The cell cycle and drug discovery: the promise and the hope

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In recent years, there have been major developments in the understanding of the cell cycle. It is now known that normal cellular proliferation is tightly regulated by the activation and deactivation of a series of proteins that constitute the cell cycle machinery. The expression and activity of components of the cell cycle can be altered during the development of a variety of diseases where aberrant proliferation contributes to the pathology of the illness. Apart from yielding a new source of untapped therapeutic targets, it is likely that manipulating the activity of such proteins in diseased states will provide an important route for treating proliferative disorders, and the opportunity to develop a novel class of future medicines.

The cell division cycle is an evolutionarily conserved process used by all eukaryotic cells to control growth and division (see Refs 1–4 for reviews). The cell cycle consists of four distinct stages: two gap-phases (G1 and G2), where RNA and protein syntheses occur, an S-phase, where DNA synthesis and replication occur, and an M-phase, where the cell undergoes mitosis and divides into two daughter cells (Fig. 1). In the G1-phase, cells monitor both their internal and external environments to determine whether to divide or to remain in a state of quiescence (also known as G0). Once initiated, the cell progresses from the G1- to the S-phase, and then to the G2-phase, before progressing into the M-phase. Normal cellular proliferation is ordered and tightly controlled by a series of regulatory mechanisms that either permit or prevent cell-cycle progression through each phase and thus play an important role in maintaining the balance between 'old' and 'new' cells within an organism.

Considerable progress has been made in understanding the molecules and mechanisms that control and coordinate cell-cycle progression (see Refs 1–6 for reviews). One such control point is the restriction (R)-point (Fig. 1) that functions in the G1-phase and regulates entry into the cell cycle. In mammalian cells, the R-point is widely believed to be analogous to START that has been operationally defined in experimental yeast[6]. Once yeast cells have passed START, they are committed to the division cycle. Other control points, known as checkpoints, maintain the order of events and integrity of the cell cycle. They operate as surveillance systems, for example during the S-phase, to ensure the cellular environment is favourable for continued progression through the cell cycle by limiting the detrimental effects of DNA damage and enabling repair mechanisms to be activated[6,7].

Importance of cyclins and cyclin-dependent kinases in the cell cycle

Molecules that play key roles in controlling cell-cycle progression are the cyclin-dependent kinases (CDKs; protein complexes that are composed of a regulatory cyclin subunit

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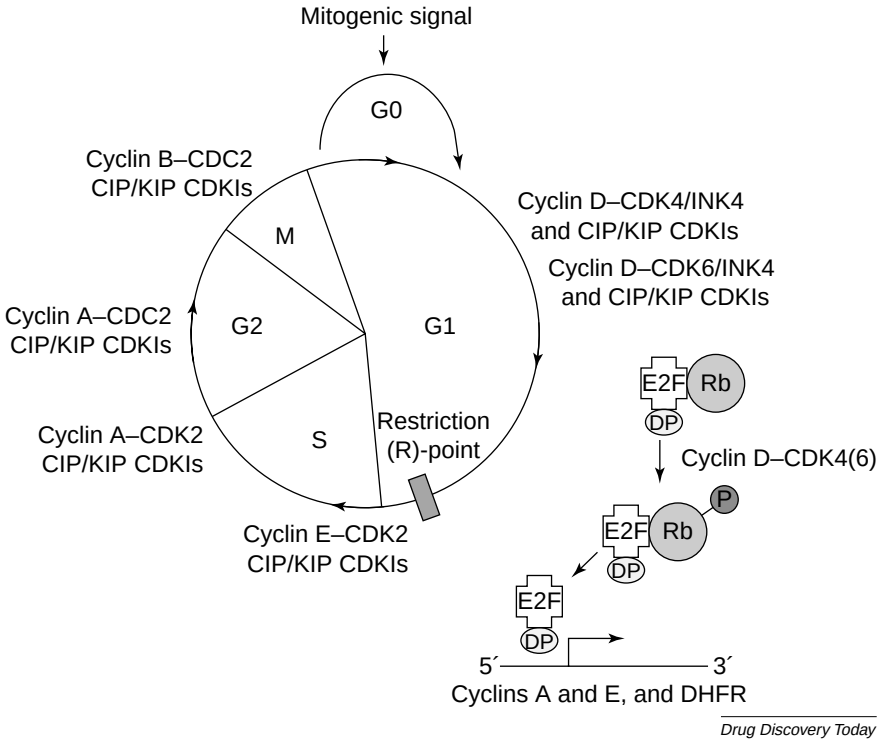

Figure 1. *The cell cycle. The normal cell division cycle is sub-divided into four distinct phases: two gap phases (G1 and G2) where RNA and protein synthesis occur, S-phase (DNA synthesis) and M-phase (mitosis, where the cell divides into two daughter cells). An additional gap phase, known as G0, exists in some cell types that undergo a stage of quiescence where RNA and protein syntheses are minimal. Each phase of the cycle is under the control of specific positive [cyclin–cyclin-dependent kinase (CDK) complexes] and negative [inhibitor of CDK4 (INK) and/or CDK2 interacting protein (CIP) and CDK inhibitory protein (KIP) CDKIs] regulators that either promote or arrest cell-cycle progression depending on their relative levels of expression. The restriction (R)-point is a position of control that functions in late G1. For a cell to pass the R-point, cyclin D–CDK4(6) complexes need to be activated (e.g. following a mitogenic signal being received at the cell surface) to phosphorylate the retinoblastoma (Rb) protein that normally is found in a hypophosphorylated form bound to the transcription factor, E2F – that itself comprises the subunits, E2F and the heterodimeric partner proteins, DP. Phosphorylation of Rb (shown as P) leads to its dissociation from E2F, the release of transcriptionally active E2F and the synthesis of genes necessary for S-phase progression, e.g. cyclins A and E and dihydrofolate reductase (DHFR).*

and a catalytic partner), their substrate proteins and the CDK inhibitors (CDKIs). Most cyclins show dramatic fluctuations in their expression during the cell cycle. For example, cyclin E levels peak during the G1/S-phase and rapidly disappear thereafter, whereas cyclins A and B accumulate transiently at the onset of the S-phase and in the late G2-phase, respectively, which is followed by their degradation[1,2]. By contrast, expression of the various CDK molecules remains relatively constant throughout the cell cycle[2].

Cyclins form complexes with specific CDKs at distinct points in the cell cycle to phosphorylate target proteins and promote cell-cycle progression. By contrast, CDKI molecules bind to, and inhibit the activities of, specific cyclin–CDK complexes, thereby causing cell-cycle arrest[4,8] (Fig. 1). CDKI molecules are categorized into two separate families, the CDK2-interacting protein (CIP)/CDK-inhibitory protein (KIP)-family comprising $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$, and the inhibitor of CDK4 (INK4)-family comprising $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$. Members of the CIP/KIP family are structurally unrelated to the INK4 family and, *in vitro* at least, the CIP/KIP family shows broad kinase specificity whereas INK4 family members only inhibit CDK4 and CDK6 (Refs 4,8).

The importance of the various cyclins and CDKI molecules in controlling normal cellular growth and differentiation has been demonstrated in knockout mouse studies, a technique that allows the endogenous gene to be genetically inactivated through homologous recombination[9]. For example, *Cyclin D1*-knockout mice display a marked defect in breast epithelial development during pregnancy[10], suggesting that cyclin D1 plays a significant role in regulating proliferation of breast cells[10,11]. In addition, mice carrying a targeted deletion of the *Ink4A* gene are viable but develop spontaneous tumours at an early age[12], and mice that carry a deficiency in the *Kip1* gene are larger than their wild-type litter mates and have a proportional enlargement of many organs of the body[13–15]. In a similar manner to *Ink4A* deletion, homozygous *Kip1*-knockout mice develop premature tumours leading to early death[13,14].

Proteins that act downstream of, and are substrates for, the cyclin–CDK complexes are critical for controlling cell-cycle progression. Of particular importance is a family of proteins (referred to as pocket proteins) exemplified by the first member of the family, the tumour suppressor protein, retinoblastoma protein (pRb). The phosphorylation status of pRb is important for controlling progression of normal cells through the G1-phase as hypophosphorylated pRb inhibits cell-cycle progression, whereas phosphorylation of pRb by cyclin D-dependent kinases enables cells to enter the S-phase[5] (see Fig. 1). Other members of the pocket protein family, p107 and p130, also serve as substrates for the G1-phase acting cyclin D–CDK4 and cyclin D–CDK6 complexes[5].

Importance of E2F and p53 transcription factors in the cell cycle

Another important group of cell-cycle regulatory proteins are the E2F transcription factors, which form complexes with pRb-family pocket proteins[5,16,17]. Phosphorylation of the pocket protein by cyclin D-dependent kinase leads to dissociation of the pocket protein from E2F, enabling the transcription of genes necessary for S-phase progression. A large number of E2F responsive genes are known, including dihydrofolate reductase (*DHFR*), *CYCLIN E* and *CYCLIN A* (Refs 5,16,17) and, in general, it is believed that most E2F regulated genes encode proteins required for cell-cycle progression. In cells, E2F comprises a family of at least six distinct DNA binding activities, composed of the proteins E2F1 to E2F6, together with heterodimeric partner proteins, termed DP[5,16,18].

Other molecules that act to regulate the cell cycle include p53, which is a tumour suppressor that functions in DNA-damage checkpoint control by causing cell-cycle arrest[19–21]. The *p53* gene is mutated in approximately half of the human tumours, and is likely to be one of the most frequently altered genes in cancer cells. In normal circumstances, the p53 protein remains in a latent state but, following genotoxic or other forms of cellular stress, post-transcriptional mechanisms result in an accumulation of the p53 protein, leading to cell-cycle arrest or apoptosis, contributing to the suppression of the malignant disease[19,20]. A primary function of p53 is to act as a sequence-specific transcription factor[21,22], and this is attributed to its capacity to limit proliferation. This has led to the identification of a variety of target genes that influence cell growth, including *Waf1/Cip1*, *Bax*, *Mdm2* and *Gadd45* (Ref. 19). Mutations in p53 that occur in human tumour cells frequently affect the crucial regions encoding the DNA binding domain, thus inactivating p53 as a sequence-specific transcription factor[23] and supporting a role for transcriptional control in mediating the biological consequences of p53 activation.

The importance of these molecules in controlling cellular growth has been demonstrated in a series of over-expression and knockout studies. Thus, $Rb^{-/-}$ mice are non-viable, showing defects in neurogenesis and haematogenesis. However, transfer of the human *RB* mini-transgene into mutant mice rescues this phenotype, thereby demonstrating that pRb is essential for normal mouse development[24]. In contrast to $Rb^{-/-}$ mice, $p53^{-/-}$ mice appear normal but are prone to the spontaneous development of a variety of tumours[25]. The importance of E2F activity for cell-cycle progression has been demonstrated by a variety of approaches, such as the over-expression of the *E2f1* gene in quiescent cells, which causes cells to enter the S-phase[26]. However, in knockout mice, deletion of the *E2f1* gene leads to reduced levels of apoptosis[27], and in older animals, an increased incidence of tumours[28]. These results suggest that E2F not only promotes proliferation, but in some circumstances can act negatively. Studies such as these have demonstrated the crucial importance of cell-cycle regulators in modulating normal cell growth and the impact that deregulation of these molecules can have in proliferative disease. The ability to target the expression and activity levels of these molecules offers new hope for the treatment of proliferative disorders.

Why target the cell cycle?

Proliferative disorders such as cancer are recognized as diseases of the cell cycle. It has generally been found that in tumour cells, the mechanisms that normally function to restrain cell division are defective, whilst those that promote division become more active. The genes responsible for these changes in growth potential are generally named the tumour suppressors and the oncogenes, respectively.

The significance of such events for human cancer is underscored by the almost invariable mutational events that disrupt the pRb or p53 pathways of cell-cycle control. Loss of pRb function is believed to promote abnormal proliferation as a result of the deregulation of the E2F transcription factors[5,16,17,23], followed by increased expression of the S-phase specific genes. Both positive (cyclins and CDKs) and negative (CDKIs) regulators of the cell cycle that function upstream of pRb, can also be aberrantly controlled during proliferative disease. For example, cyclin D1, CDK4 and the CDKI molecules, $p15^{INK4B}$ and $p16^{INK4A}$, are subject to mutational events in human tumour cells[11,29,30].

Table 1. Examples of cell-cycle molecules that are recognized targets for the treatment of proliferative diseases

Cell-cycle targets	Type of proliferative disease	Refs
$p16^{INK4A}$, $p27^{KIP1}$	Cardiovascular disease	4,8,52,64,65
p53, CDK inhibitors	Cancer, cardiovascular disease, psoriasis	19–21,29,43, 46,47,68–73, 84,85
pRb	Cancer, cardiovascular disease	4,29,64,66
CDK molecules	Cancer, cardiovascular disease, fungal infections	4,6,8,29,43
Cyclin D1/ER	Breast cancer	11,31–33
E2F	Cancer, cardiovascular disease, psoriasis	4,56–61

Cell-cycle regulatory molecules are pivotal in the modulation of aberrant cellular proliferation, and it has been argued that they provide ideal targets for therapy for a range of proliferative disorders (Table 1). Obviously, the therapeutic value of these interventions is reliant upon the fact that aberrantly proliferating cells are more sensitive to such interventions than normal cells. One way of minimizing side-effects would be to deliver the therapeutic agent locally and specific examples of these approaches will now be discussed.

For which diseases will cell-cycle drugs be of maximum benefit?

Cancer

It is clear that major diseases where there are unmet clinical needs often have aberrant cell-cycle control as a critical component of their pathology. Cancer is the cell-cycle disease *par excellence*. Invariably, components of the cell cycle are deregulated in cancer (see Table 2) resulting in uncontrolled cellular proliferation. For example, although G1/S-phase control mechanisms are aberrant in almost all human tumours, certain types of malignancies result from the preferential inactivation of one particular component of the pathway[29]. Inactivation of a specific cell-cycle gene theoretically allows tumour-specific events to be targeted by reinstating the expression and/or activity of the affected gene to levels found in normal tissues. This would have significant advantages over conventional therapies for cancer, which are not usually tissue- or cell-type specific and are frequently highly toxic to non-tumourigenic cells.

For example, carcinoma of the breast is the most common malignancy in women, accounting for approximately 20% of all female cancer deaths in the UK [*Martindale: The Extra Pharmacopoeia* (1999) (32nd edn), The Pharmaceutical Press]. Breast cancer is often hormone-regulated, being dependent on the stimulation of the oestrogen receptor (ER), a transcription factor of crucial importance for the growth of breast epithelium[11]. Up to 50% of breast carcinoma cells show increased expression of the *CYCLIN D1* gene[31–33]. The relevance of *CYCLIN D1* over-expression in this context is highlighted by the finding that tissue-specific expression of *CYCLIN D1* in transgenic mice produces mammary hyperplasia and adenocarcinoma[34], whereas *Cyclin D1*-knockout mice display a marked defect in breast development during pregnancy[10]. Moreover, cyclin D1 is over-expressed in ER-positive breast cancers with the percentage of cyclin D1-positive cells being especially high in node-negative ER-positive tumours, probably as a consequence of cyclin D1 induction by oestrogens in steroid-responsive tumours[33]. Additional evidence has suggested that at least part of the oncogenic activity of cyclin D1 is derived from interacting with and activating ER independently of functioning as a regulatory subunit of CDK4 kinase[11,35]. Thus, approaches that might be taken to limit ER-dependent breast cancer include targeting cyclin D1 expression levels directly or inhibiting the interaction of ER with cyclin D1 (Fig. 2).

Cardiovascular disease

Atherosclerosis and restenosis are together responsible for more than 50% of all deaths in the Western world and are the major causes of heart attack, stroke and gangrene of the extremities[36,37]. Whereas atherosclerosis can take several years to develop, restenosis can occur within one to six months following the unsuccessful treatment of a primary atherosclerotic plaque by procedures such as percutaneous transluminal coronary angioplasty (PTCA)[38]. It is estimated that between 30–50% of the approximately one million angioplasties performed each year will fail within the first six months because of the formation of a restenotic lesion[39]. To overcome this problem, stents are increasingly being used in PTCA procedures, and this has led to a reduction in the number of restenoses down to 15–25%. Of considerable importance is the additional problem caused by insertion of a stent, whereby the stent itself produces a proliferative reaction that leads to re-occlusion of the affected vessel, named in-stent stenosis. The number of patients presenting with in-stent stenosis is increasing such that more than 20% of patients who receive a stent are likely to be affected within 3–6 months of treatment[40]. One of the major mechanisms responsible for both restenosis and in-stent stenosis is hyperproliferation

Table 2. Examples of aberrations in cell-cycle genes that might alter cancer susceptibility

Syndrome	Cell-cycle gene	Primary tumour involved	Associated tumour traits	Refs
Ataxia telangiectasia	*ATM*	None	Leukaemia, lymphoma	86
Familial retinoblastoma	*RB*	Retinoblastoma	Osteosarcoma	29
Li–Fraumeni syndrome	*p53*	Sarcomas, breast cancer	Brain tumours, leukaemia	29
Wiedmann–Beckwith syndrome	*$p57^{KIP2}$*	Wilm's tumour	Organomegaly	29
Familial melanoma	*$p16^{INK4A}$*	Melanoma	Pancreatic cancer, dysplastic nevi, atypical moles	29
Mantle cell lymphoma	*$p15^{INK4B}$*, *$p16^{INK4A}$*, *CDK4*, *RB*, *p53*	Lymphoma	Not known	87
Not known	*E2F4*	Gastrointestinal tumours	Not known	83
Not known	*$p27^{KIP1}$*, *CYCLIN E*	Breast cancer	Not known	88
Not known	*CDC2*	Leukaemia	Not known	89

Abbreviations: ATM, ataxia telangiectasia gene mutation; CDC, cell-division cycle; CDK, cyclin-dependent kinase; RB, retinoblastoma.

of vascular smooth muscle cells (VSMCs). Thus, approaches to target VSMC proliferation and/or promote VSMC apoptosis offer therapeutically viable treatment regimes.

Fungal diseases

The numbers and types of fungal infections affecting the human population have increased in the past decade because of the emergence of widespread resistance to conventional antifungal drugs, such as ketoconazole, miconazole and fluconazole[41,42]. This has prompted a continued search for new therapeutic options to treat azole-resistant fungal diseases. A therapeutic strategy that might offer such an alternative is to target the cell cycle. In fact, much of the early work that led to our current understanding of the eukaryotic cell cycle was conducted in experimental yeasts and this has remained the model system of choice for many investigators[1,6]. Accordingly, our understanding of the yeast cell cycle is considerable and the fact that these molecules and their substrates are conserved in pathogenic fungi provides a suitable platform for the development of drugs that modulate the cycle in infectious diseases caused by, for example, *Candida*, *Aspergillus* and *Tinea*.

Viral diseases

Certain types of cancers are associated with infection by specific viruses[43,44]. For example, high-risk human papillomavirus (HPV) is frequently associated with cervical carcinoma[45]. Such viruses override growth-suppressive signals that control cell-cycle progression in non-transformed cells, causing the activation of growth-promoting genes such as *CYCLIN E*, *CYCLIN A*, *CDC25A* and *CDK2* (Ref. 43). Furthermore, complexes between E2F and pRb are disrupted by HPV16 E7 leading to transcriptionally active E2F (Ref. 44), whilst HPV16 E6 can promote the degradation of p53 (Ref. 43). It has also been demonstrated that functions of the CDKI molecules, $p21^{CIP1}$ and $p27^{KIP1}$, are inactivated by interaction of these proteins with the HPV16 E7 oncoprotein[46,47], which uncouples cellular differentiation and proliferation in human keratinocytes by abrogating $p21^{CIP1}$-mediated inhibition of CDK2 activation[48]. The protein domains involved in these interactions are known and, by designing small molecules to block these interactions, it might be possible to inhibit the oncogenic activity of E7 by releasing molecules such as pRb and $p21^{CIP1}$.

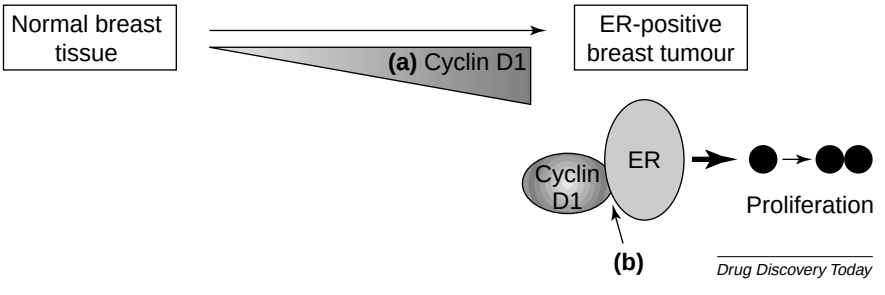

Figure 2. *Cyclin D1 as a possible therapeutic target for the treatment of breast cancer. Studies have shown that cyclin D1 is over-expressed in oestrogen receptor (ER)-positive breast cancers and evidence has suggested that cyclin D1 derives at least part of its oncogenic activity by physically interacting with, and activating, ER. Thus, strategies that could be taken to limit ER-dependent breast cancer include (a) inhibiting cyclin D1 mRNA expression with antisense oligodeoxynucleotides and/or (b) inhibiting the function of cyclin D1 as an activator of ER by blocking the cyclin D1–ER interaction.*

Table 3. Examples of agents that target the cell cycle that are in, or are close to being in clinical trials

Drug	Cell-cycle arrest	Mechanism of action	Clinical trial	Refs
Paclitaxel (Taxol®)	G2/M	Stabilizes microtubules and inhibits depolymerization back to tubulin	Breast cancer*, ovarian cancer*, non-small cell lung cancer, prostate cancer, leukaemia, lymphoma	74,90
Docetaxel (Taxotere®)	G2/M	Stabilizes microtubules and inhibits depolymerization back to tubulin	Ovarian cancer, paediatric tumours, lung cancer, melanoma, prostate, bladder and cervical cancers, non-Hodgkin's lymphoma	74,90
Camptothecin (Topotecan, CPT11, SN38, GI147211)	G2/M	Topoisomerase I inhibitor	Paediatric malignancies (neuroblastoma, rhabdomyosarcoma), ovarian cancer, metastatic breast cancer, advanced colorectal cancer	74,91–93
Flavopiridol	G1, S, G2/M	General CDK inhibitor Downregulates *BCL-2* mRNA and protein expression	Phase 1 trials in patients with refractory malignancies	79
Tyrphostins (AG490, AG494)		Inhibits CDK2 activation	Potential for treatment of a variety of proliferative diseases	81,82
E2F decoy ODN	G1	Inhibits E2F DNA binding and transcriptional activity	Prevention of infrainguinal bypass graft failure	57,58
AdCMV-p53	G1	Adenovirus carrying wild-type *p53* gene	Non-small cell lung cancer, local and metastatic head and neck squamous cell carcinoma	69,94
ONYX-015	Cell lysis	Replicates in $p53^{-/-}$ tumour cells	Head and neck cancer, pancreatic cancer, ovarian cancer	70

*In clinical use. Abbreviations: BCL, B-cell leukaemia; CDK, cyclin-dependent kinase; CMV, cytomegalovirus; ODN, oligodeoxynucleotides.

Validation of the cell cycle as a target for drug discovery

In addition to conventional pharmaceutical approaches (Table 3), a number of techniques are now being developed that directly alter the expression and activities of genes. Some of these techniques, such as the use of antisense oligodeoxynucleotides (ODNs), peptides and gene transfer, can be applied to the cell cycle.

Antisense ODNs

Major abnormalities of CDK expression and regulation have been described in human tumours[49] and during VSMC proliferation leading to restenosis[50–52]. Animal studies in the rat carotid artery balloon-injury model using antisense ODNs directed against either cell-division cycle protein 2 (CDC2), CDK2 or proliferating cell nuclear antigen (PCNA), have shown a significant reduction in neointimal formation up to eight weeks following balloon injury[50,51]. The efficiency of delivery of these ODNs was improved significantly by incorporating them into liposomes with viral coat proteins, for example those originating from the Haemagglutinating Virus of Japan (HVJ). Thus, Dzau and colleagues have demonstrated that intra-luminal delivery of antisense phosphorothioate ODNs directed against CDK2 or CDC2 significantly inhibited neointimal formation compared with injured vessels treated with sense ODNs or left untreated[51]. Furthermore, the combination of two antisense ODNs (CDK2 and CDC2) abolished neointimal formation completely. This therapy has also been shown to prevent atherosclerosis in rabbit jugular vein grafts[53,54] and an antisense ODN to CDK2 was found to prevent coronary graft arteriosclerosis for up to 30 days following cardiac transplantation into mice[55].

Morishita and colleagues have shown that VSMC proliferation is inhibited both *in vitro* and *in vivo* by a double-stranded decoy ODN sequence that competes for E2F DNA binding activity[56]. A 14-mer ODN, containing the 8-bp DNA binding site for E2F taken from the c-*myc* promoter, and a 30-mer ODN, containing the 8-bp binding site from the adenovirus E2A gene promoter, inhibited the

growth of rat aortic VSMCs in culture in a concentration-dependent manner and blocked the formation of intimal hyperplasia for up to eight weeks in rat carotid arteries injured with a balloon catheter. The effects of this E2F decoy approach have now been evaluated in a large-scale clinical trial in the US, involving 2000 patients undergoing vascular bypass graft operations[57]. This prospective, randomized, double-blind clinical trial involved transfecting the vein grafts *ex vivo* with the decoy sequence prior to transfer into the patient, and tested the efficacy of intraoperative vein graft engineering via E2F decoy ODN transfection in preventing infra-inguinal bypass graft failure. The preliminary results obtained from a small group of patients indicated that treatment of vein grafts with an E2F decoy ODN could decrease the levels of cell-cycle regulatory gene expression in vascular cells[58]. However, whether this approach will lead to a clinical benefit in terms of a reduction in graft failure rate remains to be determined.

The E2F decoy ODN strategy has also been used by two independent groups of investigators to examine the effectiveness of either a 14-mer ODN (Ref. 59, as already described) or a 25-mer ODN (Ref. 60) as inhibitors of mesangial cell proliferation, both *in vitro* and *in vivo*. Mesangial cell proliferation is a characteristic of several types of glomerulonephritis and is central to the development of glomerulosclerosis and renal failure. Thus, strategies aimed at limiting the progression of glomerular diseases would provide benefit for a substantial number of patients. Both E2F decoy ODNs inhibited the growth of human mesangial cells *in vitro* as a result of inhibiting E2F activity[59,60], whilst the 25-mer ODN additionally suppressed the proliferation of mesangial cells in a rat glomerulonephritis model[60].

Peptides

A region of the DP protein that is essential for binding to E2F, named the DP/E2F homology region (DEF) box, has been used to design specific inhibitors of heterodimer formation[61]. Active peptides that inhibit the DNA binding activity of the E2F heterodimer both *in vitro* and *in vivo* prevent cellular proliferation in various cell types from both rodents and humans[61]. Studies to evaluate the effectiveness of these peptides in a variety of relevant cell-based and animal models of restenosis will demonstrate their therapeutic potential for the treatment of this disease *per se*. Furthermore, these peptides could be used as a coating for stents, where they would be released directly into the damaged vessel wall to inhibit the intimal hyperplasia that occurs shortly after PTCA and stent implantation.

In addition, peptides containing a short motif representing a docking site for CDK2 kinase complexes taken from the E2F protein can inhibit the phosphorylation of substrates by cyclin A–CDK2 and cyclin E–CDK2. When these peptides are introduced into cells, only the transformed tumour cells undergo apoptosis[62]. Importantly, deregulated E2F expression was found to sensitize cells to the inhibitory effects of the peptides, suggesting that the regulation of E2F activity is instrumental in influencing the effects of the peptides.

Synthetic peptides, based on the carboxy-terminal sequence of $p21^{CIP1}$, have been described that bind to and inhibit the activity of cyclin D1–CDK4 complexes[63]. These peptides display *in vitro* IC_{50} values in the nanomolar range and, when introduced into cells, inhibit the phosphorylation of pRb and induce a potent G1/S-phase growth arrest. The development and effects of these small and potent peptides that inhibit cell-cycle targets underscores the potential importance of these targets in drug discovery.

Gene transfer

$p16^{INK4A}$, $p21^{CIP1}$, $p27^{KIP1}$. Both $p21^{CIP1}$ and $p27^{KIP1}$ are transiently and differentially downregulated during the log-phase of intimal hyperplastic growth that occurs following balloon injury in the rat carotid artery[52]. Furthermore, $p16^{INK4A}$ is not present in normal porcine arteries but is induced transiently following balloon injury, such that maximal levels of this CDKI are achieved seven days after intervention[52]. Retroviral and adenoviral gene transfer of these CDKI molecules into the pig coronary artery at the time of balloon injury lead to a dramatic inhibition of VSMC proliferation and neointimal formation[4,52,64]. These encouraging results have sparked interest in exploring the effectiveness of these molecules against restenosis and other disorders in clinical trials[65].

pRb. The tumour suppressor gene product, pRb, has also been proposed as a candidate to inhibit neointimal formation following balloon angioplasty[64]. Transfection of cultured rat VSMCs with an adenoviral vector expressing a constitutively active form of pRb inhibited cellular proliferation significantly[66]. Furthermore, local transfection of activated pRb into balloon-injured rat carotid and porcine femoral arterial walls inhibited neointimal formation by up to 45–50% as compared with arterial walls transfected with only the vector[66]. Although clinical trials have not been initiated, over-expression of pRb in human VSMCs during PTCA could limit the subsequent restenotic process.

p53. The p53 protein is a nuclear transcription factor that plays a crucial role in controlling the cellular response to stress[19–21], and almost 50% of human cancers contain a p53 mutation[67]. Studies in mammalian cells have shown that p53 acts as a potent tumour suppressor and restores some level of normal growth to cancerous cells *in vitro*[68]. The crucial role that p53 plays in limiting aberrant cell growth has led to a recent clinical trial examining the effects of a recombinant p53 adenovirus on nine patients whose conventional treatments for lung cancer had failed[69]. The results were promising with tumour regression being noted in three patients and tumour growth stabilized in three others. Interestingly, apoptosis was more frequent in post-treatment biopsies than in pre-treatment biopsies.

An alternative potential therapeutic strategy to prevent cancer cell growth that relies upon p53 has been to use the 55 kDa protein from the E1B-region of the adenovirus that binds to, and inactivates, p53 (Ref. 70). In a recent study, it was shown that normal human cells expressing wild-type p53 are highly resistant to an adenovirus lacking E1B (termed ONYX-015), but that a wide range of human tumour cells were efficiently destroyed by such a modified adenovirus[70]. Furthermore, intratumoural or intravenous administration of ONYX-015 to nude mouse–human tumour xenografts demonstrated potent anti-tumoural activity. Although ONYX-015 has entered clinical trials for the treatment of p53-deficient cancers, recent studies have shown that the multiplicity of infection influences the outcome of infecting cells[71,72].

Targeting the *p53* gene could also be valuable in the treatment of restenosis. For example, Yonemitsu *et al.* have shown that HVJ-liposome-mediated transfer of human wild-type p53 cDNA into bovine aortic VSMCs *in vitro* leads to a decrease in thymidine incorporation and in the S-phase, and these responses are associated with a transient increase in the G2/M-phase two days after transfection. Most of the cells arrested in the G1-phase by five days after transfection, with no evidence of apoptosis[73]. Thus, p53 might represent a valuable and potent therapeutic agent for the treatment of a variety of human tumours and other proliferative diseases.

Conventional drug therapy

The discovery of inhibitors of the cell cycle such as paclitaxel, olomoucine and flavopiridol (see Table 3) has led to rapid advances in the investigation and design of a variety of anti-mitotic compounds that are either being used clinically or have the potential for development as anti-proliferative agents. Although many of these compounds fail to exhibit specificity and possess widespread toxicity, they do validate cell-cycle control as a relevant drug target.

Paclitaxel. Paclitaxel is a natural product obtained from the bark of the Pacific Yew tree, *Taxus brevifolia*, and can now be produced by a semi-synthetic route thereby saving considerably on production costs[74]. Both paclitaxel and the analogue, docetaxel, are in use clinically or are in clinical trials for a variety of cancers. In addition to inhibiting the growth of a variety of cancer cells, paclitaxel is reported to inhibit medial VSMC proliferation and neointimal VSMC accumulation in the rat carotid artery balloon injury model[75]. Indeed, this effect was shown to occur at plasma levels approximately two orders of magnitude lower than that used clinically to treat human malignancy, suggesting that paclitaxel might be of therapeutic value in preventing human restenosis with minimal toxicity. Paclitaxel is also under investigation as a coating for stents for the treatment of restenosis and/or in-stent stenosis[76].

The main mechanism of action of paclitaxel and docetaxel is to inhibit DNA synthesis and cause cell-cycle arrest in the G2/M-phase of the cycle as a result of tubulin polymerization and the formation of stable microtubules[74]. Stable microtubules are resistant to disassembly, eventually leading to cell death by apoptosis from the activation of the mitotic checkpoint[74]. However, despite promising clinical results, side-effects with paclitaxel and docetaxel treatment can be severe, with the most significant being depression of the bone marrow stem cells. Thus, there is an increasing need to find alternative, less toxic and more specific inhibitors of the cell cycle.

Olomoucine. Olomoucine is a selective inhibitor of the cell-cycle kinases, CDC2, CDK2 and CDK5, and exhibits inhibitory effects on the mitogen-activated protein kinase pathway enzyme, ERK1 ($IC_{50} \approx 30\ \mu M$), but is a poor inhibitor of CDK6 and CDK4 (Ref. 49). Exposure of cells to olomoucine leads to cell-cycle arrest both in the late G1-phase and at the G2/M-phase transition. An analogue of olomoucine, called roscovitine, exhibits a tenfold higher level of inhibition for both CDC2 and CDK2 compared with olomoucine[77]. Both agents might be of therapeutic benefit in the treatment of a variety of human malignancies and other proliferative disorders that occur as a consequence of overexpression of CDC2, CDK2 and/or CDK5, although widespread toxicity will remain a significant problem.

Flavopiridol. Flavopiridol is a novel, potent inhibitor of the CDKs (Table 3). It is a synthetic flavone that exhibits antitumour activity in murine and human tumour cell lines both *in vitro* and *in vivo*[78,79]. Flavopiridol is currently undergoing Phase I evaluation in patients with refractory malignancies with prior disease progression[79].

Tyrphostins. The tyrphostins are a group of protein tyrosine kinase inhibitors that are effective anti-proliferative agents and as such, are potential drugs for the treatment of certain cancers, psoriasis and restenosis[80]. Certain tyrphostins that block epidermal growth factor (EGF)-receptor phosphorylation in cell-free systems fail to do so in intact cells despite inhibiting both EGF- and serum-induced cell growth and DNA synthesis[81,82]. It has subsequently been demonstrated that these latter tyrphostins selectively inhibit CDK2 activity leading to a potent G1/S-phase arrest[83]. However, the precise mechanism of action of these CDK2 inhibitors remains unclear, although they do not affect the association of CDK2 with cyclin E, cyclin A, $p21^{CIP1}$ or $p27^{KIP1}$. These inhibitors might therefore represent a novel class of inhibitors of cellular proliferation that target CDK2 activation.

Concluding remarks

It is clear from the examples described above that targeting the cell cycle as an approach to developing novel, specific and perhaps more effective treatments for proliferative disease could offer significant advantages over current therapies. In particular, it is now understood that the cell-cycle control machinery is frequently altered in hyperproliferative cells such that regulatory mechanisms and checkpoint controls are either overcome or disabled. Inhibiting the activities of crucial cell-cycle molecules that are aberrantly regulated in these disease situations provides an exciting and plausible strategy for treating such disorders.

However, it should be noted that, whilst cell-cycle targets for treating proliferative diseases offer exciting therapeutic opportunities, much work remains to be done in validating their potential as drug targets. For example, how will normal cells be affected by a cell-cycle drug? Recent evidence, however, suggests that this might not necessarily be as problematic as envisaged. Thus, small peptides that block the interaction of cyclin A–CDK2 kinase with its substrates, such as E2F1, only have dramatic apoptotic effects in tumour cells[62]. Given the generality of cyclin A–CDK2 kinase, and its role in cell-cycle control in normal cells, this somewhat surprising result does imply that in the longer term, cell-cycle drugs will prove to be viable therapeutic entities.

Whilst still being at the beginning of a new era in drug discovery, it is clear that the information that is accumulating concerning the basic mechanisms that govern the cell cycle offers new hope and promise for developing a novel class of future medicines that specifically target aberrant proliferation. In this respect, there can be little doubt of the value of targeting the cell cycle in drug discovery.

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In short…

The Imperial College of Science and Technology and Medicine (London, UK) has launched a new Government report entitled *The Biotechnology Clusters Report*, which sets out a ten-point action plan to help the UK's biotechnology industry become a major global force. The Science Minister, Lord Sainsbury presented the report, which has been based on information gathered from a fact-finding mission to identify the limitations and success factors in major UK and US biotechnology clusters. These ten critical factors for successful biotechnology cluster development were identified as:

- Strong science base
- Entrepreneurial culture
- Growing company base
- Ability to attract key staff
- Business support services and large companies in related industries
- Availability of finance
- Skilled workforce
- Effective networking
- Supportive policy environment
- Premises and infrastructure.