. Transplantable animal cancers became accepted as the best basis for the screening of such drugs. This was made possible by the availability of inbred mouse strains, which had their beginnings in the early part of the 20th century. Three inbred strains of particular importance to anticancer drug screening—DBA, BALB/c, and C57BL—were introduced in 1909, 1916, and 1921, respectively. Spontaneous or carcinogen-induced tumors in these strains could be transplanted from one inbred mouse to another, allowing repeated testing of potential anticancer drugs (Stock, 1954). A detailed description of the role of animal testing in drug development is provided in Chapter 16. In the 1950s and 1960s, most testing programs used the transplantable L1210 and P388 murine leukemia models for primary screening and transplantable solid tumors for more advanced testing (Goldin *et al.,* 1981). The discovery of the athymic "nude" mouse, which had lost its ability to mount a cell-mediated immune response, allowed the testing of new drugs against human tumor material growing as xenografts in such mice (Rygaard and Povlsen, 1969).

B. Mitotic Poisons

Many of the early drugs that were screened for anticancer activity were derived from natural products. The plant product colchicine, isolated from the autumn crocus, was one of the first of these to demonstrate activity against experimental murine tumor models. It was found to induce arrest of cultured cells in mitosis and demonstrated a new mode of induction of genomic damage: that of disturbing the correct distribution of genetic material into daughter cells at mitosis. Colchicine, although useful at lower doses for the treatment

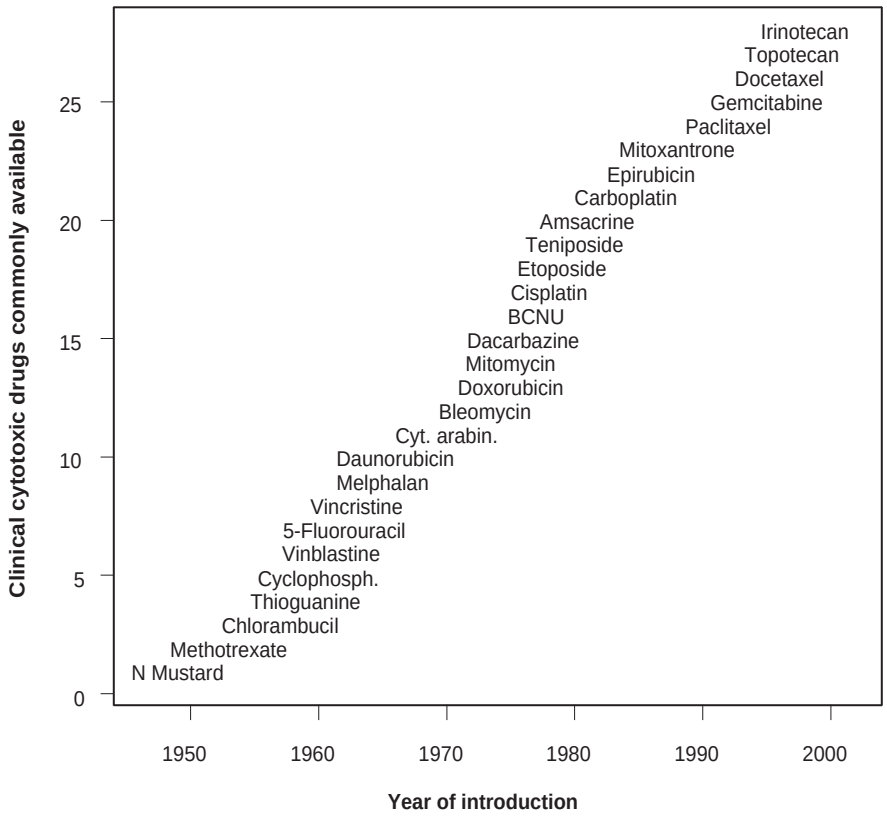

FIGURE 1 Chronology for the development of some of the anticancer drugs currently in use today. The abbreviations are N mustard (nitrogen mustard), cyt. arabin. (cytosine arabinoside), and BCNU (bischloroethylnitrosourea).

of gout, proved too toxic for use as an anticancer drug, and early attention focused on the *Vinca* alkaloids from the periwinkle plant (Johnson *et al.,* 1963). Two such alkaloids, vincristine and vinblastine, had a major impact on the early treatment of patients with malignancy (Rowinsky and Donehower, 1991). The protein tubulin was identified as the target for colchicine and the *Vinca* alkaloids (reviewed by Uppuluri *et al.,* 1993) and is the subject of considerable anticancer drug research. The broadening of the spectrum of tumor types susceptible to spindle poisons resulted from the discovery of the taxane class of compounds. Paclitaxel, discovered as a component of some *Taxus* species (Wani *et al.,* 1971), escaped detailed investigation until it was found to have a novel biochemical action distinct from that of the *Vinca* alkaloids, involving promotion rather than inhibition of microtubule assembly (Schiff *et al.,* 1979). Paclitaxel (Rowinsky *et al.,* 1990) and docetaxel (Bissery *et al.,* 1991) have a prominent place in cancer therapy today.

C. DNA-Reactive Drugs

Nitrogen mustard was the basis for the synthesis of a large series of clinically useful derivatives, including melphalan and cyclophosphamide, all found to exert their antitumor effects by alkylation of DNA. Natural products also yielded a number of clinically useful compounds that reacted chemically with DNA, such as mitomycin C (Whittington and Close, 1970), and bleomycin, which required the presence of oxygen and ferric ions to react (Crooke and Bradner, 1976). A particularly important development in DNA-reactive drugs came with the discovery of cisplatin, which had its origins in the chance observation that bacterial growth was inhibited around one of the platinum electrodes of an electrophoresis apparatus containing ammonium chloride in the buffer (Rosenberg *et al.,* 1965). Platination of DNA became a new mode of DNA damage induction and formed the basis for developing new analogues of cisplatin with reduced host toxicity.

D. Inhibitors of DNA Replication

A consequence of the elucidation of the structure of DNA was the rational design of analogues of the DNA bases, which were hypothesized to exert their anticancer activity by disruption of DNA replication. These included the thymine analogue 5-fluorouracil (Heidelberger *et al.,* 1957) and the purine analogues 6-mercaptopurine and 8-azaguanine (Hitchings and Elion, 1954). The cytotoxic effects of aminopterin and methotrexate were traced to their inhibition, through their

effect on the enzyme dihydrofolate reductase, of the conversion of deoxyuridine monophosphate to thymidine monophosphate. The phenomenon of "thymineless death," whereby bacteria unable to synthesize the DNA base thymine died in its absence, was found to have a parallel in mammalian cells and shaped the rationale for the development of the antimetabolite class of drugs. As the individual enzymes responsible for DNA replication were identified it became clear that the successful operation of the DNA replicase complex relied on a constant supply of the triphosphate precursors of DNA and that interruption of this supply resulted in damage to newly synthesized DNA. Natural products also played a role in the development of anticancer drugs acting on DNA replication. Arabinose nucleosides from the sponge *Cryptothethya* were found to have experimental antitumor activity, and one of them, the antimetabolite cytosine arabinoside, has found extensive use in the treatment of persons with leukemia (Ellison *et al.,* 1968). The testing of chemical analogues of cytosine arabinoside led more recently to drugs such as gemcitabine, which has activity against carcinoma (Plunkett *et al.,* 1996).

E. DNA Topology as a Target for Drug Development

In the 1960s, two logical problems concerning DNA structure became evident. The first was that the unwinding of DNA associated with DNA replication appeared to be thermodynamically impossible in the time frame involved, since the average chromosome would have tens of millions of helical twists. The second, familiar to anyone who has tried to untangle a fishing line, was that the separation of daughter DNA strands produced by DNA replication, prior to cell division, was thermodynamically impossible. Closed circular duplex DNA in some bacterial and mammalian viruses provided smaller molecular weight models for the study of these problems. Such DNA was found to exist in several distinct forms, each with the same sequence but with a different number of helical twists, and these were called topoisomers, based on topology (the branch of mathematics dealing with such differences in shape). In 1976, a new ATP-requiring enzyme, DNA gyrase, with the property of being able to change the topology of closed circular duplex DNA, was discovered in bacterial cells (Gellert *et al.,* 1976). DNA gyrase was found to have an essential role in DNA replication, and because it could pass one strand of DNA through another, it elegantly solved the problems of how DNA could be rapidly unwound during replication and how the daughter DNA strands could be separated after replication. Subsequent studies of mammalian cells demonstrated two main classes of enzymes. The first, topoisomerase I, changed DNA topology by breaking and rejoining a single DNA strand (Been and Champoux, 1980). The second, topoisomerase II, with some of the characteristics of bacterial gyrase, broke both strands of one double-stranded DNA to allow passage of a second double-helical strand through the breakage point (Miller *et al.,* 1981).

The discovery of the DNA topoisomerases also solved a problem concerning the activity of a number of natural products with anticancer activity that caused DNA damage but did not appear to react chemically. Actinomycin D, identified from *Streptomyces* cultures, found extensive early clinical use particularly in pediatric tumors (Farber *et al.,* 1960) and was found to bind DNA by intercalating its polycyclic chromophore between the base pairs of the DNA double helix (Müller and Crothers, 1968). It was of great biochemical interest because of its potent inhibition of RNA synthesis, but this did not appear to explain its antitumor activity. Subsequently, two anthracycline derivatives, daunorubicin and doxorubicin, were also found to bind DNA by intercalation of their chromophores, but their effects on RNA synthesis were less than those of actinomycin D. The clinical activity of daunorubicin was generally confined to hematologic malignancies but that of doxorubicin was broader (Arcamone, 1985). The synthetic compound amsacrine, which had clinical activity against acute leukemia (Arlin, 1989), bound DNA by intercalation of its acridine chromophore (Wilson *et al.,* 1981) but had little or no effect on RNA synthesis. Both amsacrine and doxorubicin were found to induce covalent links between DNA and proteins (Zwelling *et al.,* 1981), and subsequent work demonstrated that this protein was in fact the enzyme topoisomerase II (Nelson *et al.,* 1984). The drugs acted as poisons of this enzyme, subverting its normal function to one of inducing DNA damage.

A parallel development in plant natural product research provided podophyllotoxin analogues derived from the mandrake root (Stahelin and Von Wartburg, 1991). Podophyllotoxin itself, like colchicine, bound to tubulin, but some semisynthetic glycosidic derivatives, termed epipodophyllotoxins, were found to have superior experimental antitumor activity to podophyllotoxin itself. Etoposide, first tested clinically in 1971, was found to be useful against a variety of malignancies (Issell and Crooke, 1979). Investigation of the action of etoposide and of the related drug teniposide revealed that they had reduced binding to tubulin but induced DNA damage and poisoned the enzyme topoisomerase II. The plant product camptothecin, which did not bind DNA and previously had no known function, was found to be a specific poison for topoisomerase I (Hsiang *et al.,* 1985). Water-soluble analogues of camptothecin, such as topotecan and irinotecan, have clinical potential, and topoisomerase I is now an established tumor target (Pommier, 1993).

F. The Search for Selectivity

While the selectivity of radiotherapy was progressively increased by localization of the radiation field to specific areas

of tumor growth, the selectivity of cytotoxic therapy was dependent on particular properties of cancer tissue. The use of microbial models gave rise to the important concept that alkylating drugs killed cells in an exponential fashion, with a certain percentage of the cell population killed with each dose (Pittillo *et al.,* 1965). For some drugs, cytotoxicity was found to be maximal at a particular phase of the cell cycle. Skipper and colleagues (Skipper, 1967) used animal models to select administration schedules with optimal cytotoxicity for the cell-cycle-selective agents, and drug combination schedules that allowed optimal intensity of treatment. The spacing between treatments and the rate of appearance of resistant populations could make the difference between success and failure of treatment (Carl, 1989). Such reasoning was applied with success to hematologic malignancies, which often had a high rate of cell division, but was less successful in the management of solid tumors.

Another basis for selectivity was to exploit an enzyme or drug transport mechanism that was present to a different extent in tumor and normal cells. Many antimetabolites were found to exert their selectivity by such mechanisms. Topoisomerase enzymes provided a particularly good example of such selectivity since high cellular activity, which tended to occur in rapidly dividing cell populations, was associated with greater sensitivity to topoisomerase-directed anticancer drugs (Pommier, 1993). More recently, selectivity has been generated by the development of prodrugs, which have no cytotoxicity until an enzyme or other agent activates them. Initially, naturally occurring cellular enzymes, such as nitroreductases, were considered as candidates for activating prodrugs, but more recently the concept of introducing a different activating enzyme by means of a localizing antibody or gene therapy has been investigated. These concepts are discussed in Chapters 8 and 11.

3. Growth Control Pathways

While cytotoxic agents dominated the development of clinical cancer chemotherapy, the alternative approach of altering the signals that determine cancer growth was not forgotten. The demonstration by Beatson in 1896 of the role of the ovary in the progression of some types of breast cancer raised the question of whether the growth of all cancer types might be controlled by circulating hormones. Subsequent surgical studies showed that, apart from the case of prostate cancer, removal of endocrine glands was generally ineffective in cancer treatment. It was another 30 years before estrogen, one of the main hormones accounting for Beatson's result, was identified (Frank *et al.,* 1925), but the biochemical pathways linking steroid hormones to cell growth stimulation remained a mystery. During the 1960s and 1970s, studies of cultured cells, both normal and tumor, indicated that a diverse series of polypeptide growth factors were essential for cell growth, many specific for certain tissue types (reviewed by James and Bradshaw, 1984). An understanding of the action of such factors first required the elucidation of the molecular mechanism of regulation DNA replication and cell division.

A. The Cell Cycle Clock

One of the most fascinating questions posed by dividing normal and cancer cells was the nature of the molecular clock that instructed the cell as to when it would replicate its DNA and when it would divide. Early studies of cancer tissue identified mitotic cells by their morphology and DNA-synthesizing (S-phase) cells by their uptake of tritium-labeled thymidine, and these phases were found to be separated by periods of cell enlargement, termed G_1 and G_2 phase (reviewed by Tannock, 1978). The first clues to the nature of the oscillator that ran the molecular clock were provided by the discovery in developing sea urchin eggs of a protein termed *cyclin*. The cellular concentration of this protein increased up to the time of cell division and then abruptly decreased (Evans *et al.,* 1983). Studies of the division of fertilized frog eggs also indicated the presence of a cyclin, the synthesis of which was necessary to cell division (Cross *et al.,* 1989). A second component of the clock was identified from two lines of research, one using frog embryos and one using yeast mutants (Cross *et al.,* 1989). A specific enzyme, termed a cyclin-dependent kinase (cdk), was found both to associate physically with a cyclin and to be activated by it, providing a link between the oscillator and the actuator. Several distinct cdk's and cyclins were found to be present in mammalian cells. A third component of the clock mechanism was a protease (in the form of a proteasome) that was responsible for the degradation of the cyclin and thus the resetting of the clock (Glotzer *et al.,* 1991; King *et al.,* 1996). In the general scheme of the cell cycle (Fig. 2), the multiple functions required for cell division and DNA replication are exquisitely coordinated by cdk's 1 and 2, respectively, each activated at the appropriate time by specific associated cyclins. The cell cycle time for human tumors varies from around 2 days to several weeks (Wilson *et al.,* 1988).

B. Stopping and Starting the Cell Cycle Clock

While the majority of the body's cells are in a nondividing state, certain cells, such as blood cell precursors in the bone marrow and epithelial cells in the gut, are capable of dividing rapidly. Some mechanism must therefore regulate the passage of cells from a quiescent state to a dividing state. Most early studies utilized cultured fibroblasts to investigate this process. Time-lapse studies (Smith and Martin, 1973) indicated that the commitment to DNA replication and mitosis was determined by a stochastic (random) mechanism, and a

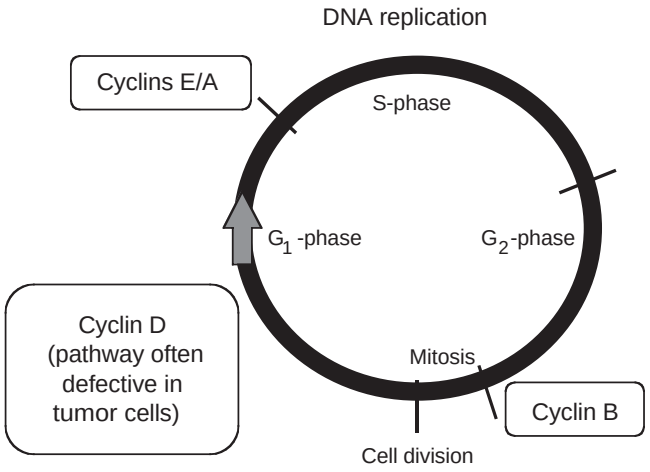

FIGURE 2 The cell cycle clock, with two key alternating processes: DNA replication and cell division. The timing of these processes is controlled primarily by oscillations (one per cell cycle) in the cellular levels of cyclin E/A and cyclin B, respectively. Activation of these processes is controlled by cyclin-dependent kinases 2 and 1, respectively. Normal cells have a further control on the decision to enter the cell cycle, timed by cyclin D and activated by cyclin-dependent kinases 4/6. This control system is deficient in cancer cells.

"restriction point" in the G_1 phase of the cell cycle was defined (Pardee, 1974), past which cells were irreversibly committed. A clear requirement was established for the presence of external polypeptide growth factors to allow passage of cells past the restriction point. Such factors were found to interact with membrane-bound surface receptors on target cells, and by the end of the 1970s it was established that at least some of these receptors, including that for epidermal growth factor, became phosphorylated as a consequence of growth factor engagement (Carpenter *et al.,* 1979). Internal cellular proteins were also phosphorylated in response to growth factors, but the identification of the complex linkages between growth factor receptors and commitment to DNA replication and cell division required new findings.

A major step forward in the identification of the pathway for initiation of cell growth had its origins in Peyton Rous's study of cancer-causing viruses in birds, mice, and rats in the early part of the 20th century (Rous, 1983). In 1983, the extraordinary finding was made that a gene in a tumor-transforming virus of simian apes was similar or identical to that specifying a known growth factor for cultured cells (Doolittle *et al.,* 1983; Waterfield *et al.,* 1983). Subsequent work defined a variety of genes that could be transmitted by retroviruses to the tissue of a variety of birds and animals, causing a tumorigenic change. As the functions of these corresponding gene products were elucidated, it was found that they mapped to biochemical pathways linking the binding of growth factors to the commitment of cells to DNA replication and cell division.

The above work led to identification not only of a network of regulatory proteins but also of new cyclins (D-cyclins) and cdk's (4 and 6) that control the passage of the cell from a quiescent phase into the cell cycle. The retinoblastoma protein and the transcription factors E2F and c-myc were also implicated in a complex control system that resulted in the up-regulation of the E and A cyclins and subsequent initiation of DNA replication.

As the elements of this control system were identified, it also became clear that the function of one or more of these elements was defective in cancer. For instance, many human cancers were found to be associated with a mutated *ras* oncogene such that the cells behaved as though they were being continuously stimulated by growth factors (Pronk and Bos, 1994). Furthermore, many cancer cells lacked the proper function of proteins, such as the retinoblastoma protein, that regulated entry into S phase (Herwig and Strauss, 1997).

C. Programmed Cell Death

In 1972, a pivotal hypothesis was advanced that cell death was, like progress through the cell cycle, a product of precise cellular programming (Kerr *et al.,* 1972). This hypothesis was to have a profound effect not only in explaining the loss of cells during the development of the embryo but in advancing our understanding of cancer growth. Apoptosis was found to be an energy-dependent process whereby a cell was converted to fragments that could be absorbed by surrounding tissue without the initiation of an inflammatory response (Wyllie, 1993). The molecular mechanisms of apoptosis are described in Chapter 4.

In a multicellular organism, loss of a single cell is generally unimportant. On the other hand, loss of growth control in a single cell could lead, in the absence of any protective mechanism, to unrestricted and catastrophic growth. The body appears to have a protective mechanism to ensure that any such cells losing growth control are eliminated. The mechanistic links are not yet fully defined, but transcription factors such as E2F and c-myc, which are involved in driving cells into the cell cycle, are also involved in driving cells into apoptosis (Evan and Littlewood, 1998; King and Cidlowski, 1998). Thus, cancer growth is a balance between cell birth and cell death, each initiated by the same pathway (Fig. 3). Cancer cells (as well as normal cells) can be prevented from undergoing apoptosis by so-called survival factors (Evan and Littlewood, 1998), such as insulin-like growth factor 1 (Juin *et al.,* 1999) and cell–matrix interactions (Meredith *et al.,* 1993).

D. The Cell Cycle Calendar

The pioneering studies of Hayflick showed that when human fibroblasts were cultured, they would die after a certain

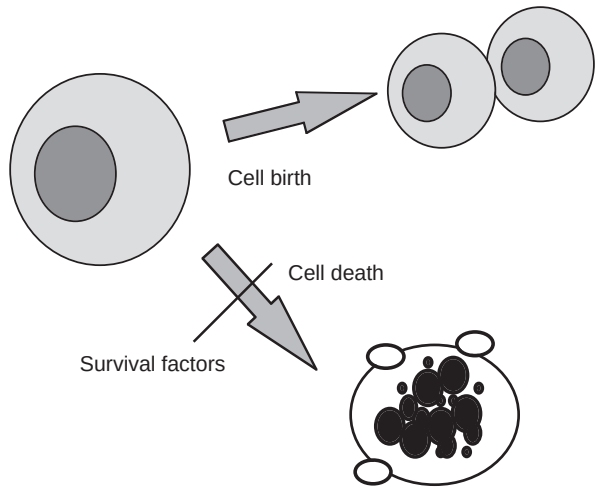

FIGURE 3 The balance of cell birth and death in a tumor cell population. In human tumors there is a high rate of turnover such that the overall doubling time of tumor tissue is generally considerably longer than the cell cycle time of the tumor cells. The rate of cell death is controlled to some extent by external factors called survival factors.

number of generations by a process called senescence (Hayflick and Moorhead, 1961; Hayflick, 1965). The number of generations of doublings, typically 60–80 for fetal cells, progressively reduced as the age of the donor of the fibroblasts increased, suggesting that some kind of calendar was running during the lifetime of an individual cell. More recently, evidence for a slightly different calendar mechanism has been obtained in breast epithelial cells (Romanov *et al.,* 2001).

Tumor cells in culture, in contrast to normal cells, appeared to grow indefinitely, suggesting that the lack of a functioning calendar may be a common characteristic (Goldstein, 1990). An understanding of the mechanism behind the calendar was provided by research on the question of how a linear chromosome could be replicated. Characterization of the DNA replication machinery indicated that DNA polymerase had to start from a priming strand, either DNA or RNA, bound to a DNA strand in a duplex conformation (Lingner *et al.,* 1995). Thus, DNA polymerase could not replicate the ends of chromosomes, and with each successive round of DNA replication the chromosomes would become progressively shorter. The solution to this "end-replication" problem was provided by the discovery of the enzyme telomerase, which added repetitive sequences to the ends or telomeres of chromosomes (Morin, 1989). Normal tissues, unlike embryonic tissues, generally lack telomerase activity. Thus, loss of telomeric DNA sequences might provide a mechanism to ensure that cells that mutate to allow inappropriate cell division, even if not killed directly by apoptosis, die as a result of telomeric DNA loss. It appears that the acquisition of an active telomerase is a very common feature of cancer tissue (Kim *et al.,* 1994).

E. Therapeutic Possibilities

One of the earliest therapeutic successes in cell growth control was the discovery of triphenylethylene derivatives that antagonized the effects of estrogens (Ward, 1973). These so-called antiestrogens revolutionized the management of some types of breast cancer. The basis for the antitumor action of antiestrogens is complex and multifactorial. Estrogen was found to bind to an internal protein that acted as a transcription factor (McKnight *et al.,* 1975), and further work indicated that the products of transcription had multiple effects on target cells, including changes in the expression of growth factor receptors (Curtis *et al.,* 1996). Thus, one possible mechanism of action of the antiestrogens is to decrease the action of survival factors and thus increase the rate of cell death.

More recently, research suggesting that cancer growth represents a balance between cell birth and cell death suggested new approaches. Thus, either a drug-induced decrease in cell birth rate or an increase in cell death rate might result in tumor regression. Low-molecular-weight drugs have been developed that either reduce the progress of cells through the cell cycle, thereby decreasing the cell birth rate, or increase the rate of apoptosis. One important consequence of this research was the finding that cytotoxic drugs, discussed in earlier sections of this chapter, could increase the rate of apoptosis. Cisplatin was one of the first cytotoxic drugs demonstrated to induce cultured cells to undergo apoptosis (Barry *et al.,* 1990). Damaged DNA appears to activate specific enzymes, such as Ataxia Telangiectasia Mutated (ATM) kinase, which in turn initiate the series of steps leading to apoptosis (Meyn, 1995). This topic is further discussed in Chapters 2–4.

The reinstatement of cancer cell mortality constitutes another possible therapeutic approach to cancer treatment. If the "calendar" could be reactivated, such as by inactivation of telomerase activity, cancer cells might once again have a finite lifetime. The design of therapies aimed at inhibiting telomerase activity is still at an early stage and has not been reviewed specifically in this volume. However, a number of excellent reviews can be found (Neidle and Kelland, 1999; Perry and Jenkins Thomas, 1999).

4. Host–Tumor Interactions

Cancer cells do not exist as isolated units but as components of a tissue containing both host and tumor cells. Host cells not only provide, through the vascular endothelium, the precursors of cancer cell growth but also secrete factors that have profound effects on the life and death of tumor cells. The early finding that a mixture of bacterial exotoxins and endotoxins present in "Coley's toxins" were effective in some types of cancer, particularly lymphomas and sarcomas (reviewed by Wieman and Starnes, 1994), prepared the way for

both vascular and immune approaches to the treatment of patients with cancer (Pardoll, 1993). Early studies of the action of bacterial lipopolysaccharides in mice led to the discovery of their induced effects on tumor capillaries, leading to vascular collapse and tumor necrosis (Algire *et al.,* 1947). Based on these results, a variety of clinical studies using nonspecific immune stimulants such as *Corynebacterium parvum* and bacillus Calmette-Guérin were instituted (reviewed by Mathé *et al.,* 1973). In the 1960s, polypeptides were identified that stimulated the proliferation of various types of blood cells (Metcalf, 1971). This was followed by the identification of cytokines that mediated the communication between cells in the immune system and other types of cells. One of these cytokines, a protein termed tumor necrosis factor (TNF), was found to mediate the effects of bacterial toxins (Carswell *et al.,* 1975), acting on capillaries to increase their permeability and induce their collapse (Watanabe *et al.,* 1988). Thus, a new approach to drug development became identified in which host cells rather than tumor cells were targeted, leading ultimately to increased tumor cell death. Some of the pathways involved in the complex interactions are shown in Figure 4.

A. Low-Molecular-Weight Inducers of Tumor Necrosis

One of the first reports on the antitumor activity of colchicine described the induction of necrosis in transplantable solid tumors. The similarity of the histologic changes to those caused by administration of bacterial toxins suggested an effect on the tumor vasculature (Ludford, 1945). Subsequently, the synthetic drug flavone acetic acid, the antibiotic fostriecin, and the plant product homoharringtonine were also found to induce hemorrhagic necrosis of experimental tumors (Baguley *et al.,* 1989). Apart from colchicine, a variety of tubulin binders, including podophyllotoxin, vincristine, vinblastine, and combretastatin, were found to induce tumor necrosis (Baguley *et al.,* 1991; Hill *et al.,* 1993; Dark *et al.,* 1997). Flavone acetic acid and the structurally related 5,6-dimethylxanthenone-4-acetic acid appeared to act as a consequence of local production of TNF (Mace *et al.,* 1990; Zwi *et al.,* 1994), while the mitotic inhibitors appeared to change the shape of vascular endothelial cells (Dark *et al.,* 1997).

One particularly important class of tumor–host interactions concerns the vascular endothelial cells that provide the blood supply to tumors. Measurement of the proliferation rates of vascular endothelial cells in tumors showed them to divide more rapidly than in normal tissues (Hobson and Denekamp, 1984), leading to antivascular strategies for tumor management (Denekamp, 1990). At the same time, the factors responsible for tumor angiogenesis were being elucidated, and a basis for antiangiogenic drugs for cancer treatment was formulated (Folkman, 1985). This is discussed in Chapter 7.

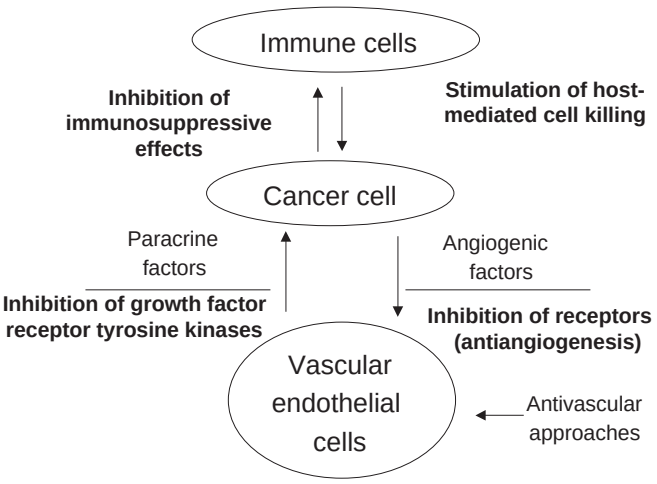

FIGURE 4 Relationships between tumor and host and their possible modulation in therapy. Factors secreted by vascular endothelial cells, together with other stromal components, contribute to the survival of tumor cells. Immune cells may contribute to increased rates of tumor cell death. The tumor cells secrete factors that support their own growth, the proliferation and remodeling of the vascular endothelium, and the inhibition of immune responses. All of these signals constitute potential targets for the development of new anticancer strategies.

B. Low-Molecular-Weight Modulators of Cytokine Effects

Research on cytokines led not only to clinical trials of individual cytokines but to the development of low-molecular-weight compounds that might modulate cytokine production. The anthelminthic drug levamisole was initially investigated as an immunostimulant (Amery *et al.,* 1977), has found clinical use in the treatment of colon and other tumors, and is currently thought to act by changing the balance of cytokine actions (Szeto *et al.,* 2000). Tilorone, a low-molecular-weight inducer of interferon (Zschiesche *et al.,* 1978), has been tested in phase II anticancer trials (Cummings *et al.,* 1981). Muramyl dipeptide was investigated as a low-molecular-weight inducer of TNF and appeared to act in concert with endotoxin to induce antitumor effects (Bloksma *et al.,* 1985). Thalidomide, infamous for its teratogenic effects in humans, was found to inhibit the synthesis of TNF (Sampaio *et al.,* 1991) and, more recently, has shown promising activity in the management of multiple myeloma (Singhal *et al.,* 1999). A complete understanding of how cytokine-modulating drugs act on tumor tissue requires further research.

5. Conclusions

In many ways, the scientific objectives of research into the drug-mediated treatment of cancer have not changed over the

last few decades. We still search for selective poisons for cancer cells, we still wish to change, selectively, the regulation of growth of cancer cells, and we still wish to encourage the body's normal tissues to reject cancer tissue. While the incidence of radically new ideas in cancer treatment is relatively sparse, our understanding of the molecular makeup of the cancer cell has changed dramatically and the available technology is extraordinarily different. For instance, recent studies using microarray technology combined with the sequencing of the human genome provide the potential to investigate the activity of huge numbers of genes in response to anticancer agents (Scherf *et al.,* 2000). The challenge is to devise, from a deep understanding of the action of known anticancer drugs on human cancers, new strategies for cancer treatment.

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CHAPTER 2

NOVEL TARGETS IN THE CELL CYCLE AND CELL CYCLE CHECKPOINTS

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Summary

Our knowledge of the functional relationship between the molecules that constitute the cell cycle and checkpoint pathways is expanding rapidly, thus providing us with new opportunities to further our understanding of the mechanisms of action of anticancer agents. This knowledge will undoubtedly lead to the development of novel strategies for the management of cancers based on the specific alterations in any given tumor. This chapter will outline the current knowledge in cell cycle and checkpoint control, discuss the rationale for targeting these pathways, and review the pharmacology of the agents that are currently known to act on the cell cycle control and checkpoint pathways.

1. Introduction

In the past decade, important milestones have been identified in the molecular pathways that control cell cycle progression. Knowledge of the functional relationships between the molecules that constitute the cell cycle and checkpoint pathways is expanding rapidly, providing us with a framework for understanding the potential effects of therapeutic intervention. New avenues for tumor-specific therapeutic intervention are now at close reach. The molecular characterization of tumors should identify the specific defects that drive cell cycle progression in particular cancers and provide us with a rationale for the type of treatment that would be most appropriate for a particular patient. New therapeutic approaches can now be validated genetically (e.g., gene knockout, antisense strategy, gene transfer) prior to a search for (a screening for) small-molecule inhibitors and for validating the activity of such inhibitors against a biochemical target in a given pathway. We will outline the molecular pathways that drive cell cycle progression and regulate the cell cycle checkpoints. We will next address the rationale for developing therapeutics that interfere with cell cycle progression and cell cycle checkpoint. Finally, we will review the various therapeutic

approaches that are being developed for cancer treatment. This chapter will focus on the potential therapeutic aspects of the regulation of the G_1/S and G_2/M transition.

2. Molecular Regulation of Cell Cycle Progression

Cellular proliferation and division requires an orderly progression through the cell cycle, primarily driven by protein complexes composed of cyclins and cyclin-dependent kinases (Cdks) (Fig. 1). Initiation of the cell cycle takes place when cells pass the "restriction point," as defined by Arthur Pardee 30 years ago (Pardee, 1974), after which cells are committed to complete their cell cycle progression. Progression through the G_1-S transition requires the activity of at least two different types of kinases, cyclin D-Cdk4/6 and cyclin E/A-Cdk2 (Figs. 1 and 2). (For Fig. 2, see color insert.) At the G_1-S transition, Cdk4/6 and Cdk2 govern the entry into S phase. Cdk2 continues to be active through S phase, with its decline in activity signaling exit from S phase. Lastly, Cdk1 (Cdc2) becomes active in G_2 and its activity persists through mitosis (Figs. 1 and 3). (For Fig. 3, see color insert.)

The activity of the cyclin-Cdk kinases is regulated by two families of cyclin-dependent kinase inhibitors (CKI): Cip/Kip and INK4. The members of the Cip/Kip family—$p21^{Cip1}$, $p27^{Kip1}$, and $p57^{kip2}$—can associate with and inactivate cyclin E/A-Cdk2 and cyclin B-Cdk1 complexes (Fig. 1). Binding of $p27^{KIP}$ to cyclin E-Cdk2 prevents cells from entering into S phase, and binding of $p27^{KIP}$ to cyclin A-Cdk2 prevents passage through S phase. $p21^{Cip1}$ has a broader specificity. It can bind and inhibit both cyclin E/A-Cdk2 and cyclin B-Cdk1. In contrast, association of Cip/Kip family with cyclin D-Cdk4 or cyclin D-Cdk6 complexes appears to have a stimulatory effect (Blain *et al.,* 1997; Cheng *et al.,* 1999; LaBaer *et al.,* 1997; Soos *et al.,* 1996). Binding of Cip/Kip proteins to cyclin D-Cdk4/6 kinases can also prevent their interaction with cyclin E/A-Cdk2, thus facilitating the role of these kinases in completing the G_1 phase of the cell cycle and initiating DNA synthesis (Sherr and Roberts, 1999). The members of the INK4 family—$p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$—specially target the Cdk4 and Cdk6 kinases, inhibiting their catalytic activity by preventing their binding to their regulatory cyclin D subunits (Sherr and Roberts, 1999) (Fig. 2).

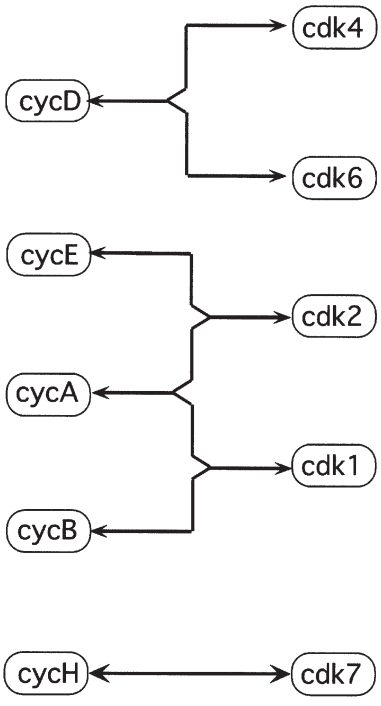

FIGURE 1 The binary association capabilities between the major cyclins and cdk's (cyclin-dependent kinases) involved in the mammalian G_1-S and G_2-M cell cycle transitions. (Modified from Kohn, 1999.)

The retinoblastoma tumor suppressor protein (pRb) is the primary substrate for cyclin-D-dependent kinases. pRb plays a critical role in regulating G_1 progression and is a key component of the molecular network controlling the restriction point. pRb can bind and suppress the transcriptional activity of various members of the E2F family (Chen *et al.,* 1996; Flemington *et al.,* 1993; Qin *et al.,* 1995). Cyclin-D-dependent Cdk's phosphorylate pRb in late G1, which disrupts the association of pRb, and E2F activation allows the coordinated expression of many genes that encode proteins necessary for S-phase entry and progression (Kato *et al.,* 1993) (Fig. 2). Among the E2F-regulated genes are cyclins E and A, whose association with Cdk2 is required for cells to make a transition from G_1 into S phase (Girard *et al.,* 1991; Pagano and Draetta, 1991).

The capacity of E2F to induce cyclin E, which in turn regulates Cdk2 to enforce pRb phosphorylation at additional sites, creates a positive-feedback loop for the accumulation of active E2F that helps contribute to the irreversibility of the G_1/S transition (Sherr, 1996; Taya, 1997; Weinberg, 1995). Cyclin-A- and B-dependent Cdk's activated later in the cell cycle maintain pRb in an hyperphosphorylated form until cells exit mitosis when pRb is returned to a hypophosphorylated state in the next G_1 phase (Ludlow *et al.,* 1993) (Fig. 2). Overexpression INK4 proteins arrest the cell cycle in G_1, which is consistent with the notion that INK4 proteins can antagonize the assembly of cyclin-D-dependent kinases by binding to Cdk4 or 6, thereby maintaining pRb in a growth-suppressive, hypophosphorylated state.

It is widely accepted that the onset of mitosis is driven by cyclin-B-dependent kinase Cdk1 (Cdc2) activation (Fig. 3). Cdk1 activation requires binding to its positive regulatory

subunit, cyclin B. The kinase activity of Cdk1 is also regulated by phosphorylation. Phosphorylation on threonine 161 activates Cdk1, whereas phosphorylation on tyrosine 15 and threonine 14 by the Cdk1 inhibitory kinases Myt1 and Mik1 inhibits its activity. Cdc25C activates Cdk1 by removing the inhibitory phosphorylations of Cdk1 (Fig. 3). The activity of Cdc25C itself can be enhanced by Cdk1/cyclin B1-mediated phosphorylation (Hoffmann *et al.,* 1993; Izumi and Maller, 1993). Therefore, activation of Cdk1/cyclin B has been proposed to result in an autocatalytic feedback loop to ensure rapid activation of these complexes at the G_2-M transition (Hoffmann *et al.,* 1993).

3. Molecular Regulation of the Cell Cycle Checkpoints

Cell cycle checkpoints are mechanisms that monitor cell regulatory pathways and DNA structure before the cells enter the next phase of the cell cycle. Their activation in response to DNA damage either leads to cell cycle arrest, so as to allow repair of DNA damage, or leads to cell death by apoptosis or terminal growth arrest. In recent years, many genes involved in the regulation of DNA damage responses have been identified and related to the DNA damage checkpoint pathways (Figs. 2 and 3).

A. PI3K-Related Kinases: Key DNA Damage Signal Transducers

The mammalian DNA damage response pathway consists of several families of conserved protein kinases. Two members of the phosphoinositol kinase (PIK) family, Ataxia Telangiectasia Mutated (ATM) and ATR, share a COOH-terminal kinase domain bearing significant sequence homology to the catalytic domain of mammalian and yeast PIK family and are central to this signal transduction cascade (Fig. 3). ATM can bind directly to free DNA ends (Smith *et al.,* 1999) and act as a DNA damage sensor that signals to cell cycle checkpoint control. The ATM gene is mutated in the familial neural degeneration and cancer predisposition syndrome ataxia telangiectasia (AT) (Savitsky *et al.,* 1995). Cells derived from AT patients are defective for DNA damage checkpoints at G_1, S, and G_2 and are very sensitive to agents that cause DNA double-strand breaks, such as γ irradiation ((Morgan and Kastan, 1997; Morgan *et al.,* 1997; Rotman and Shiloh, 1998). A second mammalian PIK, ATR, carries out checkpoint-related functions that partially overlap with those performed by ATM (Cliby *et al.,* 1998; Wright *et al.,* 1998). The central roles of ATM and ATR in DNA damage response pathways have been demonstrated by their ability to activate p53 and other checkpoint kinases as discussed below.

B. Role of p53 and p21 in G_1-S Checkpoint Control

Of the various mammalian checkpoints, the G_1 checkpoint is now relatively well understood. Arrest in the G_1 phase of the cell cycle in mammalian cells exposed to DNA damage is mediated by the p53 gene product (Kastan *et al.,* 1991; Levine, 1997). The dependence of G_1 checkpoint on p53 is demonstrated by the observation that cells with wild-type p53 display a dose-dependent G_1 arrest in response to γ irradiation. However, cells lacking p53 function enter S phase due to a defective G_1 arrest. The p53 gene is a tumor suppressor gene most frequently mutated in human cancers (Cox and Lane, 1995). Loss of G_1-S checkpoint function has become a hallmark of human cancers with p53 mutations. p53 is a short-lived protein present at very low levels in the nuclei of normal cells. A variety of cellular insults, including DNA damage, hypoxia, and aberrant oncogenic signaling, elevate the levels of p53 due to enhanced stabilization. Stabilization of p53 in cells exposed to ionizing radiation (IR) or ultraviolet (UV) light is due to dissociation from Mdm2 (Shieh *et al.,* 1997), a protein that targets p53 for degradation through the ubiquitin pathway (Haupt *et al.,* 1997; Kubbutat *et al.,* 1997; Lane and Hall, 1997). p53 is a sequence-specific transcription factor that induces expression of several cell-cycle- and cell-death-related genes, including p21, Bax, and MDM2. G_1-S arrest results, at least in part, from p53-mediated p21 transactivation (Harper *et al.,* 1993). p21, also known as Cip1/Waf1, binds G_1-specific cyclin-CDK complex and acts as a CDK inhibitor, thus preventing cell cycle transition from G_1 to S phase (Deng *et al.,* 1995; Waldman *et al.,* 1995).

The mechanism that governs the p53 activation in response to DNA damage has been elucidated. Cells lacking ATM show reduced and delayed p53 activation in response to DNA damage, implicating the role of ATM in the regulation of p53 (Morgan and Kastan, 1997). Recently, it has been shown that ATM and ATR can phosphorylate p53 on Ser15 *in vivo* (Khanna *et al.,* 1998; Tibbetts *et al.,* 1999). They also contribute to the phosphorylation of p53 on Ser20 through activation of the checkpoint kinases Chk1 and Chk2 (Chehab *et al.,* 2000; Shieh *et al.,* 2000) (see below). Phosphorylation on Ser20 has been reported to decrease p53 binding to MDM2 and thereby contribute to the p53 stabilization (Chehab *et al.,* 1999; Unger *et al.,* 1999). Thus, ATM/ATR, p53, and p21 are three important genes that regulate the G_1-S checkpoint.

C. Role of Cdc25C in G_2-M Checkpoint Control

Cdc25 is one of the best characterized regulatory components of the eukaryotic cell cycle machinery. There are three human Cdc25 homologues: Cdc25A, B, and C. The three phosphatases share approximately 50% homology at the

amino acid sequence level (Nilsson and Hoffmann, 2000). It appears that the phosphatases function differently in the cell cycle regulation. Cdc25A activity is required for S-phase entry, while Cdc25B and C activation is necessary for the regulation of entry into mitosis (Lammer *et al.,* 1998; Nilsson and Hoffmann, 2000). Cdc25C is best characterized for its function in G_2-M regulation by activation of Cdk1 (Fig. 3). In both human and yeast, inhibitory phosphorylation of Cdk1 is required for cell arrest in G_2 after DNA damage or DNA replication block (Blasina *et al.,* 1997; Rhind *et al.,* 1997). Although DNA-damage-induced activation of Wee1 kinase may be responsible for this inhibitory phosphorylation of Cdk1 (Den Haese *et al.,* 1995; O'Connell *et al.,* 1997; Rhind *et al.,* 1997), much evidence in both yeast and human cells points to the negative regulation of Cdc25C as a major event in G_2 checkpoint control.

It has been shown that Cdc25C is phosphorylated on Ser216 in asynchronously growing cells and throughout the G_1 and S phases of the cell cycle (Peng *et al.,* 1997). During mitosis, Ser216 becomes dephosphorylated and Cdc25C is hyperphosphorylated on NH_2-terminal serine and threonine residues (Peng *et al.,* 1997). It appears that the mitotic hyperphosphorylation of Cdc25C increases its intrinsic phosphatase activity (Lew and Kornbluth, 1996). In contrast, phosphorylation of Cdc25C on Ser216 throughout interphase negatively regulates Cdc25C. The negative effect of Ser216 phosphorylation is mediated by 14–3–3 binding, which sequesters Cdc25C in the cytoplasm and results in a decrease of Cdc25C access to Cdk1 (Peng *et al.,* 1997; Yang *et al.,* 1999). Thus, it has been proposed that DNA damage results in inactivation of Cdc25C by increased Ser216 phosphorylation. Two checkpoint kinases, Chk1 and Cds1, have been described to phosphorylate Cdc25C on Ser216 *in vitro* in response to genotoxic stress (Blasina *et al.,* 1999a; Brown *et al.,* 1999; Chen *et al.,* 1999a; Matsuoka *et al.,* 1998; Sanchez *et al.,* 1997), suggesting that Chk1 and Cds1 may regulate the DNA-damage-induced G_2 checkpoint by inactivation of Cdc25C to prevent mitotic entry (Fig. 3).

D. Checkpoint Effector Kinases Chk1 and Chk2

Studies in different species suggest that the two checkpoint kinases perform distinct roles in various checkpoint responses. In fission yeast, Cds1 is phosphorylated and activated when replication is inhibited by the DNA replication inhibitor hydroxyurea (HU) or when DNA is damaged in S phase (Brondello *et al.,* 1999; Lindsay *et al.,* 1998; Murakami and Nurse, 1999). However, Chk1 is normally only responsive to DNA damage but not to HU treatment (Walworth and Bernards, 1996). HU-induced Chk1 phosphorylation can only be detected when Cds1 is activated (58-59), indicating a cross-talk between the two checkpoint kinases.

However, human Chk1 and Cds1 (also called Chk2), appear to respond differently from their counterparts in fission yeast. In human cells, Chk2 is phosphorylated and activated upon exposure to a variety of signals, including ionizing and ultraviolet (UV) radiation, and hydroxyurea. Among them, the response to ionizing radiation is strongest (Brown *et al.,* 1999; Matsuoka *et al.,* 1998). By contrast, Chk1 phosphorylation appears to be most pronounced in cells treated with DNA-replication-interfering agents such as HU or UV, and, to a lesser extent, with ionizing radiation (Liu *et al.,* 2000). In addition, Chk1 activity is increased following UV irradiation (Mailand *et al.,* 2000) but displays no change following ionizing irradiation (Falck *et al.,* 2001; Kaneko *et al.,* 1999; Lukas *et al.,* 2001). It has been shown that Chk2 is regulated by ATM (Brown *et al.,* 1999; Matsuoka *et al.,* 1998), whereas Chk1 is regulated by ATR (Liu *et al.,* 2000; Zhao and Piwnica-Worms, 2001). These findings favor a model in which ATM-Chk2 and ATR-Chk1 represent two parallel pathways that respond to different types of DNA damage signals in mammalian cells. The ATM-Chk2 pathway primarily responds to DNA damage caused by ionizing radiation/double-strand DNA breaks, whereas the ATR-Chk1 pathway responds primarily to DNA-replication-blocking agents such as UV radiation and HU. In support of this model, a recent study showed that Chk1 is involved in the replication checkpoint through proteasome-dependent degradation of Cdc25A, whose activity is required for S-phase entry (Mailand *et al.,* 2000). However, both Chk1 and Chk2 pathways could overlap and cooperate with each other to ensure sufficient enforcement of checkpoints after DNA damage. When Chk2 is down-regulated by antisense inhibition, defective S and G_2 delays have been observed upon exposure, respectively, to the topoisomerase I inhibitor camptothecin that blocks DNA replication and to the topoisomerase II inhibitor etoposide (VP-16) that causes DNA double-strand breaks, suggesting that Chk2 is involved in both the DNA replication and damage checkpoints (Yu and Pommier, unpublished). Both Chk2-deficient mouse embryonic stem (ES) cells (Hirao *et al.,* 2000) and antisense Chk2-expressing human 293 cells (Yu and Pommier, unpublished) display normal onset of G_2 arrest but are defective in sustaining it, indicating that Chk2 is only required for maintaining the G_2 arrest. In contrast, Chk1 has been suggested to be required to initiate the G_2 arrest (Liu *et al.,* 2000). Therefore, human Chk1 and Chk2 appear to jointly enforce the G_2 checkpoint in response to DNA damage. It remains unclear whether Chk1 and Chk2 share the same substrate(s) *in vivo.* UCN-01 has been identified recently to inhibit Chk1 (Busby *et al.,* 2000; Graves *et al.,* 2000) (Fig. 3). Because UCN-01 inhibits Cdc25C phosphorylation *in vivo,* it has been suggested that Chk2 may not be the dominant kinase targeting Cdc25C (Busby *et al.,* 2000; Graves *et al.,* 2000). We recently found that UCN-01 inhibits Chk2 immunoprecipitated from human cancer cells (Yu and

Pommier, unpublished). Thus, it is likely that Chk2 may regulate checkpoint response through various substrate(s), including Cdc25C and p53. It would be interesting to develop anticancer drugs that target Chk2, since Chk2 down-regulation has been shown to enhance apoptotic response in p53-inactive human cells (Yu and Pommier, unpublished).

E. Role of p53 in G_2 Checkpoint Control

Contrary to the G_1 checkpoint, p53 did not appear to be required for G_2 arrest, since cells without p53 function are capable of DNA-damage-induced arrest in G_2. Recently, however, p53 has been implicated in the regulation of the G_2 checkpoint. By using gene targeting, it was shown that p53 and its effector gene p21 are essential to sustain DNA-damage-induced G_2 arrest (Bunz *et al.,* 1998). Both p53- and p21-deficient cells display a normal initiation of G_2 arrest, but they prematurely escape the G_2 arrest (Bunz *et al.,* 1998). Since p53 leads to accumulation of p21, it was generally thought that p53-mediated p21 induction contributes to the G_2 checkpoint by inactivation of Cdk1. However, given the poor inhibition of Cdk1/cyclin B1 by p21 (Harper *et al.,* 1993, 1995), it does not appear to be a major event for the regulation of Cdk1/cyclin B1 activity. A more detailed mechanism has been described recently by Smits *et al.* showing that p21 blocks the CAK-mediated activating phosphorylation of Cdk1 on Thr161 (Smits *et al.,* 2000), a different event from what has been well described on Tyr15 dephosphorylation by Cdc25C.

Another p53-responsive gene 14-3-3σ has been shown to be required for maintaining the DNA-damage-induced G_2 checkpoint. In the human colorectal cancer cell line, HCT116, expression of the 14-3-3σ gene is induced in a p53-dependent manner after DNA damage, and its overexpression leads to G_2 arrest by sequestering cyclin B1 and Cdk1 in the cytoplasm and preventing the cells from entering mitosis (Hermeking *et al.,* 1997). Thus, cells lacking 14-3-3σ were unable to maintain G_2 arrest following DNA damage and underwent mitotic catastrophe as they entered mitosis (Chan *et al.,* 1999).

Alternatively, p53 has been shown to transcriptionally down-regulate Cdk1 and cyclin B1 (perhaps indirectly) to modulate the G_2 checkpoint (Park *et al.,* 2000; Passalaris *et al.,* 1999). Treatment of p53 wild-type cells with the DNA damaging agent doxorubicin resulted in an arrest in G2, the maintenance of which correlated with down regulation of Cdk1 and cyclin B1 mRNA and protein (Park *et al.,* 2000; Passalaris *et al.,* 1999). Conversely, constitutive activation of Cdk1 kinase overrides p53-mediated G_2 arrest (Park *et al.,* 2000). It appears that p53's role lies in the transcriptional repression of the cyclin B1 promoter (Innocente *et al.,* 1999).

Taken together, current evidence indicated that Cdk1/cyclin B1 is regulated by multiple mechanisms, including p53-dependent and independent pathways (Fig. 3). However, deregulation of Cdk1 and cyclin B1 might not be the only events that modulate the G_2 checkpoint. In contrast to p53 wild-type cells that abolish Cdk1-associated kinase activity when cells are arrested in G_2, cells lacking p53 function show a G_2 arrest despite high levels of cyclin B1 and Cdk1 kinase activity (Park *et al.,* 2000; Passalaris *et al.,* 1999). This observation suggests that there may exist a Cdk1/cyclin-B1-independent pathway(s) that maintains G_2 arrest in p53-deficient cells. Accordingly, it is possible that $p53^+$ and $p53^-$ cells use distinct pathways to regulate G_2 arrest. In cells with functional p53, p53-dependent regulation of Cdk1/cyclin B1 is a primary pathway that regulates G_2 arrest. However, in cells with defective p53 function, the p53-, Cdk1-independent pathway may become critical for G_2 arrest. Understanding of these mechanisms will better serve us in our search for novel therapeutic strategies to abrogate the G_2 checkpoint more efficiently and enhance cell killing by cancer therapeutic agents.

4. Rationale for Targeting Cyclin-Dependent Kinases and Cell Cycle Checkpoint Pathways

Uncontrolled cell proliferation and decreased apoptosis are basic dynamic mechanisms underlying cancer growth. In addition, genetic instability and defective checkpoints are key factors for carcinogenesis. Our understanding of the molecular elements that regulate these mechanisms has increased in the recent years. Cell cycle pathways and checkpoint controls have been found to be deregulated in human tumors and by oncogenic viruses. Genetic systems have also been used to demonstrate how the restoration of these pathways and/or interference with their molecular effectors can block cell proliferation. The fact that cyclin/Cdk's and checkpoint inhibitors, such as flavopiridol and UCN-01, demonstrate anticancer activity in animal models and in clinical trials is a demonstration that this strategy can work. Understanding how these agents achieve their selectivity for cancer cells should provide us with new approaches for cancer treatment.

1. Oncogenic viruses commonly activate cyclin-dependent kinases and inactivate the checkpoint pathways.
Tumor viruses commonly inactivate the pRb and p53 pathways to force cell division and block apoptosis. For instance, two genes (E6 and E7) are essential for replication of the human papillomaviruses (HPVs) that are associated with human cervical carcinomas. The E6 and E7 proteins bind and promote the degradation of p53 and pRb, respectively. The E1A and E1B gene products of adenoviruses inactivate pRb and p53, respectively. In the case of the papillomaviruses, p53 and pRb are inactivated by a single polypeptide, the SV40 T antigen.

Some tumor viruses can also activate the G_1 cyclins independently from p53. The herpesvirus associated with Kaposi's sarcoma (KSHV/HHV8) encodes a cyclin D2 homo-

logue that forms a complex with Cdk6. This complex promotes the degradation of the CDK inhibitor, $p27^{KIP1}$, and thereby lifts the inhibition of cyclin D/Cdk complexes (Ills *et al.,* 1999; Mann *et al.,* 1999). The HPV E7 protein, in addition to inactivating pRb, also inactivates the other major CKIs, including $p21^{WAF1/CIP1}$ (Funk *et al.,* 1997; Jones *et al.,* 1997).

2. The $G_1 \rightarrow S$ transition pathways are commonly activated in human cancer.

There are numerous examples implicating enhanced/deregulated G_1-S cell cycle transition pathways in human cancer. The retinoblastoma gene protein, pRb, has been among the first tumor suppressor genes discovered. Inactivation and mutations of pRb that prevent the E2F binding and its inactivation by pRb predispose to cancers both in animal models and in humans. Cyclin-D-dependent kinases are activated directly or indirectly (through their negative regulators) in a large fraction of human cancers (Bartek *et al.,* 1999; Bartkova *et al.,* 1997; Donnellan and Chetty, 1999; Hall and Peters, 1996; Kaelin, 1997; Lavia and Jansen-Durr, 1999; Mansuri *et al.,* 1997; Sellers and Kaelin, 1997). For instance, cyclin D1 is overexpressed in 70% of mantle cell lymphomas as a result of a translocation (t11:14) (Callanan *et al.,* 1996). $p27^{KIP1}$ is low in aggressive carcinomas (breast, colon, stomach, lung, and prostate) (Loda *et al.,* 1997). Other genetic changes found in human cancers affect Cdk4, Cdk2, cyclin E, and the CKIs $p15^{INK4B}$, $p16^{INK4A}$, and $p57^{KIP2}$ (Donnellan and Chetty, 1999). Up-regulation of the cyclin- D-dependent kinase pathways results in enhanced phosphorylation of pRb, which suppresses the negative regulatory effect of pRb on E2F and activates the E2F transcription factor (Fig. 2).

The proof of principle (validation) that inactivation of the G_1 Cdk's (Cdk4 and Cdk6) can effectively block cell proliferation has been demonstrated by overexpression of $p16^{INK4A}$ *in vitro* and *in vivo* (McConnell *et al.,* 1999; Nguyen *et al.,* 1995; Schreiber *et al.,* 1999; Serrano *et al.,* 1993; Sumitomo *et al.,* 1999; Wu *et al.,* 1996). A 36-residue peptide (amino acid residues 84–103 of $p16^{INK4A}$) coupled with a peptide carrier has also been shown to be sufficient for inhibition of Cdk4 and of cell proliferation (Fahraeus *et al.,* 1996). Because $p16^{INK4A}$ selectively blocks Cdk4 and Cdk6, but not the other Cdk's (Serrano *et al.,* 1993), it appears that selective targeting of the Cdk4 and Cdk6 kinases can selectively arrest cells that have functional pRb. Thus, assuming that pRb is the primary target for cyclin-D-dependent kinases, Cdk4 and Cdk6 inhibitors might be useful against cancers with normal pRb, and also possibly in patients with pRb deficiency for protecting normal tissues against the activity of cell-cycle-dependent anticancer agents.

Cyclin E is commonly amplified in human tumors. High cyclin E is correlated with low $p27^{KIP1}$ levels and poor prognosis in young breast cancer patients (Donnellan and Chetty, 1999). A recent study indicated that inhibition of Cdk2 using dominant negative forms of Cdk2 or oligonucleotides antisense to Cdk2 results in G_1 arrest and differentiation in some cells (Lee *et al.,* 1999). However, because Cdk2 is associated with both cyclin E and the S-phase cyclin, cyclin A, it is questionable how selective Cdk2 inhibitors would be. It was recently observed that small peptides that block the activity of cyclin A and cyclin E were selectively toxic to transformed cells with pRb inactivation (Chen *et al.,* 1999b). Because pRb-deficient cells have elevated E2F, which is negatively controlled by cyclin A/Cdk2, inhibition of Cdk2 would lead to high levels of E2F-1 and cell death by apoptosis.

To our knowledge there is no evidence that hyperactivation of the G2/M cyclin/Cdk's is a primary factor in human cancers and that the G2/M cyclin/Cdk's are differentially regulated in human cancers. Therefore, it is difficult to rationalize the development of cyclin B/Cdk1 inhibitors as anticancer agents. However, one interesting finding is that phosphorylation of survivin (Li *et al.,* 1999), an inhibitor of apoptosis (IAP) by cyclin B/Cdk1, is necessary for binding of survivin to caspase 9 and inhibition of apoptosis. Thus, inhibition of Cdk1 might abrogate the antiapoptotic activity of survivin, which is selectively expressed in malignant tumors but not in adult tissues.

3. The cell cycle checkpoint pathways are commonly defective in human cancer.

A significant fraction of cancers are associated with cycle checkpoint deficiencies. The tumor suppressor gene p53 is defective (mutated or not expressed) in approximately 50% of human cancers, and patients with hereditary p53 mutations (Li–Fraumeni syndrome) have a high incidence of cancers (Lane, 1999). The sites of interaction of p53 can be related in two main functions: cell cycle checkpoint (including cell cycle arrest and DNA repair) and induction of apoptosis (Levine, 1997). Restoration of p53 activity has been validated for anticancer therapeutics by experiments showing that restoration of p53 activity in p53-negative tumors is effective both in experimental systems and in cancer patients (Ries *et al.,* 2000).

Whether inhibition rather than restoration of checkpoint pathways is a valid approach for cancer chemotherapy is more debatable. It is plausible that the robustness of the cell cycle checkpoints relies on their redundancy. Since cancer cells are commonly defective in one or more checkpoint, it is plausible that abrogation of one checkpoint pathway might compromise cell survival. For instance, abrogation of the G_2- or S-phase checkpoints by caffeine and UCN-01 selectively sensitizes p53-deficient cells to genotoxic agents (Powell *et al.,* 1995; Yao *et al.,* 1996). Furthermore, even in the absence of genotoxic stress (associated DNA-damaging treatment), it is conceivable that cell cycle checkpoint abrogation might be selectively toxic for cancer cells that exhibit genomic instability and spontaneous DNA lesions.