The history and future of targeting cyclin-dependent kinases in cancer therapy

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Abstract | Cancer represents a pathological manifestation of uncontrolled cell division; therefore, it has long been anticipated that our understanding of the basic principles of cell cycle control would result in effective cancer therapies. In particular, cyclin-dependent kinases (CDKs) that promote transition through the cell cycle were expected to be key therapeutic targets because many tumorigenic events ultimately drive proliferation by impinging on CDK4 or CDK6 complexes in the G1 phase of the cell cycle. Moreover, perturbations in chromosomal stability and aspects of S phase and G2/M control mediated by CDK2 and CDK1 are pivotal tumorigenic events. Translating this knowledge into successful clinical development of CDK inhibitors has historically been challenging, and numerous CDK inhibitors have demonstrated disappointing results in clinical trials. Here, we review the biology of CDKs, the rationale for therapeutically targeting discrete kinase complexes and historical clinical results of CDK inhibitors. We also discuss how CDK inhibitors with high selectivity (particularly for both CDK4 and CDK6), in combination with patient stratification, have resulted in more substantial clinical activity.

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Fundamentally, the cell cycle process is conserved from unicellular eukaryotes to complex metazoans[1], and distinct phases of the cell cycle are responsive to physiological cues that dictate the appropriateness of cell division. Cyclin-dependent kinases (CDKs) are critical regulatory enzymes that drive all cell cycle transitions[1–6], and their activity is under stringent control to ensure successful cell division. In particular, all mitotic cell division requires that faithful DNA replication occurs in S phase and that the requisite machinery to divide chromosomes is in place during mitosis, leading to the production of daughter cells. In unicellular eukaryotes, cell cycle progression is predominantly controlled by the availability of nutrients to ensure the completion of successful duplication. Cell cycle progression in unicellular eukaryotes is also dependent on the absence of genetic damage that would preclude the viability of daughter cells. In multicellular organisms, more complex regulatory mechanisms that reflect cell–cell communication have evolved.

Many of the key concepts of CDK biology (FIG. 1) were discovered >20 years ago through the study of yeast and the synchronous cycles of division seen in embryo extracts; indeed, the findings from studies led to the award of a Nobel Prize for these researchers[7,8]. In particular, CDK1 emerged as a key determinant of mitotic progression, and CDK2 emerged as being more relevant for DNA replication in higher eukaryotes. In metazoans, much of the control over cell cycle entry is elicited at the level of CDK4 and CDK6, which are responsive to numerous growth regulatory signals. Subsequently, in addition to the CDKs that directly promote cell cycle progression (for example, CDK4, CDK6, CDK2 and CDK1), an additional family of CDKs that regulate transcription was identified, which include CDK7, CDK8 and CDK9 (REFS 3,9–11). CDKs with post-mitotic functions in specialized tissue settings, such as CDK5, were also identified. Owing to the central role of CDKs in the control of cell division, it is perhaps not surprising that all cancers exhibit some features that derange the normal controls over the cell cycle[12], and over the past 20 years, numerous drugs that target CDK activity have emerged and have been tested in the clinic. Here, we review the biology of CDKs and their suitability as therapeutic targets in cancer, the key mechanisms through which CDKs become deranged in cancer and the challenges that have, until recently, complicated attempts to bring CDK inhibitors through to successful clinical application.

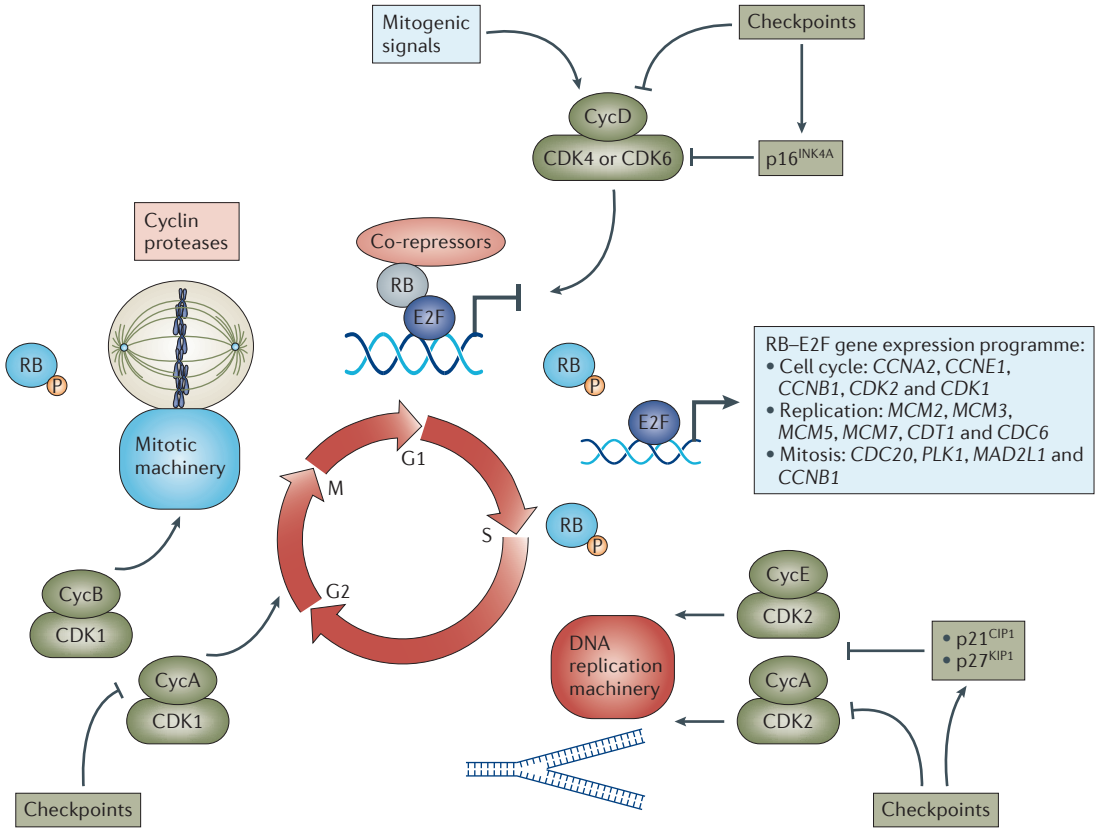


Figure 1 | **Progression of the cell cycle driven by CDKs.** Mitogenic signals stimulate cyclin-dependent kinase 4 (CDK4) and CDK6 and promote entry into the cell cycle, whereas antiproliferative checkpoints inhibit CDK4 and CDK6 activity or induce the expression of the CDK4 and CDK6 inhibitor $p16^{INK4A}$. Active CDK4 and CDK6 complexes initiate the phosphorylation (P) of key substrates, including the tumour suppressor retinoblastoma protein (RB), thereby unleashing a gene expression programme that is coordinated by the E2F family of transcription factors. In this context, CDK4 and CDK6 initiate transcription and stability of E-type and A-type cyclins (CycE and CycA, respectively) and the subsequent activation of CDK2 that contributes to the further phosphorylation of RB and the initiation of DNA replication. Further checkpoints can directly inhibit CDK2 activity or induce the CDK-interacting protein/kinase inhibitory protein (CIP/KIP) class of inhibitors ($p21^{CIP1}$ and $p27^{KIP1}$) that bind to and inhibit CDK2–cyclin complexes. With the completion of DNA replication, CDK1–Cyc A and CDK1–Cyc B complexes form to phosphorylate targets in G2 phase. In the absence of DNA damage and following appropriate preparation for chromosomal segregation, the cellular default is to activate CDK1–CycB complexes and progress into mitosis. However, there are potent checkpoints that limit CDK1 activity and halt mitotic progression. Subsequent degradation of CycB is required for anaphase progression and the production of two daughter cells in G1 phase of the cell cycle. During this transition from M phase back into G1 phase, RB is dephosphorylated, and the cycle is once more responsive to mitogenic and antiproliferative signalling. *CCN*, cyclin; *CDC*, cell division cycle; *CDT1*, chromatin licensing and DNA replication factor 1; *MAD2L1*, MAD2 mitotic arrest deficient-like 1; *MCM*, minichromosome maintenance complex component; *PLK1*, polo-like kinase 1.

The biology of CDKs

Integration of multiple signalling pathways through control of CDK4 and CDK6 activation. An understanding of the biology of CDKs is critical to deciphering the clinical results seen with CDK inhibitors, particularly in regard to determining biomarker and combination strategies. In most adult tissues, the majority of cells exit the cell cycle with diploid DNA content and are maintained in a quiescent G0 state. Tissue maintenance involves cues that physiologically induce cell cycle entry in a highly regulated manner. The mechanisms through which cells initiate entry into the cell cycle have been comprehensively described. Extracellular signals — including those activated by peptide growth factors (for example, RAS, mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR)) and nuclear receptors (for example, the oestrogen receptor (ER) in mammary epithelia) — converge on the cell cycle to drive progression from G0 or G1 phase into S phase through regulation of the metazoan-specific CDK4 or CDK6 complex[2,3,12,13]. CDK4 and CDK6 emerged phylogenetically with the appearance of multicellular organisms, and are subjected to multiple levels of regulation to control the transition into S phase. CDK4 and CDK6 are structurally related proteins that harbour many biochemical and biological similarities, although most published studies have focused on CDK4 (REF. 14). CDK6 is particularly important in promoting the proliferation of haematological precursors[15,16].

The activity of CDK4 and CDK6 is primarily controlled by their association with D-type cyclins (that is, cyclin D1, cyclin D2 and cyclin D3)[17,18]. Among these, cyclin D1 is the best characterized. The expression of cyclin D1 is characterized as a 'delayed-early' response to mitogenic signalling, and intricate promoter and enhancer interactions control its transcription[19]. Although less well studied, cyclin D3 conforms to a similar pattern as cyclin D1, whereas the regulation of cyclin D2 remains more enigmatic, although cyclin D2 also drives proliferation in certain contexts[20–24]. The differential expression of paralogues of D-type cyclins is likely to reflect tissue-specific aspects of normal physiology, wherein different D-type cyclins are expressed to promote CDK4 or CDK6 activation[3,25].

In addition to the transcriptional regulation of CDK4 and CDK6, the stability, intracellular localization and association of cyclin D with CDK4 and CDK6 are tightly regulated (FIG. 2a). In particular, cyclin D1 is unstable and actively shuttles between the cytoplasm and the nucleus. Phosphorylation of threonine 286 on cyclin D1 actively promotes its export and ubiquitin-mediated degradation[26,27]. In contrast to other CDKs, for which cyclin association seems to occur relatively spontaneously, for CDK4 and CDK6 this process is regulated by multiple mechanisms[28]. The inhibitor of CDK4 (INK4) proteins, which include $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$, represent CDK4- and CDK6-interacting proteins that seem to solely function as inhibitors of CDK4 and CDK6 (REFS 3,29,30). The INK4 proteins weaken the binding of D-type cyclins to CDK4 and CDK6, and also interact with the catalytic domains of CDK4 and CDK6 to potently suppress kinase activity[14,31,32]. These proteins therefore negatively regulate CDK4 and CDK6 in response to stress conditions[33]. For example, $p16^{INK4A}$ is induced by multiple oncoproteins to counteract transformation. Moreover, under stress conditions associated with cellular ageing[34], overexpression of $p16^{INK4A}$ results in a profound G1 arrest of the cell cycle. Similarly, $p15^{INK4B}$ is induced by transforming growth factor-β-mediated suppression of epithelial cell proliferation[35]. CDK4 and CDK6, similar to other CDK proteins, are also subjected to phosphoregulation[36,37]. Thus, CDK4 and CDK6 serve as key nodes of integration downstream of multiple signalling pathways, in which their activation initiates progression into the cell cycle (FIG. 2a).

The association of D-type cyclins with CDK4 and CDK6 can induce kinase activity with a unique substrate spectrum compared with other CDKs[38]. In particular, CDK4 and CDK6 have a specific preference for the phosphorylation of the tumour suppressor retinoblastoma protein (RB) and the related proteins p107 (also known as RBL1) and p130 (also known as RBL2)[39,40] (FIG. 2b). RB, the first tumour suppressor identified, has been extensively studied[41,42]. The RB protein does not have catalytic activity but functions through the assembly of multiprotein complexes to control the cell cycle. In particular, RB can bind to the E2F transcription factors, recruit co-repressors and repress the transcription of target genes that are regulated by E2Fs[41,42] (FIG. 2b). The E2Fs regulate the expression of a set of genes involved in cell cycle control (for example, cyclin E (*CCNE*), *CCNA* and *CCNB1*), dNTP biosynthesis (for example, dihydrofolate reductase (*DHFR*), ribonucleotide reductase M1 (*RRM1*) and *RRM2*) and mitotic progression (for example, polo-like kinase 1 (*PLK1*), BUB1 mitotic checkpoint serine/threonine kinase (*BUB1*) and spindle checkpoint protein MAD2 (*MAD2*)). The phosphorylation of RB by CDK4 and CDK6 initiates an intricate process of phosphorylation-mediated disruption of RB function that releases E2F and initiates subsequent progression through the cell cycle (FIG. 2c). CDK4 and CDK6 also phosphorylate forkhead box protein M1 (FOXM1), leading to stabilization of FOXM1 as a further mediator in the expression of genes required for progression though mitosis[38] (FIG. 3).

Deregulation of the CDK4/6–RB–$p16^{INK4A}$ pathway in cancer. The CDK4/6–RB axis is critical to cell cycle entry; therefore, it is unsurprising that the vast majority of cancers subvert this axis to promote proliferation[2,42,43] (FIG. 4). Most oncogenes promote the induction of $p16^{INK4A}$ as an intrinsic break to deregulated proliferation[34,44–46]. Overexpression of $p16^{INK4A}$ ultimately engages RB to suppress growth and cell cycle progression, and promotes oncogene-induced senescence. Oncogene-induced senescence must be subverted to enable subsequent oncogenic proliferation, which occurs through two principal means in tumours: loss of $p16^{INK4A}$ or loss of RB[29,47]. Loss of $p16^{INK4A}$ uncouples the oncogenic stress from the suppression of CDK4 or CDK6 activity, whereas loss of RB deregulates downstream signalling in the cell cycle. Consistent with this model, RB is required for the cell cycle arrest mediated by $p16^{INK4A}$ (REFS 48,49). In addition, RB-negative tumours express super-physiological levels of $p16^{INK4A}$ and are therefore insensitive to additional expression of $p16^{INK4A}$ owing to the absence of RB[29].

A contrasting mechanism of deregulating the CDK4/6–RB axis is the direct oncogenic activation of CDK4 or CDK6 activity. Deregulated cyclin D1 protein expression, gene translocation and gene amplification are observed in many tumour types[50–54], and a plethora of functional data support the specific oncogenic activity of cyclin D1 (REFS 17,18,51). Furthermore, amplification of CDK4 and CDK6 is observed in several different types of cancer[55,56]. Importantly, the distinct mechanisms of pathway dysregulation are mutually exclusive and are frequently tumour type-specific. For example, RB loss is a hallmark of small cell lung cancer, deregulation of cyclin D1 is common in breast cancer, and loss of $p16^{INK4A}$ is particularly common in glioblastoma (FIG. 4).

Distal regulation of CDK2 and its deregulation in cancer. Although all CDKs have similarities, CDK2 is structurally and functionally related to CDK1 (REF. 3). CDK2 has a considerably broader substrate profile than CDK4 and CDK6, and it phosphorylates a large number of proteins involved in cell cycle progression (for example, $p27^{KIP1}$ and RB), DNA replication (for example, replication factors A and C), histone synthesis (for example, NPAT), centrosome

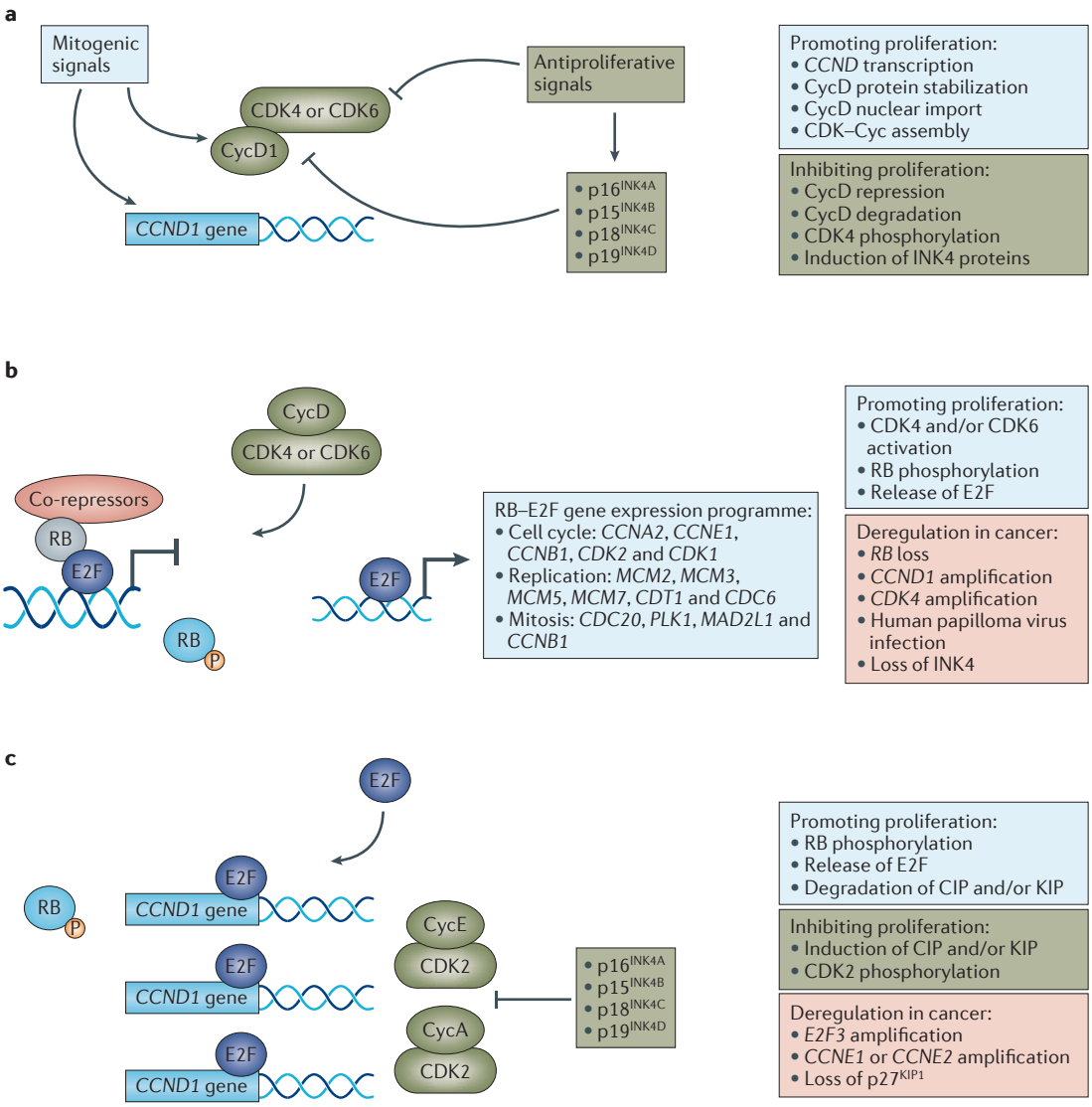


Figure 2 | **G1–S regulatory modules and relevance to cancer.** Control over the G1–S transition is coordinated by distinct regulatory modules that are dysregulated in cancer. **a** | Initially, mitogenic signals impinge on cyclin-dependent kinase 4 (CDK4) or CDK6 activity through multiple mechanisms, including the induction of cyclin D1 (CycD1) gene (*CCND1*) expression, protein stability and assembly of the CDK–Cyc complex. These steps can be individually antagonized, or the induction of CDK4 and CDK6 inhibitors (that is, the inhibitor of CDK4 (INK4) family of proteins) can function to prevent complex assembly and to inhibit assembled complexes **b** | The net activation state of CDK4 and CDK6 initiates the phosphorylation of the tumour suppressor retinoblastoma protein (RB) that contributes to activation and release of the E2F family of transcription factors. E2F proteins control the expression of a multitude of positive-acting factors that are critical for progression through the S phase and the G2–M transition. Multiple mechanisms lead to RB inactivation in cancer, such as mutations, aberrant phosphorylation or protein sequestration. **c** | E2Fs and additional signals drive the expression and activation of CDK2–CycE and CDK2–CycA complexes, which contribute to DNA replication and further phosphorylation of RB. Deregulation of this activity is found in cancer through amplification of E-type cyclins or loss of CDK inhibitors. *CCN*, cyclin; *CDC*, cell division cycle; *CDT1*, chromatin licensing and DNA replication factor 1; CIP, CDK-interacting protein; KIP, kinase inhibitory protein; *MAD2L1*, MAD2 mitotic arrest deficient-like 1; *MCM*, minichromosome maintenance complex component; *PLK1*, polo-like kinase 1.

duplication (for example, nucleophosmin (NPM)), among other processes[57–59] (FIG. 3). *In vitro*, CDK2 and its preferred E-type and A-type cyclin partners assemble spontaneously to form active kinase complexes[3,60]. Much of the control over CDK2 involves the synthesis and availability of the cyclins, with RB and E2F regulating the abundance of CDK2, cyclin E1 and cyclin E2 transcripts and proteins[61–65]. This process couples mitogen-mediated activation of CDK4 and CDK6 with the activation of CDK2 (REFS 66,67) (FIG. 2c). In contrast to CDK4 and CDK6, CDK2 is not regulated by INK4 proteins[30,68] but by the CDK-interacting protein/kinase inhibitory protein

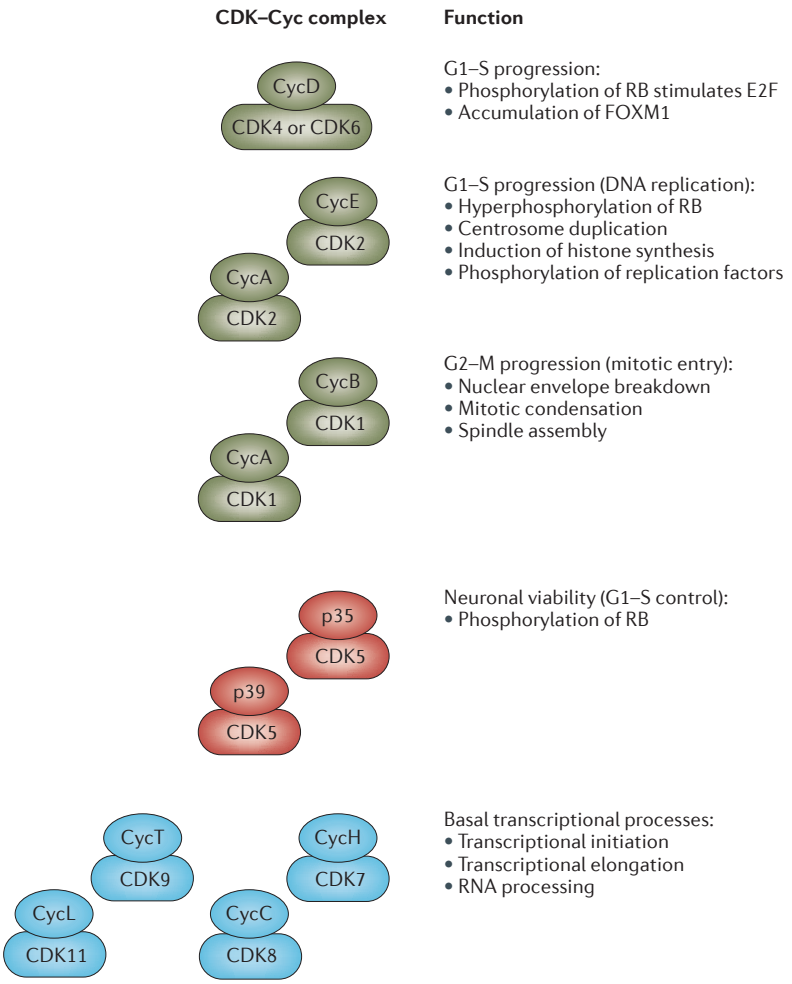


Figure 3 | **Summary of the biological functions of CDK complexes.** A summary of the different classes of cyclin-dependent kinase (CDK)–cyclin (Cyc) complexes involved in the cell cycle or in diverse biological processes is shown. CDK–Cyc complexes shown in green promote cell cycle progression, whereas those depicted in blue are generally involved in transcriptional processes. The CDK5 complexes shown in red are involved both in the control of neuronal viability and in the promotion of the cell cycle. FOXM1, forkhead box protein M1; RB, retinoblastoma protein.

(CIP/KIP) class of CDK inhibitors, which bind to CDK2–cyclin complexes and render them inactive[60,69–71]. For example, $p21^{CIP1}$ acts as a DNA damage checkpoint (it is a critical downstream target gene of p53 that inhibits DNA synthesis), whereas $p27^{KIP1}$ is responsive to mitogenic signalling as a further control on deregulated proliferation[71,72]. Additionally, CDK2 is regulated by phosphorylation events[73]. Therefore, multiple mechanisms in addition to the CDK4/6–RB axis can modulate the activity of CDK2 and subsequent DNA replication.

Recently, it has become clear that deregulation of CDK2 also occurs frequently in certain types of cancer[74]. Cyclin E1 or cyclin E2 amplifications are key oncogenic events in multiple cancers, particularly in uterine and ovarian cancers[75–77] (FIG. 4). Ectopic expression of cyclin E bypasses the need for CDK4 or CDK6 activity to initiate the S phase[78–80], and it is therefore assumed that amplification of E-type cyclins may be oncogenic in a similar manner (that is, bypassing the physiological requirement for CDK4 or CDK6 activity to initiate expression of E-type cyclins). The CDK inhibitor $p27^{KIP1}$ is downregulated in many cancers, although the genetic loss of $p27^{KIP1}$ is fairly rare[81,82].

CDK1 is a key determinant of mitotic progression. CDK1 was cloned on the basis of complementarity to the *cdc2* gene of *Schizosaccharomyces pombe*[83]. The expression of CDK1 and associated cyclins (cyclin A2 and cyclin B1) is coordinated through the activity of E2F-assembled complexes[63,65]. These include the canonical E2F and RB constituents, as well as the transcription factor FOXM1 and the DREAM (dimerization partner, RB-like, E2F and multivulval class B) complex, which are particularly relevant for the coordination of transcripts involved in mitotic progression[84–86]. The cyclins that assemble with CDK1 are intrinsically unstable and are regulated by ubiquitin-mediated degradation mechanisms, and the assembly and localization of CDK1 complexes are regulated by multiple overlapping mechanisms[87–90].

Mouse knockout experiments have shown that CDK1 is required for mammalian cell proliferation; it is the only CDK that can initiate the onset of mitosis (that is, the M phase)[91]. Premature initiation of mitosis before completion of the S phase results in chromosomal shattering and cell death[92]. Multiple factors restrain the activity of CDK1 until DNA replication is complete and there is minimal DNA damage. This integration of DNA replication and CDK1 activity is mediated by checkpoint signalling kinases such as CHK1 and WEE1, which suppress the activity of CDK1 via inhibitory phosphorylation[93], as well as through the cell division cycle 25 (CDC25) family of phosphatases. At the onset of mitosis, activation of CDK1 occurs rapidly through a positive feedback loop whereby CDK1 phosphorylates and inactivates WEE1. CDK1 subsequently phosphorylates multiple substrates, leading to nuclear envelope breakdown, chromosome condensation and mitotic spindle assembly[94] (FIG. 3). The subsequent progression from metaphase to anaphase is controlled by the spindle assembly checkpoints, and progression through anaphase is dependent on the attenuation of CDK1 activity through the degradation of cyclin B1 by the anaphase-promoting complex[95,96].

Interestingly, in contrast to the genetic deregulation of the CDKs that coordinate the S phase, there is limited evidence to show that CDK1 activity is dysregulated by direct genetic alteration in tumorigenesis. Derangement of p53 signalling or of DNA damage checkpoints indirectly leads to the deregulation of CDK1 (REFS 97,98), and high cyclin B1 expression is generally associated with a more aggressive cancer phenotype[99,100]. However, the requirement that CDK1 activity must be attenuated to exit mitosis and the lethal aspects of uncoordinated CDK1 activity are likely to limit its potential as a direct oncogenic driver.

The role of cell cycle-independent CDKs. In addition to the well-known CDKs involved in regulating the cell cycle, there is an equivalent number of CDKs involved in

basal transcriptional regulation[3,10,11] (FIG. 3). In particular, CDK8 is part of the mediator complex that regulates a plethora of genes[101,102]. CDK7 has a general role in the phosphorylation of the RNA polymerase II carboxy-terminal domain that contributes to the initiation of transcription[103], and CDK9 also phosphorylates RNA polymerase II, thereby promoting elongation of transcription. Finally, CDK11 acts on the splicing machinery. In each of these contexts, the CDK activity is directed by specific cyclin interactions. Accumulating evidence suggests that these transcription-regulating CDKs may represent therapeutic targets in cancer.

In addition to the CDKs involved in transcriptional regulation, there remains a class of CDKs, including CDK3 and CDK5, for which the underlying functions are elusive. CDK3 was found to be intrinsically important for cell cycle control based on cell-based experiments that used a dominant-negative version of CDK3 (REF. 72) (FIG. 3). However, it was subsequently revealed that many mouse strains harbour an inactive CDK3, suggesting that its role in the cell cycle can be readily compensated[104]. CDK5 was largely viewed as a neuronal kinase implicated in the protection of the nervous system from injury[105]. However, recent work suggests that it harbours many functions similar to CDK4 and CDK2 in driving progression from G1–S and in RB phosphorylation in medullary thyroid cancer models[106]. CDK5 might therefore have potential as a therapeutic target in this thyroid cancer subtype[106].

Development of pan-CDK inhibitors

The first generation of CDK inhibitors. Over the past 20 years, several CDK inhibitors have been developed as potential cancer therapeutics and tested in numerous trials and in several tumour types (FIG. 5). The first-generation CDK inhibitors developed were relatively nonspecific and may therefore be referred to as 'pan-CDK' inhibitors (an example of which is flavopiridol (also known as alvocidib; developed by Sanofi-Aventis), although some compounds, such as olomucine (not commercially developed) and roscovitine (also known as seliciclib; developed by Cyclacel), have relatively low affinity for CDK4 and CDK6). As the field of CDK biology progressed in parallel with the development of these agents, our understanding of their targets and interpretation of their behaviour have also evolved. For example, it was initially believed that roscovitine was a relatively specific inhibitor of CDK1, CDK2 and CDK5; however, subsequent data demonstrated that it also inhibited transcription, probably through the inhibition of CDK7 and CDK9 (REF. 107).

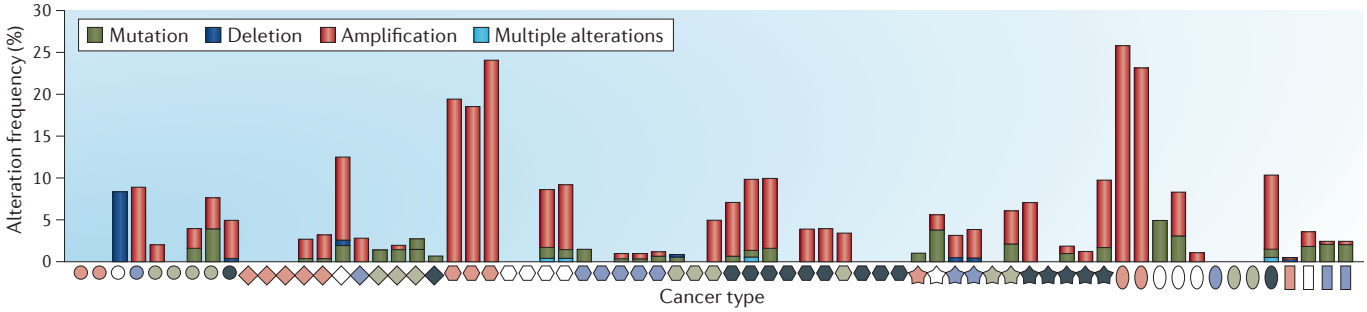
Of these first-generation inhibitors, flavopiridol is the most extensively investigated CDK inhibitor so far, with >60 clinical trials carried out between 1998 and 2014 (see Supplementary information S1 (box)). Flavopiridol is a semi-synthetic flavonoid derived from rohitukine, a chromone alkaloid, and has been shown to inhibit CDK1, CDK2, CDK4, CDK6, CDK7 and CDK9 (REFS 108,109). Although flavopiridol can induce cell cycle arrest in G1 and G2 phases, in certain contexts it also induces a cytotoxic response, probably as a result of CDK7 and CDK9 inhibition that leads to suppression of transcription[110]. Although the underlying broad-spectrum nature of flavopiridol results in substantial *in vitro* activity, substantially less activity was observed *in vivo*[110]. Consequently, flavopiridol did not meet the initial high expectations for a CDK inhibitor, and low levels of clinical activity were seen in Phase II studies in several solid tumour types (see Supplementary information S1 (box)). There is evidence to indicate that flavopiridol may have clinical activity in haematological malignancies, such as chronic lymphocytic leukaemia (CLL) and mantle cell lymphoma[111,112], in which scheduling also seems to influence flavopiridol efficacy. For example, a relatively short infusion time (4 hours) resulted in response rates of 41% in 22 assessable patients with CLL[113,114]. Patients with a high disease burden and high-risk genetic features achieved durable responses, and tumour lysis syndrome was reported in approximately 40% of patients with CLL treated with flavopiridol[115]. Despite these reports and extensive investment, no Phase III studies have emerged and drug development of flavopiridol was consequently discontinued in 2012.

In parallel with flavopiridol, roscovitine, a purine-based CDK inhibitor, was among the first agents to be evaluated in the clinic (see Supplementary information S1 (box)). Of the 56 patients treated in the Phase I setting, 1 patient achieved a partial response[116]. A subsequent blinded, randomized Phase II trial (APPRAISE) that compared roscovitine with the best supportive care was performed in patients with advanced non-small cell lung cancer. The APPRAISE study was terminated after 187 patients were enrolled; although results were never published, roscovitine did not seem to improve progression-free survival in this patient population (see Cyclacel press release). Currently, only a single trial is ongoing for roscovitine in Cushing disease.

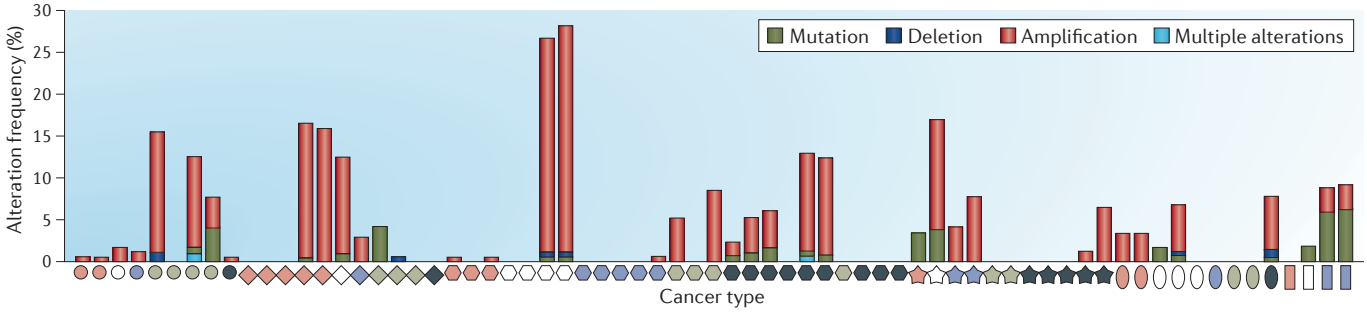
Second-generation inhibitors of multiple CDKs. Following on from flavopiridol and roscovitine, other CDK inhibitors were developed with the aim of increasing selectivity for CDK1 and CDK2 and/or increasing overall potency (FIG. 5). Again, numerous CDK inhibitors seemed to be particularly promising in preclinical studies, but only a few progressed past Phase I clinical trials[117–120] (see Supplementary information S1 (box)).

Of these second-generation CDK inhibitors, dinaciclib (also known as MK-7965 and SCH727965; developed by Merck) has been most extensively studied in the clinic. Dinaciclib was specifically developed as a highly potent inhibitor of CDK1, CDK2, CDK5 and CDK9 (half-maximal inhibitory concentration (IC_{50}) values in the range of 1–4 nM), with less activity against CDK4, CDK6 and CDK7 (IC_{50} values in the range of 60–100 nM). Compared to flavopiridol, dinaciclib exhibited superior activity with regard to suppression of RB phosphorylation in cell-based assays[118]. Moreover, dinaciclib inhibited cell cycle progression in >100 tumour cell lines of various tumour types and induced the regression of established solid tumours in a range of mouse models[118]. Initial results from Phase I studies were promising:

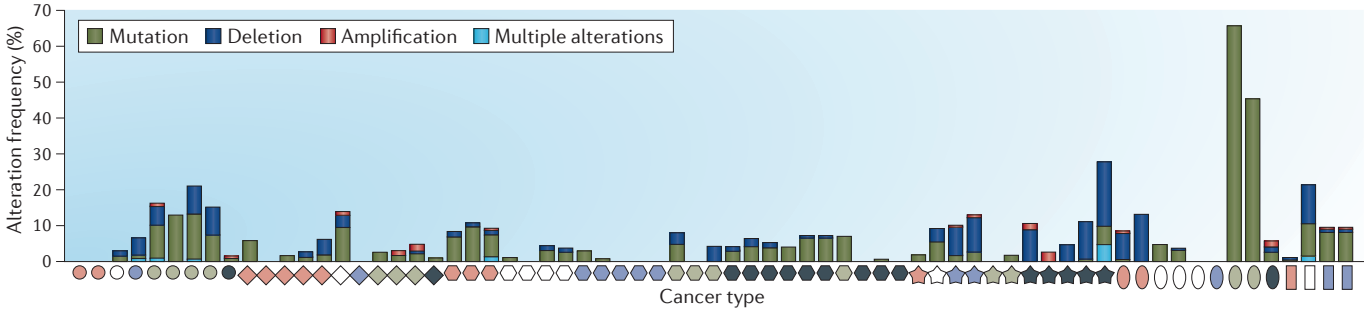
CDK4* and *CDK6



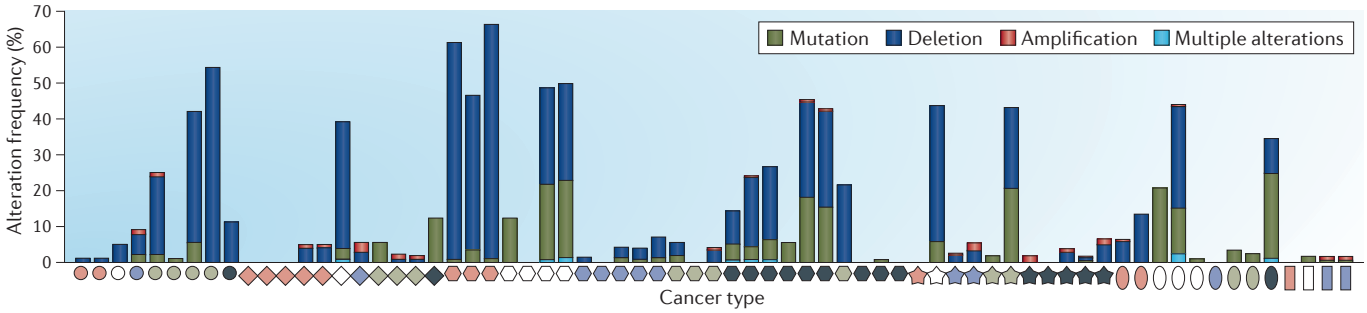
CCND1



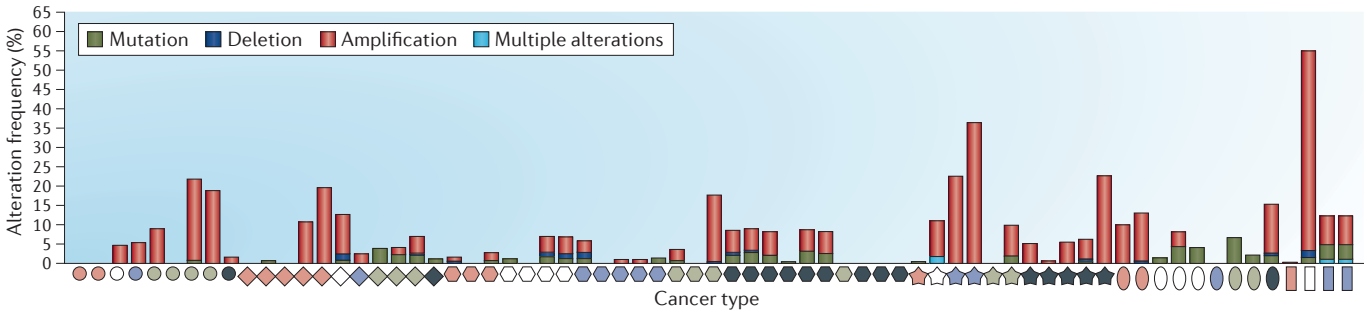
RB1

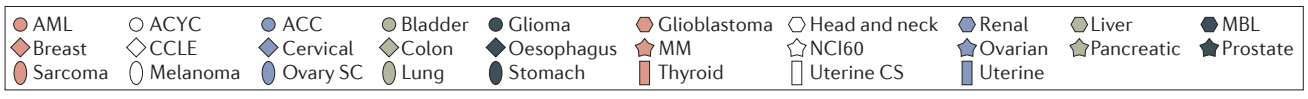


CDKN2A



CCNE1* and *CCNE2





◂ Figure 4 | **Deregulation of CDK regulatory genes in cancer.** The frequencies of genetic amplification of cyclin-dependent kinase 4 (*CDK4*) and *CDK6*; cyclin D1 (*CCND1*); retinoblastoma 1 (*RB1*); cyclin-dependent kinase inhibitor 2A (*CDKN2A*); and cyclin E1 (*CCNE1*) and *CCNE2* are summarized across multiple disease sites. For each of the indicated cancer types, the frequencies of mutation (green), amplification (red) and homozygous deletion (dark blue) were determined using genetic data from >2,000 cancer cases obtained through cBioPortal for Cancer Genomics. As shown, different types of cancer exhibit distinct predominant mechanisms of genetic alterations in cell cycle control. In many cases, the same cancer type has been evaluated in multiple independent studies. Detailed information about each case and disease is accessible through the cBioPortal for Cancer Genomics. ACC, adrenocortical carcinoma; ACYC, adenoid cystic carcinoma; AML, acute myeloid leukaemia; CCLE, Cancer Cell Line Encyclopedia; CS, carcinosarcoma; MBL, medulloblastoma; MM, multiple myeloma; NCI60, US National Cancer Institute (NCI) 60 human tumour cell line anticancer drug screen; SC, serous cystadenocarcinoma.

dinaciclib induced stable disease in a range of malignancies and displayed tolerable toxicity[121]. However, results from recent randomized Phase II trials of dinaciclib in solid tumours have been disappointing. A randomized Phase II trial of dinaciclib versus the chemotherapeutic agent capecitabine in advanced breast cancer was stopped after 30 patients were enrolled because interim analysis showed that the time to disease progression was inferior with dinaciclib treatment[122]. In a study evaluating dinaciclib monotherapy in patients with non-small cell lung cancer, dinaciclib showed no activity in previously treated patients[123]. In adult patients with advanced acute myeloid leukaemia (patients ≥60 years of age) or acute lymphoblastic leukaemia, no objective responses were observed with dinaciclib[124]. However, in patients with relapsed multiple myeloma, preliminary encouraging single-agent activity was reported in a Phase I/II trial, with 2 patients of 27 achieving partial responses[125], and dinaciclib, similar to flavopiridol, demonstrated clinical activity in pretreated patients with CLL[126]. Based on these data, a randomized Phase III study of dinaciclib in refractory CLL is ongoing. Therefore, dinaciclib may prove useful in the treatment of certain haematological malignancies, in which flavopiridol also had evidence of activity.

Other CDK inhibitors include AT7519 (developed by Astex), a pyrazole 3-carboxyamide compound that acts as an inhibitor of CDK1, CDK2, CDK4, CDK6 and CDK9. AT7519 has been evaluated in combination with bortezomib in a Phase II clinical trial enrolling patients with previously treated multiple myeloma. The combination was well tolerated, and more than one-third of patients achieved partial response[127]. R547 (developed by Hoffman-La Roche) is an inhibitor of CDK1, CDK2 and CDK4 with less potency for CDK7, glycogen synthase kinase 3α (GSK3α) and GSK3β. R547 was tested in a Phase I study in 2007 as an intravenous weekly infusion[128]. Although reported to have manageable side effects, there have not been further clinical trials with this compound. SNS-032 (also known as BMS-387032; developed by Bristol-Myers Squibb), which was initially described as having greater selectivity for CDK2 than CDK1 and CDK4, is now recognized to also target CDK7 and CDK9. Two Phase I clinical studies with SNS-032, one in 2010 in advanced lymphoid malignancies[129] and one in 2008 in selected advanced tumours[130], have been reported, but no further developments are apparent. The development of AZD5438 (developed by Astra Zeneca) — an orally bioavailable, potent inhibitor of CDK1, CDK2 and CDK9 with less selectivity for CDK5 and CDK6 (REF. 131) — was discontinued after it was reported to be intolerable when administered continuously in patients with advanced solid tumours[132]. AG-024322 (developed by Pfizer) — a potent inhibitor of CDK1, CDK2 and CDK4 — was also discontinued in 2007 after the Phase I study was terminated, as it failed to achieve an acceptable clinical end point (see Supplementary information S1 (box)).

Reasons for failure of CDK inhibitors with low selectivity. The general failure of non-selective CDK inhibitors in the clinic can be partly explained by at least three key underlying principles. First, there was a lack of clear understanding of the mechanism of action. For many of the CDK inhibitors with low specificity, there remains a lack of clarity with regard to which CDKs are actually being inhibited *in vivo* and therefore the corresponding mechanism that could underlie the therapeutic effect. For example, flavopiridol has been associated with diverse distal cellular effects, including cell cycle inhibition, transcriptional suppression, apoptosis, autophagy and endoplasmic reticulum stress[133–135]. This lack of understanding confounds the ability to develop these agents as targeted therapies and to design effective combination strategies.

Second, there was a lack of appropriate patient selection. The vast majority of studies conducted with CDK inhibitors with low specificity were in unstratified patient cohorts. This is because there are essentially no biomarkers that may select for sensitive subpopulations for this class of inhibitors. The potential activity of both flavopiridol and dinaciclib in CLL and the rare 'extraordinary' responders suggest that there are molecular-based reasons that some tumours are vulnerable to such agents. Although the molecular underpinnings for these responses are unknown, it is tempting to speculate that the inhibition of CDKs that control transcription could underlie at least part of this activity.

Third, there is a lack of a therapeutic window. Many of these CDK inhibitors target several proteins that are critical to the proliferation (for example, CDK1) and survival (for example, CDK9) of normal cells. This limits the ability to achieve therapeutic levels of these drugs because of their intrinsic inability to discriminate between cancerous and healthy tissues. Consistent with this point, the toxicities associated with non-selective CDK inhibitors include diarrhoea, myelosuppression, anaemia and nausea[116,121,136].

A case for selectivity of CDK inhibitors. The ascribed weaknesses of pan-CDK inhibitors suggest that improved selectivity for certain CDKs is the key to the successful development of CDK inhibitors as therapeutic cancer agents. Selective inhibitors of CDK2 might provide the ability to target genetic and/or driver events

in tumours driven by cyclin E amplification. Emerging data suggest that targeting CDK1 is toxic in certain contexts, and it may be challenging to achieve a therapeutic window. For example, synthetic lethal screens against *KRAS* mutations have indicated a potential sensitivity to CDK1 knockdown, although follow-up studies are required[137]. Similarly, CDK1 or CDK9 inhibition is synthetically lethal with *MYC*[138,139]. Pharmacologically, CDK1 inhibitors seem to potently cooperate with inhibitors of poly(ADP-ribose) polymerase[140] by

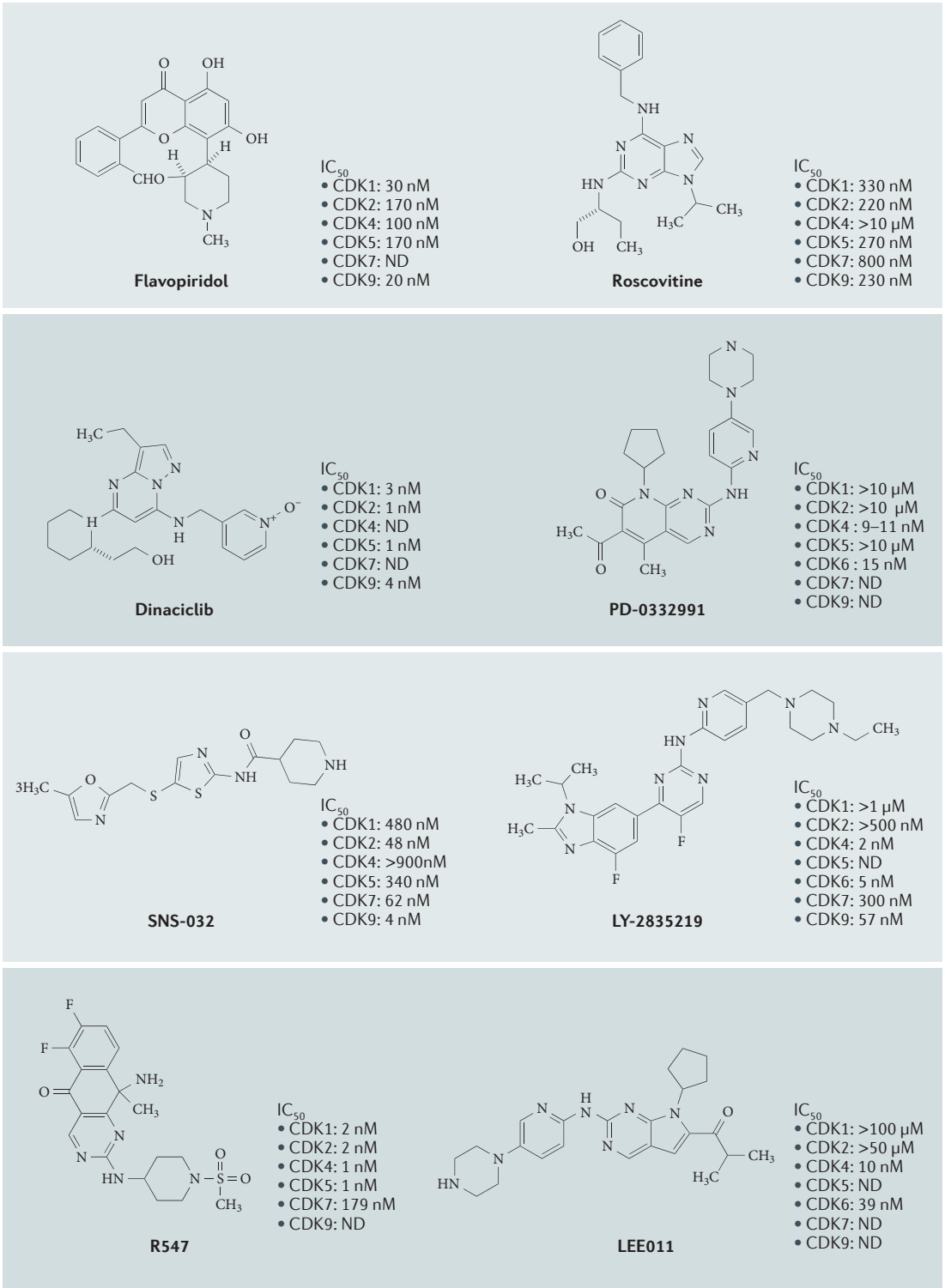


Figure 5 | **Selected CDK inhibitors.** The chemical structures of several pan-cyclin-dependent kinase (CDK) and CDK4- and CDK6-selective inhibitors are shown. The published half-maximal inhibitory concentration (IC_{50}) values against selected CDK complexes are shown. ND, not determined.

compromising DNA repair pathways. An alternative strategy is to selectively target CDK7, CDK8 or CDK9, which are associated with basal transcription, because cancer cells may harbour unique vulnerabilities to selective suppression. CDK8 may function as an oncoprotein in colorectal cancer by regulating the transcription of β-catenin target genes[141]. A covalent inhibitor of CDK7 (THZ1), which is relatively specific for CDK7 compared with other CDKs, has shown activity in multiple cancer cell models[142]. Similarly, a specific inhibitor for CDK9 (CDKI-73) that exhibited activity in animal models of leukaemia was recently developed[143].

Targeted inhibition of CDK4 and CDK6

Rationale for targeting CDK4 and CDK6. Based on the myriad findings from mechanistic studies and studies of CDK4 and CDK6 deregulation in cancer, three important features and expectations arose for CDK4 and CDK6 inhibitors in the clinic. First, one would expect that a pure CDK4 or CDK6 inhibitor would elicit a single phenotype in tumours: cytostatic G0/G1 arrest. Second, this effect would be a direct reflection of the engagement of RB to suppress gene expression and proliferation. Third, such effects would be particularly actionable in tumours that exhibit deregulation of CDK4 and CDK6 activity as opposed to other CDKs. Initial data from mouse models seeded confusion as to whether CDK4 and CDK6 were therapeutic targets because many tissues in the mouse developed normally despite the absence of CDK4 and/or CDK6 (REFS 15,144) and in the absence of D-type cyclins[145]. These data reflected substantial compensatory plasticity with other CDKs. Despite uncertainties arising from the mouse knockout models, it subsequently became clear that attenuation of CDK4 and CDK6 activity could prevent the development of specific mouse tumour types. For example, cyclin D1 is crucial for the development of mammary tumours driven by HER2 (also known as ERBB2)[146], and similar observations were obtained in NOTCH-driven T cell leukaemia mouse models[147] and cell lines[147,148].

Pharmacological approaches. The development of selective inhibitors of both CDK4 and CDK6 has markedly changed the perception of CDKs as therapeutic targets in cancer. Through a combination of chemical screening and optimization, it was found that pyrido[2,3-*d*]pyrimidin-7-one compounds with a 2-aminopyridine side chain at the *C*2 position act as CDK inhibitors with a high degree of selectivity for CDK4 and CDK6 relative to other CDKs[149] (FIG. 5). Subsequent optimization resulted in the compound PD-0332991 (also known as palbociclib; developed by Pfizer) that induced potent G1 arrest in cell culture and xenograft models[150,151]. As anticipated from the basic biology of G1–S transition, the effects of palbociclib were dependent on the presence of a functional RB protein, thus demonstrating a degree of biological specificity that had not been previously described for CDK inhibitors[150,151]. Subsequently, multiple independent groups have demonstrated that specific CDK4 and CDK6 inhibitors arrest the cell cycle through the downstream blockade of phosphorylation of RB, as well as the related p107 and p130 proteins. This blockade results in the loss of expression of S-phase cyclins, nucleotide biosynthesis, DNA replication machinery and mitotic regulatory genes[152,153]. Dual CDK4 and CDK6 inhibitors have been shown to be active in multiple preclinical models, including xenografts, genetically engineered mouse models and primary human tumour explants[152,154–156] (TABLE 1). Parallel drug discovery efforts at Eli Lilly and Novartis resulted in the development of the drugs LY-2835219 (also known as abemaciclib) and LEE011, respectively[157–162]. Each drug is structurally similar and chemically distinct from the less specific pan-CDK inhibitors (FIG. 5). The selectivity of all of these compounds is likely to reflect binding to the specialized ATP-binding pocket of CDK4 and CDK6 and specific interactions with residues in the ATP-binding cleft, although this has not been proven by structural analysis. In contrast to other CDK4- and CDK6-selective compounds, abemaciclib inhibits CDK9 in *in vitro* kinase assays, although there is no evidence of functional inhibition in cellular models[158].

Single-agent clinical outcomes. There are numerous emerging clinical studies with dual CDK4 and CDK6 inhibitors (TABLE 2). Results from a Phase I study with palbociclib monotherapy indicated promising clinical efficacy and a well-tolerated toxicity profile in patients with RB-positive advanced solid tumours and non-Hodgkin lymphoma. Of the 33 patients enrolled and treated with palbociclib (once daily for 14 days followed by 7 days off), 1 patient with testicular cancer achieved a partial response and 9 patients achieved stable disease[163]. The anticipated cytostatic nature of dual CDK4 and CDK6 inhibitors resulted in prolonged stable disease duration of 18, 22 and 24 months in 3 men with growing teratoma syndrome with inoperable tumours[164]. In patients with relapsed mantle cell lymphoma (which exhibits frequent cyclin D1 amplification) receiving palbociclib monotherapy, 5 of the 17 patients had >1 year progression-free survival, with 1 complete response and 2 partial responses[165]. A Phase II study in liposarcoma, a disease with frequent CDK4 amplification, also reported favourable progression-free rates in patients with CDK4 amplification and RB expression[165,166]. The recently reported Phase I study with LEE011 monotherapy in patients with advanced solid tumours demonstrated that LEE011 was well tolerated, with 2 confirmed partial responses and 40% of patients with stable disease[167]. Similarly, abemaciclib exhibited single-agent activity associated with delayed disease progression and particularly robust activity in metastatic ER-positive breast cancer, although data are from a relatively small study with one group of patients[168]. There are multiple ongoing Phase II trials evaluating dual CDK4 and CDK6 inhibitors as monotherapy in various tumour types (TABLE 2).

These early trials defined several key clinical hallmarks of inducing CDK4 and CDK6 inhibition in patients with cancer. Most importantly, it seems that neutropaenia is the principal dose-limiting toxicity of

Table 1 | **Preclinical outcome analysis of CDK4 and CDK6 inhibitors**

Indication	Agent	Cell culture	Animal model	Markers	Clinical	Refs
Mantle cell lymphoma	PD-0332991	Yes	Yes	–	Yes	165,187
Acute lymphoblastic lymphoma	PD-0332991	Yes	Yes	–	–	147,148
Multiple myeloma	PD-0332991	Yes	Yes	–	Yes	188
Liposarcoma	PD-0332991 and LEE011	Yes	Yes	CDK4	Yes	159,166
Hepatocellular carcinoma	PD-0332991	Yes	Yes	RB and $p16^{INK4A}$	Yes	153
Ovarian cancer	PD-0332991	Yes	Yes	RB and $p16^{INK4A}$	Yes	175
Breast cancer	PD-0332991, LEE-011	Yes	Yes	RB, $p16^{INK4A}$ and luminal subtype	Yes	152,154, 155,169
Lung adenocarcinoma	PD-0332991	Yes	Yes	–	Yes	150,189
Prostate cancer	PD-0332991	Yes	Yes	–	Yes	190
Glioma	PD-0332991	Yes	Yes	RB and $p16^{INK4A}$	Yes	156,191,192
Renal cancer	PD-0332991	Yes	Yes	RB and $p16^{INK4A}$		176
Melanoma	PD-0332991 and LY2835219	Yes	Yes	–	Yes	183,193,194
Medulloblastoma	PD-0332991	Yes	Yes	–	–	195
Colon cancer	PD-0332991	Yes	Yes	–	Yes	150
Oesophageal cancer	PD-0332991	Yes	–	–	–	196
Neuroblastoma	LEE011	Yes	Yes	–	Yes	157
Pancreatic cancer	PD-0332991	Yes	Yes	–	–	184,197

CDK, cyclin-dependent kinase; RB, retinoblastoma protein.

palbociclib and LEE011. Although neutropaenia is a common side effect of cytotoxic agents, the neutropaenia associated with palbociclib and LEE011 is distinct in that it is rapidly reversible, reflecting a cytostatic effect on neutrophil precursors in the bone marrow. Consequently, both palbociclib and LEE011 are dosed intermittently to accommodate a break for haematological recovery[167]. Interestingly, abemaciclib exhibits more prominent gastrointestinal-associated toxicity, whereas neutropaenia is less evident, enabling continuous dosing. The reasons behind these observations and implications for future development remain unclear.

Hormone therapy combination strategies. Preclinical investigation suggested that dual CDK4 and CDK6 inhibitors positively interact with other therapeutic agents. In particular, synergy was observed when palbociclib was combined with hormone therapy in ER-positive breast cancer cell lines, although the observed effects can range from additive to synergistic depending on the model[155]. Additionally, CDK4 and CDK6 inhibition has shown activity in multiple ER-positive breast cancer models that have acquired resistance to ER antagonists[155,169,170]. Importantly, resistance to endocrine therapy is associated with the deregulation of proliferation-associated genes that are regulated by the CDK4/6–RB–E2F axis, suggesting a basis for cooperativity in the clinic[43,171]. These observations triggered a series of randomized Phase II studies that have consequently transformed the CDK field.

The PALOMA-1 Phase II clinical trial randomized 165 women with advanced ER-positive breast cancer into two treatment groups: the aromatase inhibitor letrozole versus letrozole plus palbociclib. Data from this trial showed that the combination of palbociclib plus letrozole elicited significant improvement in median progression-free survival compared with letrozole alone (20.2 months compared with 10.2 months; hazard ratio (HR) = 0.488 (95% CI: 0.319–0.748) and one-sided $p = 0.0004$). The overall survival analysis after 61 deaths demonstrated a trend in favour of the letrozole plus palbociclib combination (37.5 months versus 33.3 months, respectively; HR = 0.813; $p = 0.2105$)[172–174]. Consequently, palbociclib received Breakthrough Therapy designation by the US Food and Drug Administration in April 2013. ER-positive breast cancer is characterized by frequent dysregulation of CDK4 and CDK6 activity due to the overexpression and amplification of the gene encoding cyclin D1 (*CCND1*). Those cancers with amplification of *CCND1* seemed to derive no greater benefit from

Table 2 | **Reported clinical trials with targeted CDK4 and CDK6 inhibitors**

Tumour type	Phase	Dosage	Response rate	Refs
Palbociclib (PD-0332991)				
Advanced melanoma, breast cancer, renal cancer, ovarian cancer, liposarcoma and colon cancer, among others	• Phase Ia (dose escalation) • *N* = 41	• Administered over 6 cohorts (standard 3 + 3) • 3 weeks on, 1 week off • RP2D: 125 mg PO OD	SD: 27% (10/37)	178
Liposarcoma, colon cancer and melanoma, among others	• Phase Ia • *N* = 33 (RB positive)	• Administered over 4 cohorts • 2 weeks on; 1 week off • RP2D: 200 mg PO OD	• PR: 3% (1/31; testicular cancer) • SD: 29% (9/31)	163
Relapsed mantle cell lymphoma with ≥1 of: *CCND1* positivity by immunostaining, t(11;14) translocation on cytogenetic analysis and molecular evidence of *CCND1–IGH* rearrangement	• Phase I • *N* = 17	• 100–125 mg PO OD	• CR: 6% (1/16) • PR: 12% (2/16) • SD: 43% (7/16)	165
ER-positive and HER2-negative metastatic breast cancer	• Phase Ib • *N* = 12	• Palbociclib (125mg PO OD; 2 weeks on; 1 week off) + letrozole (2.5mg PO OD; continuous)	• PR: 25% (3/12) • SD: 75% (9/12)	198
ER-positive and HER2-negative metastatic breast cancer	• Phase II (PALOMA-1; TRIO-18) • Palbociclib + letrozole versus letrozole alone in 1:1 randomization • *N* = 165	• Palbociclib (125 mg; 3 weeks on; 1 week off) + letrozole (2.5 mg; continuous)	• PFS: 20.2 months for palbociclib + letrozole versus 10.2 months for letrozole alone (HR = 0.488 (95% CI: 0.319–0.748) and 1-sided $p = 0.0004$) • OS: 37.5 months for palbociclib + letrozole versus 33.3 months for letrozole alone (HR = 0.813; $p = 0.2105$)	173
Metastatic breast cancer (64% ER-positive; 7% ER-positive and HER2-positive; 29% TNBC)	• Phase II • *N* = 37 (RB positive)	• 125 mg PO • 3 weeks on; 1 week off	• PR: 7% (2/28) • SD: 50% (14/28) • PFS: 4.1 months for ER-positive (95% CI: 2.3–7.7) • PFS: 18.8 months for ER-positive and HER2-positive (95% CI: 5.1–∞) • PFS: 1.8 months (95% CI 0.9–∞) for TNBC	199
Well-differentiated (17%) and dedifferentiated (83%) liposarcoma with *CDK4* amplification detected by FISH and RB expression detected by IHC	• Phase II • *N* = 30	• 200 mg PO OD • 2 weeks on; 1 week off	• PR: 3% (1/ 30) at 74 weeks; 19/30 were progression-free at 12 weeks • PFS: median 18 weeks • PFS: 66% (90% CI: 51–100%) at 12 weeks • Met primary end point of exceeding PFS rate of 40% at 12 weeks for active second-line agent	166
LEE011				
RB-positive advanced solid tumours and lymphomas	• Phase I • *N* = 132	• Stage 1 (*N* = 85): Treatment arm 1: escalating LEE011 doses (3 weeks on; 1 week off) Treatment arm 2: escalating LEE011 doses (continuous) • Stage 2 (*N* = 47): RP2D expansion MTD: 900mg; RP2D: 600 mg using 3 weeks on; 1 week off schedule	• PR: 2.9% (2/70) at 600 mg per day • SD: 26% with >4 cycles and 14% with >6 cycles	167
Post-menopausal ER-positive, HER2-negative metastatic breast cancer	• Phase Ib • LEE011 + everolimus (mTOR inhibitor) + exemestane (aromatase inhibitor) • *N* = 6	• Treatment arm 1: escalating LEE011 doses (starting 200 mg per day; 3 weeks on; 1 week off) + everolimus (2.5 mg per day, fixed continuous) + exemestane (25 mg per day; continuous) • Treatment arm 2: safety run-in with LEE011 (600 mg per day; 3 weeks on; 1 week off) + exemestane (25 mg per day; continuous)	• Preliminary results indicate that triple combination is tolerable • Efficacy data not yet available	200

Table 2 (cont.) | **Reported clinical trials with targeted CDK4 and CDK6 inhibitors**

Tumour type	Phase	Dosage	Response rate	Refs
Post-menopausal ER-positive, HER2-negative locally advanced or metastatic breast cancer	• Phase Ib • LEE011 + BYL719 (PI3Kα inhibitor) + letrozole • *N* = 11	• Treatment arm 1: LEE011 (3 weeks on; 1 week off) + letrozole (2.5 mg; continuous); 4 week cycle • Treatment arm 2: BYL719 (continuous) + letrozole (2.5 mg; continuous); 4 week cycle • Treatment arm 3: LEE011 + BYL719 (continuous) + letrozole (2.5 mg; continuous); 4 week cycle	Efficacy data not yet available	201
NRAS-mutant metastatic melanoma	• Phase Ib (single arm) • LEE011 + binimetinib (MEK inhibitor) • *N* = 14	• LEE011 (starting 200 mg per day OD; 3 weeks on; 1 week off) + binimetinib (45 mg PO BD)	• PR: 43% (6/14) • SD: 43% (6/14) • Promising preliminary antitumour activity	202
Abemaciclib (LY2835219)				
Non-small cell lung cancer (*KRAS* wild type and *KRAS* mutant)	• Phase I • *N* = 49	• MTD already established at 200 mg in earlier stage of study • Treatment arm 1 (*N* = 25): 200 mg PO BD continuous (28-day cycle) • Treatment arm 2 (*N* = 24): 150 mg PO BD continuous (28-day cycle)	• RR: 2% PR (1/49) • Overall DCR: 51% • DCR 37% (19/49) for *KRAS* wild type and 54% (26/49) for *KRAS* mutant • PFS: 2.1 months	203
Hormone receptor-positive metastatic breast cancer	• Phase I • Abemaciclib + fulvestrant • *N* = 60	• Treatment arm 1 (*N* = 47): abemaciclib (200 mg BD PO; continuous; 28-day cycle) • Treatment arm 2 (*N* = 13): abemaciclib + fulvestrant (500 mg IM every 4 weeks)	• PR: 17% (8/47) with 6% (3/47) unconfirmed • Single-agent activity demonstrated; acceptable safety profile in combination with fulvestrant • Further evaluation required	204

BD, twice daily; *CCND1*, cyclin D1; CDK, cyclin-dependent kinase; CR, complete response; DCR, disease control rate (CR+PR+SD); ER, oestrogen receptor; FISH, fluorescence *in situ* hybridization; HR, hazard ratio; IHC, immunohistochemistry; *IGH*, immunoglobulin heavy locus; IM, intramuscularly; MTD, maximum tolerated dose; mTOR, mammalian target of rapamycin; *N*, number of patients; OD, once daily; OS, overall survival; PFS, progression-free survival; PI3Kα, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), catalytic subunit-α; PO, oral route; PR, partial response; RB, retinoblastoma protein; RP2D, recommended Phase II dose; RR, response rate; SD, stable disease; TNBC, triple-negative breast cancer.

palbociclib, an observation that is likely to reflect the central nature of cyclin D1 in promoting ER-positive breast cancer proliferation regardless of whether high *CCND1* expression was due to amplification or other mechanisms.

As a follow-up to these findings, multiple Phase II and III trials of combination therapies were initiated. The combination therapies tested each include a dual CDK4 and CDK6 inhibitor (abemaciclib, LEE011 or palbociclib) and a hormone therapy (letrozole, anastrazole or fulvestrant) (TABLE 2).

CDK4 and CDK6 inhibitor biomarker strategies. Preclinical work has defined a series of biomarkers that may be used in the selection of tumours that may respond to dual CDK4 and CDK6 inhibitors. The most conservative and best supported of these markers is the direct assessment of the CDK4–RB–$p16^{INK4A}$ pathway. Data from multiple groups have demonstrated that RB is necessary for the arrest induced by CDK4 and CDK6 inhibition[152–157,175,176], and loss of RB is therefore a marker of resistance to CDK4 and CDK6 inhibition. Loss of RB results in supra-physiological expression of $p16^{INK4A}$, which may also be a biomarker of resistance. For example, high levels of $p16^{INK4A}$ are identified in malignancies caused by human papilloma virus, a virus that can inactivate RB in cervical and in head and neck cancers[29,177]. Whether dual assessment of RB loss and induction of $p16^{INK4A}$ expression is better than either biomarker alone is uncertain. There remains a considerable need to identify other predictive markers for tumours with CDK4 and/or CDK6 dependence or 'addiction' that can be selectively targeted. Examples of other predictive markers could be the amplification of cyclin D1 or CDK4 and CDK6, loss of $p16^{INK4A}$ or other genetic alterations leading to the deregulation of CDK4 or CDK6 activity. This concept has been incorporated into 'basket trial' designs with palbociclib (LUNG-MAP) and LEE011 (SIGNATURE), in which patients with specific signature mutations that would be expected to deregulate CDK4 and CDK6 activity can be enrolled for treatment with these inhibitors.

The future of CDK4 and CDK6 inhibitors. Preclinical and clinical data suggest that dual CDK4 and CDK6 inhibitors could have broad-ranging efficacy in many cancer indications. Several questions have arisen from the published work regarding understanding which diseases would benefit the most from dual CDK4 and CDK6 inhibitors.

One important question is how to determine whether an RB-proficient tumour benefits from CDK4 and CDK6 inhibition. In some tumour types, CDK4 and CDK6 inhibition has a surprisingly modest clinical effect despite

molecular alterations indicating a robust response[163,178]. Some cancer types seem either to be innately resistant or to acquire rapid resistance to the effects of CDK4 and CDK6 inhibition. For example, CDK4 and CDK6 suppression seems to have little clinical effect in colorectal cancer, triple-negative breast cancer and melanomas. Therefore, such tumours would not benefit from monotherapy in the absence of potent combination strategies and robust predictive markers. In these cancers, other CDKs, particularly CDK2, are likely to compensate for selective CDK4 and CDK6 inhibition. However, the factors that determine whether other CDKs can compensate for CDK4 and CDK6 inhibition are poorly understood.

Another question is how to optimize schedules for treatment. CDK4 and CDK6 inhibition antagonizes the effect of cytotoxic chemotherapy and radiotherapy because the vast majority of cytotoxic chemotherapies require cells to be cycling[179–181]. Despite this requirement, several studies are underway to evaluate scheduling with CDK4 and CDK6 inhibition, following the concept that release from CDK4 and CDK6 inhibition may synchronize cells and thereby sensitize cancer cells to a subsequent cytotoxic treatment, or may prevent ongoing proliferation or re-population of cancer cells between cytotoxic administrations[182]. Although such scheduling approaches have been shown to be potentially beneficial in preclinical models, translating this to the clinic, where proliferation rates of tumours are highly variable, will be challenging. A variation of this principle in patients with a known RB-inactivated cancer is the potential use of CDK4 and CDK6 inhibition to protect normal cells from the effect of chemotherapy or radiotherapy while rendering the tumour vulnerable[179].

Finally, it is important to determine ideal combinations. Considerable interest lies in the potential for combining CDK4 and CDK6 inhibitors with other targeted therapies. Substantial preclinical work has demonstrated that CDK4 and CDK6 inhibition may be synergistic with MEK inhibition in *NRAS*-positive melanoma[183]. Similarly, in pancreatic cancer, CDK4 and CDK6 inhibition is synergistic with inhibitors of insulin-like growth factor 1 receptor and mTOR[184,185]. Furthermore, in cancers with mutated phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), catalytic subunit-α (PIK3CA), PI3K inhibitors may synergize with CDK4 and CDK6 inhibitors[160]. Studies with LEE011 have incorporated this CDK4 and CDK6 inhibitor into the triplet combinations with a PIK3CA inhibitor (BYL719) and letrozole (ClinicalTrials.gov identifier: NCT01872260), and the mTOR inhibitor everolimus with exemestane in ER-positive breast cancers (NCT01857193). Recently released data from a study in melanoma suggest that such rational combinations are effective and that CDK4 and CDK6 inhibition could represent a preferred combination agent with a range of targeted agents[186].

Conclusion

CDK complexes have critical roles in multiple aspects of biology, including proliferation control and transcription. After the generally disappointing results seen in clinical trials with non-selective CDK inhibitors, the importance of selectivity of compounds for specific CDKs and of patient selection is now widely accepted. The main challenges will be in the development of a suite of highly selective agents against specific CDKs, companion diagnostics that will enable the selection of appropriate patient populations, and a firm understanding of the intersection of pharmacology and biology that will provide the basis for rational drug combinations. Now, >10 years after Hunt, Nurse and Hartwell were awarded the Nobel Prize for the identification of CDKs, the promise of their seminal studies is finally beginning to be realized.

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Competing interests statement
The authors declare competing interests: see Web version for details.

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