

The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer

Katrien Vermeulen, Dirk R. Van Bockstaele and Zwi N. Berneman

Faculty of Medicine, Laboratory of Experimental Hematology, University of Antwerp, Antwerp University Hospital, Edegem, Belgium

Received 7 May 2003; revision accepted 2 June 2003

Abstract. The cell cycle is controlled by numerous mechanisms ensuring correct cell division. This review will focus on these mechanisms, i.e. regulation of cyclin-dependent kinases (CDK) by cyclins, CDK inhibitors and phosphorylating events. The quality checkpoints activated after DNA damage are also discussed. The complexity of the regulation of the cell cycle is also reflected in the different alterations leading to aberrant cell proliferation and development of cancer. Consequently, targeting the cell cycle in general and CDK in particular presents unique opportunities for drug discovery. This review provides an overview of deregulation of the cell cycle in cancer. Different families of known CDK inhibitors acting by ATP competition are also discussed. Currently, at least three compounds with CDK inhibitory activity (flavopiridol, UCN-01, roscovitine) have entered clinical trials.

GENERAL STRATEGY OF THE CELL CYCLE

Cell division consists of two consecutive processes, mainly characterized by DNA replication and segregation of replicated chromosomes into two separate cells. Originally, cell division was divided into two stages: mitosis (M), i.e. the process of nuclear division; and interphase, the interlude between two M phases (Fig. 1). Stages of mitosis include prophase, metaphase, anaphase and telophase. Under the microscope, interphase cells simply grow in size, but different techniques revealed that the interphase includes G_1 , S and G_2 phases (reviewed in Norbury & Nurse 1992). Replication of DNA occurs in a specific part of the interphase called S phase. S phase is preceded by a gap called G_1 during which the cell is preparing for DNA synthesis and is followed by a gap called G_2 during which the cell prepares for mitosis. G_1 , S, G_2 and M phases are the traditional subdivisions of the standard cell cycle (Fig. 1). Cells in G_1 can, before commitment to DNA replication, enter a resting state called G_0 . Cells in G_0 account for the major part of the non-growing, non-proliferating cells in the human body.

Correspondence: Zwi N. Berneman, Laboratory of Experimental Hematology, University of Antwerp, Antwerp University Hospital, Wilrijkstraat 10, B-2650 Edegem, Belgium. Tel.: +32 3821 37 80; Fax: +32 3821 42 86; E-mail: zwi.berneman@uza.be

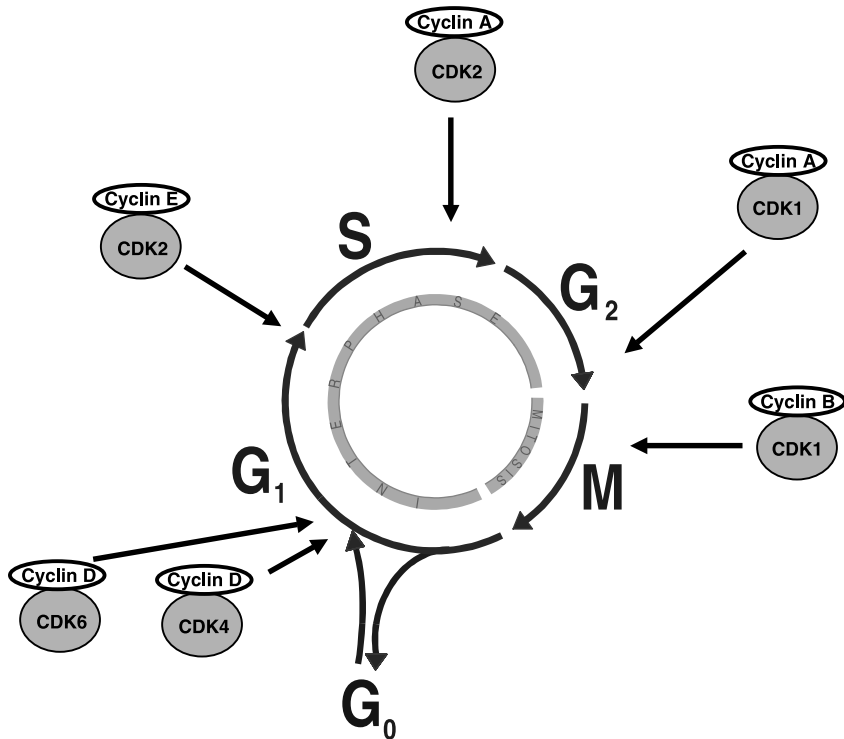


Figure 1. The stages of the cell cycle. The site of activity of regulatory CDK/cyclin complexes is also indicated.

CONTROL OF THE CELL CYCLE

Cyclin-dependent kinase (CDK) regulation

The transition from one cell cycle phase to another occurs in an orderly fashion and is regulated by different cellular proteins. Key regulatory proteins are the cyclin-dependent kinases (CDK), a family of serine/threonine protein kinases that are activated at specific points of the cell cycle. Until now, nine CDK have been identified and, of these, five are active during the cell cycle, i.e. during G₁ (CDK4, CDK6 and CDK2), S (CDK2), G₂ and M (CDK1) (Table 1, Fig. 1). When activated, CDK induce downstream processes by phosphorylating selected proteins (Morgan 1995; Pines 1995). CDK7 acts in combination with cyclin H as CDK activating kinase (CAK, see below) (Fisher & Morgan 1994). The remaining CDK have not yet been shown to have a crucial role in normal cell cycle progression (Rickert *et al.* 1996). CDK protein levels remain stable during the cell cycle, in contrast to their activating proteins, the cyclins. Cyclin protein levels rise and fall during the cell cycle and in this way they periodically activate CDK (Evans *et al.* 1983; Pines 1991). Different cyclins are required at different phases of the cell cycle (Table 1). The three D type cyclins (cyclin D1, cyclin D2, cyclin D3) bind to CDK4 and to CDK6 and CDK-cyclin D complexes are essential for entry in G₁ (Fig. 2) (Sherr 1994). Unlike the other cyclins, cyclin D is not expressed periodically, but is synthesized as long as growth factor stimulation persists (Assoian & Zhu 1997). Another G₁ cyclin is cyclin E which associates with CDK2 to regulate progression from G₁ into S phase (Ohtsubo *et al.* 1995). Cyclin A binds with

Table 1. Cyclin-CDK complexes are activated at specific points of the cell cycle. CAK, CDK activating kinase

CDK	Cyclin	Cell cycle phase activity
CDK4	Cyclin D1, D2, D3	G ₁ phase
CDK6	Cyclin D1, D2, D3	G ₁ phase
CDK2	Cyclin E	G ₁ /S phase transition
CDK2	Cyclin A	S phase
CDK1	(cdc2)	G ₂ /M phase transition
CDK1	(cdc2)	Mitosis
CDK7	Cyclin H	CAK, all cell cycle phases

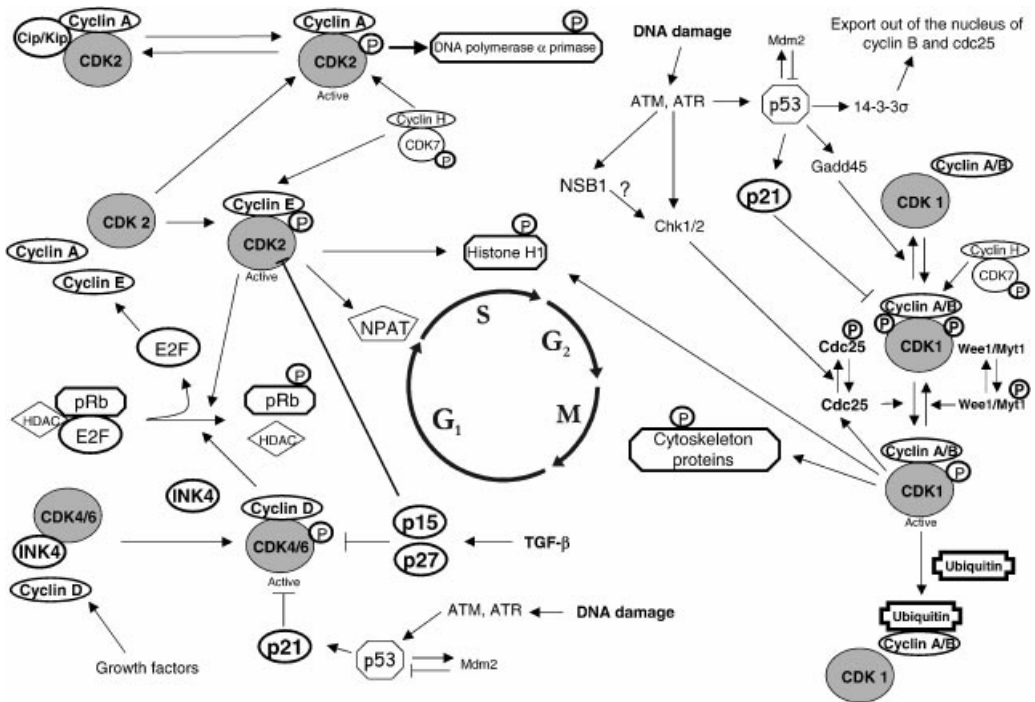


Figure 2. A schematic overview of some essential steps in cell cycle regulation. ⊕, phosphorylated site. (→: activation; —: inhibition)

CDK2 and this complex is required during S phase (Fig. 2) (Girard *et al.* 1991; Walker & Maller 1991). In late G₂ and early M, cyclin A complexes with CDK1 to promote entry into M. Mitosis is further regulated by cyclin B in complex with CDK1 (Fig. 2) (King *et al.* 1994; Arellano & Moreno 1997). Sixteen cyclins have been identified so far but, like CDK, not all of them are cell-cycle related (Peng *et al.* 1998; Okamoto & Beach 1994; Rickert *et al.* 1996). Cyclins A and B contain a destruction box and cyclins D and E contain a PEST sequence [segment rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues]: these are protein sequences required for efficient ubiquitin-mediated cyclin proteolysis at the end of a cell cycle phase (Glotzer *et al.* 1991; Rechsteiner & Rogers 1996).

Table 2. Cyclin dependent kinases inhibitors (CKI) bind to CDK alone or to the CDK-cyclin complex and regulate CDK activity. p19 (ARF) is also encoded by the INK4 locus, but has no known CKI activity

CKI family	Function	Family members	
INK4 family	Inactivation of G ₁ CDK (CDK4, CDK6)	p15	(INK4b)
		p16	(INK4a)
		p18	(INK4c)
		p19	(INK4d)
Cip/Kip family	Inactivation of G ₁ cyclin-CDK complexes and cyclin B-CDK1	p21	(Waf1, Cip1)
		p27	(Cip2)
		p57	(Kip2)

In addition to cyclin binding, CDK activity is also regulated by phosphorylation on conserved threonine and tyrosine residues. Full activation of CDK1 requires phosphorylation of threonine 161 (threonine 172 in CDK4 and threonine 160 in CDK2), brought about by the CDK7-cyclin H complex, also called CAK (Fig. 2). These phosphorylations induce conformational changes and enhance the binding of cyclins (Jeffrey *et al.* 1995; Paulovich & Hartwell 1995). The Wee1 and Myt1 kinases phosphorylate CDK1 at tyrosine-15 and/or threonine-14, thereby inactivating the kinase (Fig. 2). Dephosphorylation at these sites by the enzyme Cdc25 is necessary for activation of CDK1 and further progression through the cell cycle (Fig. 2) (Lew & Kornbluth 1996).

CDK activity can be counteracted by cell cycle inhibitory proteins, called CDK inhibitors (CKI) which bind to CDK alone or to the CDK-cyclin complex and regulate CDK activity. Two distinct families of CDK inhibitors have been discovered, the INK4 family and Cip/Kip family (Table 2) (Sherr & Roberts 1995). The INK4 family includes p15 (INK4b), p16 (INK4a), p18 (INK4c), p19 (INK4d), which specifically inactivate G₁ CDK (CDK4 and CDK6). These CKI form stable complexes with the CDK enzyme before cyclin binding, preventing association with cyclin D (Carnero & Hannon 1998). The second family of inhibitors, the Cip/Kip family, includes p21 (Waf1, Cip1), p27 (Cip2), p57 (Kip2). These inhibitors inactivate CDK-cyclin complexes (Polyak *et al.* 1994; Harper *et al.* 1995; Lee *et al.* 1995). They inhibit the G₁ CDK-cyclin complexes, and to a lesser extent, CDK1-cyclin B complexes (Hengst & Reed 1998). p21 also inhibits DNA synthesis by binding to and inhibiting the proliferating cell nuclear antigen (PCNA) (Pan *et al.* 1995; Waga *et al.* 1997). CKI are regulated both by internal and external signals: the expression of *p21* is under transcriptional control of the *p53* tumour suppressor gene. The *p21* gene promoter contains a p53-binding site, that allows p53 to transcriptionally activate the *p21* gene (el Deiry *et al.* 1993). The expression and activation of, respectively, p15 and p27, increases in response to transforming growth factor β (TGF- β), contributing to growth arrest (Hannon & Beach 1994; Reynisdottir *et al.* 1995).

The intracellular localization of different cell cycle-regulating proteins also contributes to a correct cell cycle progression. Cyclin B contains a nuclear exclusion signal and is actively exported from the nucleus until the beginning of the prophase. The CDK inactivating kinases Wee1 and Myt1 are located, respectively, in the nucleus and Golgi complex and protect the cell from premature mitosis (Heald *et al.* 1993; Liu *et al.* 1997). The 14-3-3 group of proteins regulates the intracellular trafficking of different proteins. During interphase, the CDK activating kinase, Cdc25, is kept in the cytoplasm through interaction with 14-3-3 proteins. Sequestration of the CDK1-cyclin B complex in the cytoplasm following DNA damage is also mediated by 14-3-3 proteins (Peng *et al.* 1997; Yang *et al.* 1999).

CDK substrates

When CDK is active, target proteins become phosphorylated on CDK consensus sites, resulting in changes that are physiologically relevant for cell cycle progression. The most frequently studied target is the substrate of CDK4/6-cyclin D, i.e. the product of the retinoblastoma tumour suppressor gene (pRb) (Fig. 2). During early G₁, pRb becomes phosphorylated and this leads to disruption of the complex with the histone deacetylase protein (HDAC) and release of the transcription factors E2F-1 and DP-1, which positively regulate the transcription of genes whose products are required for S phase progression, including cyclin A, cyclin E, Cdc25 (Fig. 2) (Buchkovich *et al.* 1989; Kato *et al.* 1993; Brehm *et al.* 1998). pRb remains hyperphosphorylated for the remainder of the cell cycle and CDK2-cyclin E participates in maintaining this hyperphosphorylated state. During G₁/S the CDK2-cyclin E complex also phosphorylates its inhibitor p27, inducing its proteasome-dependent degradation (Hinds *et al.* 1992; Montagnoli *et al.* 1999). NPAT (nuclear protein mapped to the ATM locus) associates with, and is also phosphorylated by, CDK2-cyclin E. The protein level of NPAT peaks at the G₁/S boundary and is thought to play a role in S phase entry (Zhao *et al.* 2000). CDK2-cyclin E phosphorylates histone H1 and this activity may be important for chromosome condensation required during DNA replication. Histone H1 is also a substrate for CDK1-cyclin B (Bradbury *et al.* 1974). Cyclin A-dependent kinases regulate initiation of DNA replication by phosphorylation of DNA polymerase alpha primase (Voitenleitner *et al.* 1997). Other CDK substrates include CDK's own regulators Wee1 and Cdc25, and cytoskeletal proteins such as nuclear lamins, microtubules and vimentin, which are required for correct mitosis (Heald & McKeon 1990; Courvalin *et al.* 1992; Hoffmann *et al.* 1993; Blangy *et al.* 1995).

Quality control of the cell cycle: restriction points and checkpoints

The restriction point (R) is defined as a point of no return in G₁, following which the cell is committed to enter the cell cycle. Experiments demonstrate that cells starved of serum before the restriction point enter a G₀-like state, while cells starved after R are unaffected and continue through mitosis (Pardee 1974). Additional controls or checkpoints exist further in the cell cycle, ensuring an orderly sequence of events in the cell cycle (Hartwell & Weinert 1989). Up to now, DNA damage checkpoints and spindle checkpoints have (partly) been elucidated. In response to DNA damage, checkpoints arrest the cell cycle in order to provide time for DNA repair. DNA damage checkpoints are positioned before the cell enters S phase (G₁-S checkpoint) or after DNA replication (G₂-M checkpoint) and there appears to be DNA damage checkpoints during S and M phases.

At the G₁/S checkpoint, cell cycle arrest induced by DNA damage is p53-dependent. Usually, the cellular level of p53 is low but DNA damage can lead to rapid induction of p53 activity (Levine 1997). p53 stimulates the transcription of different genes including *p21*, *Mdm2* and *Bax* (Agarwal *et al.* 1998). The induction of p21, a CKI, results in CDK inhibition and cell cycle arrest, preventing the replication of damaged DNA (Fig. 2) (Ko & Prives 1996). Mdm2 plays an important role in the regulation of p53: it binds to and inhibits p53 transcriptional activity and contributes to the proteolytic degradation of p53 by facilitating its ubiquitination, hereby providing a negative feedback loop (Oren 1999). Binding of regulatory proteins can also modulate p53 ubiquitination: the p19 (ARF) protein, encoded by the ARF-INK4 locus (see below), binds to Mdm2 and this prevents the Mdm2-mediated p53 proteolysis (Zhang *et al.* 1998). In the case of severely damaged cells, p53 induces cell death by activating genes (e.g. *Bax*, *Fas* and genes involved in oxidative stress pathways) that are involved in apoptotic signalling (Owen-Schaub *et al.* 1995; Polyak *et al.* 1997; Gottlieb & Oren 1998).

Different protein kinases 'recognize' DNA damage, e.g. ataxia-telangiectasia-mutated (ATM), ataxia and rad3 related (ATR). These kinases phosphorylate p53 in response to DNA damage,

resulting in p21 blocking the cell cycle, at least at the G₁/S checkpoint (Fig. 2) (Siliciano *et al.* 1997). DNA protein kinase (DNA-PK), a DNA double-strand break repair enzyme is related to ATM and ATR, but it is not known yet whether it also plays an important role at the G₁/S checkpoint (Burma *et al.* 1999; Durocher & Jackson 2001).

The mechanisms of the S phase DNA damage checkpoint are poorly understood, but some studies demonstrated suppression of both the initiation and elongation phases of DNA replication (Painter 1986; Paulovich & Hartwell 1995). There is also evidence that ATM-mediated phosphorylation of (Nijmegen breakage syndrome 1 (NBS1) is required to induce S phase arrest during the S phase checkpoint (Lim *et al.* 2000).

When DNA damage occurs during G₂, cells are able to initiate a cell cycle arrest in the presence or absence of p53. The entry into mitosis is prevented by maintaining CDK1 in its inhibited form through inhibitory phosphorylation or by sequestration of components of the CDK1-cyclin B complex outside the nucleus. This is achieved by the protein kinases Chk1 and Chk2, which are activated during DNA damage in an ATM-dependent manner and which phosphorylate Cdc25 (Fig. 2). Phosphorylation of Cdc25 inhibits its activity and promotes its binding to 14-3-3 proteins, sequestering it outside the nucleus and preventing it from activating CDK1-cyclin B and mitotic entry (Sanchez *et al.* 1997; Zeng *et al.* 1998). Besides induction of inhibitory phosphorylations on CDK, p53 may also play a role in the regulation of the G₂/M checkpoint. DNA damage-dependent increase of p53 results, as during the G₁/S checkpoint, in increased transcription of *p21* and of *14-3-3 sigma* (14-3-3 σ). Increased binding of cyclin B to 14-3-3 σ actively excludes it from the nucleus. p53 also mediates the dissociation of CDK1-cyclin B1 complexes by induction of Gadd45 (growth arrest and DNA damage inducible gene) (Hermeking *et al.* 1997; Taylor & Stark 2001).

The 'spindle checkpoint' brings about detection of improper alignment of the chromosomes on the mitotic spindle and stops the cell cycle in metaphase. Initially identified in budding yeast, several mammalian spindle checkpoint-associated proteins have recently been identified. Mitotic arrest deficient (Mad) and budding uninhibited by benomyl (Bub) proteins are activated when defects in microtubule attachment occur and inhibit the Cdc20 subunit of the anaphase-promoting complex (APC), resulting in the prevention of metaphase-anaphase transition (Fang *et al.* 1998; Amon 1999).

CELL CYCLE AND CANCER

In cancer, there are fundamental alterations in the genetic control of cell division, resulting in an unrestrained cell proliferation. Mutations mainly occur in two classes of genes: proto-oncogenes and tumour suppressor genes. In normal cells, the products of proto-oncogenes act at different levels along the pathways that stimulate cell proliferation. Mutated versions of proto-oncogenes or oncogenes can promote tumour growth. Inactivation of tumour suppressor genes like *pRb* and *p53* results in dysfunction of proteins that normally inhibit cell cycle progression. Cell cycle deregulation associated with cancer occurs through mutation of proteins important at different levels of the cell cycle. In cancer, mutations have been observed in genes encoding CDK, cyclins, CDK-activating enzymes, CKI, CDK substrates, and checkpoint proteins (reviewed by Sherr 1996; McDonald & el Deiry 2000).

CDK

Alterations of CDK molecules in cancer have been reported, although with low frequency. CDK4 overexpression, that occurs as a result of amplification, has been identified in cell lines,

melanoma, sarcoma and glioma (Wolfel *et al.* 1995). Mutations in *CDK4* and *CDK6* genes resulting in loss of CKI binding have also been identified (Easton *et al.* 1998). *CDK1* and *CDK2* have been reported to be overexpressed in a subset of colon adenomas, a greater overexpression was seen in focal carcinomas in adenomatous tissue (Yamamoto *et al.* 1998; Kim *et al.* 1999).

Cyclins

Cyclin D acts as a growth sensor and provides a link between mitogenic stimuli and the cell cycle. Cyclin D1 binds to *CDK4* and *CDK6* in early G₁. Aberrant cyclin D1 expression has been reported in many human cancers. In the first study that implicated cyclin D1 in human tumours, its gene was linked in parathyroid adenomas to the parathyroid hormone gene (Motokura *et al.* 1991). It is now clear that *cyclin D1* gene translocation is associated with B-cell malignancies, including mantle cell lymphoma. In the latter case, the characteristic t(11; 14) translocation juxtaposes the *cyclin D1* gene (initially described as *bcl-1/PRAD1*) to the immunoglobulin heavy chain gene, leading to cyclin D1 overexpression in centrocytic B-lymphocytes (Weisenburger *et al.* 1987). *Cyclin D1* gene amplification occurs in breast, esophageal, bladder, lung and squamous cell carcinomas (Hall & Peters 1996). *Cyclin D2* and *cyclin D3* have also been reported to be overexpressed in some tumours and *cyclin E* has been found to be amplified, overexpressed or both in some cases of breast and colon cancer and in acute lymphoblastic and acute myeloid leukaemias (Leach *et al.* 1993; Hunter & Pines 1994; Keyomarsi *et al.* 1995; Scuderi *et al.* 1996; Iida *et al.* 1997). Both cyclin A and cyclin E are overexpressed in lung carcinoma and elevated expression of cyclin A but not cyclin E correlated with shorter survival (Dobashi *et al.* 1998).

CDK activating enzymes

Activation of CDK is regulated through dephosphorylation by members of the Cdc25 phosphatase family. *Cdc25A* plays an important role at the G₁/S-phase transition, *Cdc25B* undergoes activation during S-phase and *Cdc25C* activates *CDK1*-cyclin B during entry into mitosis. Deregulation or overexpression of *Cdc25* allows for unscheduled activation of *CDK*-cyclins and can be associated with tumour formation. *Cdc25A* and *Cdc25B* are potential human oncogenes (Nilsson & Hoffmann 2000). *Cdc25B* is overexpressed in 32% of primary breast cancers. Transcription of *Cdc25A* and *Cdc25B* genes is activated by *c-myc*, an oncogene found to be frequently mutated in human cancers (Galaktionov *et al.* 1996). Raf, a kinase downstream of the frequently mutated *ras* oncogene, is able to bind, activate and deregulate *Cdc25* protein (Galaktionov *et al.* 1995).

CKI

The inhibitory activity of CKI results in growth suppression through activation of pRb, reflecting the tumour suppressor function of CKI. The *p16* gene is altered in a high percentage of human tumours and can be inactivated by a variety of mechanisms including deletion, point mutations and hypermethylation (Kamb 1998). Cells with altered p16 will be unrestrained to proceed through G₁. The p16 protein is a specific inhibitor of *CDK*-cyclin D, preventing phosphorylation of the pRb protein and arresting cells in G₁ phase (Table 2). As pRb, p16 and *CDK*/cyclin D are functionally interconnected, perturbations in any of these cell cycle regulators are likely to have similar consequences. Moreover, alterations of at least one of these regulators are found in nearly all human cancers (Jiang *et al.* 1993; Bates *et al.* 1994; Okamoto *et al.* 1994; Parry *et al.* 1995; Gronbaek *et al.* 1998). Deletions of *p16* have been reported in approximately 50% of gliomas and mesotheliomas, 40–60% of nasopharyngeal, pancreatic and biliary tract tumours and

20–30% of acute lymphoblastic leukaemias (reviewed by Hall & Peters 1996). The *p16* gene product is encoded by the ARF-INK4 locus. Besides the *p16* gene product, this locus also encodes p19 (ARF), following an alternative reading frame. The *p19* transcript acts independently of *p16* in regulating p53 stability. Large deletions of the ARF-INK4 locus can also affect the *p19* gene, resulting in a mutated *p19* and in deregulation of *p53*. The gene encoding p15, another CKI, is located close to the *p16* gene on chromosome 9 and is also often simultaneously deleted (Harper & Elledge 1996). Loss of *p27* expression has been reported for a number of human tumour types (lung, breast, bladder) and has been correlated with poor prognosis and tumour aggressiveness. It has been shown in colorectal carcinomas that increased proteasome-dependent proteolysis, rather than gene deletion, is responsible for p27 down-regulation (King *et al.* 1996; Pagano 1997). A few alterations have been found in *p18* and *p21* in breast tumour and leukaemia, respectively (Lapointe *et al.* 1996; Shi *et al.* 1996; Esposito *et al.* 1997; Loda *et al.* 1997; Tan *et al.* 1997). p21 has been implicated in tumorigenesis through its regulation by the p53 tumour suppressor protein. The *p53* gene is the most commonly mutated gene in human cancer and regulation of p21 in response to DNA damage is lost when p53 is inactivated (Deng *et al.* 1995).

Substrate

pRb, the most important CDK substrate during G₁, is frequently mutated in human retinoblastoma and lung cancer (Knudson 1971; Hall & Peters 1996). Deletion and mis-sense mutations result in truncated, non-functional pRb or in complete absence of pRb, while binding of certain tumour virus proteins (e.g. human papillomaviruses (HPV) E7, adenovirus E1A and simian virus 40 (SV40) large (T) inactivate pRb (Gage *et al.* 1990; Hu *et al.* 1990). Absence or loss of function of pRb is associated with unrestrained cell cycle progression and is common in acute lymphoblastic leukaemia (Horsthemke 1992; Tsai *et al.* 1996). The implications for human cancer of the interconnection of pRb with CDK4/6-cyclin D and p16 have been mentioned above. Approximately 90% of human cancers have abnormalities in some component of the pRb pathway (Hall & Peters 1996). The other pRb family members, p107 and p130, have not yet been linked with tumour-promoting mutations, neither have tumour-associated mutations of the E2F family of transcription factors been described (Bartek *et al.* 1996).

Checkpoint proteins

Mutations of checkpoint proteins are frequent in all types of cancer. The tumour suppressor protein p53 is a sequence-specific DNA-binding protein, that is able to induce either cell cycle arrest or apoptosis at the cell cycle checkpoints. The *p53* tumour suppressor gene was first discovered in SV40 transformed cells by the finding that its protein product p53 was tightly bound to the SV40 large T oncogene product. Now, the *p53* gene is known to be the most frequently mutated gene in human cancer (Miller & Koeffler 1993; Greenblatt *et al.* 1994). Point and mis-sense mutations lead to conformational changes and inactivation of the protein (Nataraj *et al.* 1995). Other mechanisms, like binding of viral oncoproteins including SV40 T antigen, HPV E6 and adenovirus E1B-55K, can alter or block p53 function (van den Heuvel *et al.* 1990; Crook & Vousden 1994). In general, tumours that retain wild type p53 have a better prognosis and have a better response to therapy (Lowe *et al.* 1994). Overexpression by gene amplification or other mechanisms of *Mdm2*, the negative regulator of p53, has been reported in leukaemia and lymphoma, breast carcinoma, sarcoma and glioma and may represent an alternative mechanism to p53 mutation for escaping p53-mediated growth control (Bueso-Ramos *et al.* 1995; Bueso-Ramos *et al.* 1996; Moller *et al.* 1999).

CDK INHIBITORS IN ANTI-CANCER DRUG DEVELOPMENT

The process of searching for new cancer drugs has undergone a major change: it has moved from a strategy identifying drugs that kill tumour cells towards a more mechanistic strategy acting on molecular targets that underly cell transformation. The evidence that CDK, their regulators and substrates are targets of genetic alteration in different types of human cancer has stimulated the search for chemical CDK inhibitors. Different strategies for therapeutic intervention can modulate CDK activity: targeting the major regulators of CDK activity (indirect strategy) or inhibiting the catalytic activity of the CDK kinases (direct strategy). Approaches for the indirect strategy include overexpression of CKI, synthesis of peptides mimicking the effects of CKI, decrease of cyclin levels, modulation of the proteasomal machinery, modulation of the phosphorylated state of CDK and of the enzymes regulating it. Details of these indirect strategies are beyond the scope of this review; for further information, readers are referred to other reviews (McDonald & el Deiry 2000; Senderowicz & Sausville 2000). Until now, direct inhibition of CDK kinase activity has been the most successful strategy for the development of potent cell cycle inhibitors. All inhibitors identified so far act by competitive inhibition of ATP binding to CDK. The potential for disruption of ATP binding in the small, defined ATP pocket of CDK is much higher than disrupting a large protein-protein interface, such as a CDK-cyclin binding surface. More than 50 inhibitors have been described and most studied families will be described below and in Table 3.

Purine analogues, plant cytokinin analogues and pyrimidine analogues

The natural phytohormones or cytokinins dimethylaminopurine and N⁶-isopentenyladenine were first identified as CDK1-cyclin B kinase inhibitors, but they were found to be non-specific kinase inhibitors (Meijer & Pondaven 1988; Neant & Guerrier 1988; Rialet & Meijer 1991). Screening of chemically synthesized aromatic cytokinin analogues for inhibition of CDK1-cyclin B kinase led to the discovery of the highly CDK-specific inhibitor olomoucine (Vesely *et al.* 1994; Schulze-Gahmen *et al.* 1995). Olomoucine has been found to inhibit cell proliferation and to induce apoptosis in tumour cells (Abraham *et al.* 1995; Schutte *et al.* 1997). Olomoucine also potentiated mitoxantrone-induced apoptosis (Ongkeko *et al.* 1995) and initiated apoptosis in a case of dog malignant melanoma (Hajduch *et al.* 1997). Among 35 highly purified kinases, only the cell cycle regulating CDK1-cyclin B, CDK2-cyclin A, CDK2-cyclin E, brain CDK5-p35 and ERK1/MAP kinase were substantially inhibited by olomoucine. Among 81 cytokinin analogues tested, only C2-, C6-, N9-substituted purines showed strong inhibitory effect on CDK1 (Vesely *et al.* 1994). Roscovitine, a more recently developed C2-, C6-, N9-substituted purine has a 10-fold more potent inhibitory activity on CDK1 than olomoucine. Roscovitine showed strong anti-proliferative effects and it has currently entered clinical trials (De Azevedo *et al.* 1997; Meijer *et al.* 1997; Clough 2002). CVT-313 is another CDK inhibitor, identified from a purine analogue library; this compound blocks proliferation of human lung fibroblasts (Brooks *et al.* 1997). Another group of purine-based structures was identified in a screening of trisubstituted purine combinatorial libraries designed for CDK inhibition. This screening led to the development of new CDK inhibitors, e.g. purvalanol A and B (Gray *et al.* 1998). Recently, different studies on synthesis and *in vitro* evaluation of C2-, C6-, N9-substituted purines have been published and research on the mechanistic basis of anti-proliferative effect is currently ongoing (Chang *et al.* 1999; Legraverend *et al.* 1999; Legraverend *et al.* 2000; Davis *et al.* 2001; Dreyer *et al.* 2001; Davies *et al.* 2002; Gibson *et al.* 2002). Our own studies show that cytokinin analogues with anti-CDK activity have marked anti-proliferative effects on leukaemic cell lines, primary myeloid cells and on primary lymphocytes, that this anti-proliferative effect is correlated with

Table 3. Chemical CDK inhibitors and their IC₅₀ (concentration at which 50% inhibition occurred) on CDK1

Inhibitor	IC ₅₀ (μM)	Reference no.
Purine analogues		
Dimethylaminopurine	120	Meijer & Pondaven 1988; Neant & Guerrier 1988
N6-isopentenyladenine	55	Rialet & Meijer 1991
Olomoucine	7	Vesely <i>et al.</i> 1994
Roscovitine	0.2–0.8	De Azevedo <i>et al.</i> 1997; Meijer <i>et al.</i> 1997
CVT-313	4.2	Brooks <i>et al.</i> 1997
Purvalanol A	0.004	Gray <i>et al.</i> 1998
Purvalanol B	0.006	Gray <i>et al.</i> 1998
New cytokinin analogues	0.1–3.8	Vermeulen <i>et al.</i> 2002a; Vermeulen <i>et al.</i> 2002b
Olomoucine II	0.02	Krystof <i>et al.</i> 2002
NU2058	5	Arris <i>et al.</i> 2000
Pyrimidine analogues		
NU6027	2.5	Arris <i>et al.</i> 2000
Butyrolactone	0.6	Kitagawa <i>et al.</i> 1993; Kitagawa <i>et al.</i> 1994
Flavonoïds		
Flavopiridol	0.4	Losiewicz <i>et al.</i> 1994
Oxoflavopiridol	0.130	Kim <i>et al.</i> 2000
Thioflavopiridol	0.110	Kim <i>et al.</i> 2000
Paullones		
Kenpaullone	0.4	Zaharevitz <i>et al.</i> 1999
Alsterpaullone	0.035	Schultz <i>et al.</i> 1999
Indolinones		
Indirubin	10	Hoessel <i>et al.</i> 1999
Indirubin-3'-monoxime	0.18	Hoessel <i>et al.</i> 1999
5-chloro-indirubin	0.4	Hoessel <i>et al.</i> 1999
Indirubin-5-sulphonic acid	0.055	Hoessel <i>et al.</i> 1999
SU9516	0.04	Lane <i>et al.</i> 2001
Staurosporine and derivatives		
Staurosporine	0.003–0.009	Gadbois <i>et al.</i> 1992
UCN-01	0.031–1	Wang <i>et al.</i> 1995; Kawakami <i>et al.</i> 1996
9-hydroxyellipticine	1	Ohasi <i>et al.</i> 1995
Suramin	4	Larsen 1993
Hymenialdisine	0.022	Meijer <i>et al.</i> 2000
Toyocamycin	0.88	Park <i>et al.</i> 1996

anti-CDK activity and that it is due, at least in part, to induction of apoptosis (Vermeulen *et al.* 2002a). Apoptosis seems to be induced through the mitochondrial pathway (Vermeulen *et al.* 2002b). Another newly synthesized compound, olomoucine II, is a potent and specific CDK1 inhibitor with 10 times higher inhibitory activity than roscovitine and with a cytotoxic activity that exceeds that of purvalanol A (Krystof *et al.* 2002). NU2058 and NU6027, respectively, a guanine and pyrimidine analogue, show potent CDK1 and CDK2 inhibition, and both have a growth inhibitory pattern distinct from flavopiridol and olomoucine (Arris *et al.* 2000; Davies *et al.* 2002; Gibson *et al.* 2002). Also, pyridopyrimidines have been identified as CDK inhibitors and the study of the structure–activity relationship of more than 60 analogues showed clear trends that might be exploited in the design of more potent inhibitors (Barvian *et al.* 2000).

Butyrolactone

Butyrolactone, isolated from *Aspergillus* strain F-25799, was found to be a CDK inhibitor in a screen of micro-organism culture media. Butyrolactone inhibits CDK1 and CDK2 and shows

good specificity against other kinases, including CDK4 (Kitagawa *et al.* 1993). Butyrolactone inhibits phosphorylation of pRb and of histone H1, inhibits G₁/S and G₂/M transition and DNA synthesis in human fibroblasts and inhibits proliferation of lung, colon and pancreatic cancer cell lines (Kitagawa *et al.* 1994; Nishio *et al.* 1996; Wada *et al.* 1998; Yamamoto *et al.* 1998).

Flavonoids

Flavonoids, like quercetin and genistein, are known to exhibit biological activity through inhibition of various kinases, of which protein kinase C (PKC) is the most prominent example. However, flavopiridol, another flavonoid and a synthetic analogue of a natural alkaloid extracted from an Indian plant, *Dysoxylum binectariferum*, specifically inhibits CDK1 and CDK2 (Maik *et al.* 1988; Losiewicz *et al.* 1994). In contrast to olomoucine, roscovitine and butyrolactone, it is also active against CDK4 (Carlson *et al.* 1996). Flavopiridol was first discovered as a potent growth inhibitor of several breast and lung cancer cell lines (Kaur *et al.* 1992). Different studies have shown that flavopiridol has the potential to inhibit the proliferation of a broad range of different types of cell lines, human tumours, leukaemias and lymphomas (Drees *et al.* 1997; Parker *et al.* 1998). Several phase I and phase II clinical trials with different regimens have been completed. Activity has been shown in some patients with non-Hodgkin's lymphoma and in renal, prostate, colon and gastric carcinoma (reviewed by Senderowicz & Sausville 2000; Senderowicz 2001). However, many questions remain: What is the best treatment schedule? Is there a 'best' combination with standard chemotherapeutic agents (Zhai *et al.* 2002)?

Recently, flavopiridol analogues thioflavopiridol and oxoflavopiridol were shown to have selective anti-CDK activity and to inhibit the colony forming ability of multiple human tumour cell lines (Kim *et al.* 2000).

Paullones

Paullones were discovered following analysis of the *in vitro* anti-proliferative profile in the National Cancer Institute (NCI) anti-cancer drug screen panel, that was performed in order to detect compounds that show a similar pattern of activity as flavopiridol. Kenpaullone was identified as an inhibitor of CDK1-cyclin B, CDK2-cyclin A, CDK2-cyclin E and CDK5-p35; it arrests breast epithelial cells at the G₁/S boundary (Zaharevitz *et al.* 1999). Derivatives of the lead compound kenpaullone were synthesized and alsterpaullone was developed. This compound showed a high CDK1-cyclin B inhibitory activity and exceeded the *in vitro* anti-tumour potency of the other paullones by one order of magnitude (Schultz *et al.* 1999).

Indolinones

Indirubin was isolated from a Chinese herbal mixture, that was used to treat chronic myeloid leukaemia (CML). Recently, indirubin and its analogues 5-chloro-indirubin, indirubin-3'-monoxime and indirubin-5-sulphonic acid were identified as potent and selective CDK inhibitors. They show inhibitory activity against CDK1, CDK2, CDK4 and CDK5 (Hoessel *et al.* 1999). Indirubin-3'-monoxime has activity in several tumour models and blocks the cell cycle at G₁/S and G₂/M (Marko *et al.* 2001). SU9516, a novel 3-substituted indolinone inhibits the activity of CDK1, CDK2 and CDK4 and induces apoptosis in colon carcinoma cells (Lane *et al.* 2001). Oxindole (2-indolinone)-based inhibitors with effect on CDK have been developed and are described by (Andreani *et al.* 2001; Bramson *et al.* 2001; Davis *et al.* 2001).

Other (non-specific) CDK inhibitors

Staurosporine is a microbial alkaloid, isolated from *Streptomyces* sp. cultures. Staurosporine is a general non-specific inhibitor of PKC and of CDK1 and it induces G₂ cell cycle arrest of normal

and transformed cells (Tamaoki 1991; Gadbois *et al.* 1992). UCN-01 or 7-hydroxystaurosporine, a staurosporine analogue that is also a non-specific CDK inhibitor, shows an anti-proliferative effect on human tumour cell lines and has now entered clinical trials (Takahashi *et al.* 1989; Wang *et al.* 1995; Kawakami *et al.* 1996). It is not yet clear whether the effect is the result of inhibition of CDK or of other kinases (reviewed by Senderowicz 2001).

Other CDK inhibitors include suramin, hymenialdisine, 9-hydroxyellipticine, toyocamycin, quinazolines and aminothiazoles (Bojanowski *et al.* 1994; Ohashi *et al.* 1995; Park *et al.* 1996; Meijer *et al.* 2000; Shewchuk *et al.* 2000; Sielecki *et al.* 2001; Kim *et al.* 2002). Suramin is a naturally occurring glycosaminoglycan currently used as an anti-helminthic, anti-protozoal and anti-tumour agent (reviewed by Larsen 1993). Suramin inhibits various enzymes including CDK1 (Bojanowski *et al.* 1994). Hymenialdisine is a compound isolated from a marine sponge and is a very potent inhibitor of CDK1, CDK2 and CDK5, of glycogen synthase kinase-3 β (GSK-3 β) and of casein kinase 1 (CK1) (Meijer *et al.* 2000). The aminothiazole 2-acetamidothiozylthio acetic ester 1 showed CDK2 inhibition but was inactive in cells. Synthesis and structure-activity relationship studies of more than 100 analogues revealed many analogues with CDK2 inhibitory activity and with potent and broad spectrum anti-proliferative activity across a panel of tumour cell lines *in vitro* (Kim *et al.* 2002).

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