Treating cancer with selective CDK4/6 inhibitors

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Abstract | Uncontrolled cellular proliferation, mediated by dysregulation of the cell-cycle machinery and activation of cyclin-dependent kinases (CDKs) to promote cell-cycle progression, lies at the heart of cancer as a pathological process. Clinical implementation of first-generation, nonselective CDK inhibitors, designed to inhibit this proliferation, was originally hampered by the high risk of toxicity and lack of efficacy noted with these agents. The emergence of a new generation of selective CDK4/6 inhibitors, including ribociclib, abemaciclib and palbociclib, has enabled tumour types in which CDK4/6 has a pivotal role in the G_1-to-S-phase cell-cycle transition to be targeted with improved effectiveness, and fewer adverse effects. Results of pivotal phase III trials investigating palbociclib in patients with advanced-stage oestrogen receptor (ER)-positive breast cancer have demonstrated a substantial improvement in progression-free survival, with a well-tolerated toxicity profile. Mechanisms of acquired resistance to CDK4/6 inhibitors are beginning to emerge that, although unwelcome, might enable rational post-CDK4/6 inhibitor therapeutic strategies to be identified. Extending the use of CDK4/6 inhibitors beyond ER-positive breast cancer is challenging, and will likely require biomarkers that are predictive of a response, and the use of combination therapies in order to optimize CDK4/6 targeting.

Dysregulated cell division, resulting in aberrant cell proliferation, is one of the key hallmarks of cancer, and identifying therapeutic targets to block cell division is a widely used approach to cancer treatment. For a cell to divide, it must first progress through a predetermined number of stages, which are under the control of a complex network of regulatory factors, termed the cell cycle — a process that is highly conserved among eukaryotes[1]. Each stage of the cell cycle must be passed through in turn, with strict control over completion of all the necessary processes exercised at signalling checkpoints, thus precluding progression in the presence of, for example, genetic damage to the cell[2]. Transition from one stage of the cell cycle to the next is controlled by the actions of cyclin-dependent kinases (CDKs), which are activated upon interaction with their partner cyclins. CDKs have, therefore, long been regarded as promising targets for cancer therapies, although many of the early, first-generation CDK inhibitors failed in clinical development[3,4], at least in part because nonselective pan-CDK inhibition was found to be toxic to noncancer cells[5].

These issues of effectiveness and toxicity seem to have been overcome by more selective targeting of CDKs 4 and 6, a pair of kinases that are similar in structure and function, which mediate transition from G_0/G_1-phase to S-phase of the cell cycle. Three of these new CDK4/6 inhibitors — abemaciclib, palbociclib and ribociclib — have emerged, following the findings of early phase trials[6–17], as agents with promising anticancer activity and manageable toxicity; phase III trials are currently in progress for each drug. Of these agents, palbociclib has progressed furthest towards the clinic, having received accelerated approval from the FDA in February 2015, with pivotal phase III data available, in the setting of hormone receptor (HR)-positive, advanced-stage breast cancer — a disease in which signalling of the cyclin D–CDK4 axis is known to be critical[6,18,19]. Further work is required to facilitate optimal selection of patients and to tackle the inevitable emergence of resistance in the metastatic setting. In this Review, we discuss the biological rationale for targeting CDK4/6, review the available clinical evidence for the agents that are furthest advanced in development, and discuss the challenges with regard to optimizing their use.

Targeting CDK4/6 in cancer

CDK4/6 and G_1–S-phase transition

The cell cycle is regulated by the interaction of cyclins with their partner serine/threonine CDKs. The importance of CDKs to the cell cycle was first elucidated by the discovery of cdc28 and cdc2 (homologues of CDK1 in humans) in budding and fission yeast, respectively[20,21],

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Key points

- The actions of cyclin-dependent kinases (CDK) 4/6, through phosphorylation of retinoblastoma-associated protein 1 (RB1) are pivotal in the transition from G1 to S phase in many cancer cells
- The effectiveness of non-selective inhibition of CDKs is hampered by toxicities, selective CDK4/6 inhibition results in fewer toxicities and also provides promising antitumour effectiveness in various tumour types
- Evidence of antitumour activity from phase III trials is currently available for palbociclib in patients with hormone-receptor (HR) positive metastatic breast cancer that have progressed on prior endocrine therapy
- CDK4/6 inhibitors are most effective in combination with endocrine therapy in patients with HR-positive breast cancer: preclinical data support the combination of CDK4/6 inhibitors with PI3K and/or MAPK inhibitors
- Loss of RB1 function is an established mechanism of primary resistance to CDK4/6 inhibitors *in vitro*, but this, and other biomarkers are yet to be validated clinically

with the specific interacting cyclins described a decade later[22,23]. A further 10 years passed before the homologues of cdc28/cdc2 were confirmed as being present in other mammalian systems and for the cyclin–CDK nomenclature to be adopted[24,25]. To enter the cell cycle, a cell must progress from G_1 to S phase via this restriction point, a transition that is in part governed by the retinoblastoma-associated protein (RB1) and is usually regulated through perturbations in a delicate balance between promitotic and antimitotic signals. Mitogenic signalling is critical for entry into the normal cell cycle, although its importance is greatly reduced once the cell has entered the S phase[26].

According to the classic view of the initiation of the cell cycle, the D-type cyclins, cyclins D1, D2 and D3, are the key drivers of G_1-to-S-phase transition[27–30] (FIG. 1a,b). The expression level of the D-type cyclins is controlled by growth factor signalling, with transcription, turnover and nuclear transport of these proteins all dependent on mitogenic signalling[31–33]. Early in the G_1 phase of the cell cycle, an overall promitotic signalling balance results in increased expression of the D-type cyclins, which complex with, and activate CDK4/6. This complex subsequently phosphorylates RB1, and the other RB1-like, 'pocket' proteins p130 and p107 (also known as retinoblastoma-like proteins 1 and 2, respectively), at a number of positions[34–36]. In its hypophosphorylated state, RB1 represses the transcription of genes that are necessary for cell-cycle progression by binding to the transactivation domain of the E2F transcription factor family of proteins[37–40]; thus, increasing phosphorylation of RB1 by the cyclin D–CDK4 complex reduces inhibitory control of the E2F transcription factor family by RB1. This reduced inhibition of E2F transcription factors initiates a positive feedback loop, as the E2F transcription factors promote transcription of the E-type cyclins, which activate CDK2 and other proteins that are important for initiation of S phase and DNA synthesis[41,42] (FIG. 1b). Cyclin E–CDK2 further phosphorylates RB1, reducing E2F inhibition and promoting S-phase entry. During S phase, CDK2 complexes with cyclin A and mediates transcriptional control of DNA synthesis[43–45]. Throughout the process of progression through S phase and G_2 phase of the cell cycle, RB1 remains hyperphosphorylated, returning to its hypophosphorylated state only following mitosis[46–48].

Regulation of the E2F family of transcription factors remains the best-described mechanism through which RB1 exerts control over the cell cycle; however, other mechanisms are also likely to exist because RB1 interacts with more than 100 other proteins, and most of these interactions are currently poorly understood[49]. Furthermore, evidence exists that RB1 exerts transcriptional control through chromatin remodelling; phosphorylation of RB1 leads to a weakening of its interaction with histone deacetylases and modulates cyclin E and cyclin A transcription through the formation of regulatory complexes between RB1 and SWI/SNF chromatin-remodelling proteins[50,51].

Members of the inhibitor of CDK4 (INK4) and cyclin-dependent kinase inhibitor 1/kinase inhibitory protein (CIP/KIP) protein families also regulate and control cyclin D–CDK4/6 activity, and are known collectively as the cyclin-dependent kinase inhibitors (CKIs)[31]. The INK4 group consists of four structurally-related proteins, $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$, which specifically bind to CDK4 and CDK6 and have limited affinity for other CDKs[52–55]. Of the INK4 group, $p16^{16INK4A}$ is the best described and its expression is induced by a number of cellular processes, such as oncogenic signalling, senescence, transforming growth factor-β (TGFβ) signalling, and contact inhibition[56–58] (FIG. 1a). Increased expression of $p16^{16INK4A}$ is a hallmark of tumours in which RB1 function has been lost[59–62]. The CIP/KIP family is comprised of three proteins, the ubiquitously expressed $p27^{KIP1}$ and $p21^{CIP1}$, and a third member, $p57^{KIP2}$, which is expressed in a limited number of tissues[63–68]. In contrast to the members of the INK4 family, the CIP/KIP proteins are able to bind to all of the CDKs involved in the cell cycle to varying degrees, and can have both a positive and negative regulatory role depending on the proteins that are complexed. The control of G_1-to-S-phase transition exerted by these two groups of proteins is complex and interlinked, incorporating a number of feedback loops. $p16^{INK4A}$ is the best known inhibitor of cyclin D–CDK4, and contributes to G_1 arrest in two ways. Firstly, to become functional, CDK4 requires cytoplasmic, post-translational folding in a complex involving heat shock protein (HSP) 90, an interaction that is disrupted by $p16^{INK4A}$ (REFS 69–71). In addition, $p16^{16INK4A}$ can bind to CDK4 directly and inhibit its catalytic activity[52,71]. The combination of these two mechanisms results in G_1 arrest in cells with functional RB1, but not in RB1-deficient cells[72]. By contrast, the CIP/KIP proteins $p21^{CIP1}$ and $p27^{KIP1}$ can stabilize the formation of cyclin D–CDK4 complexes, thus sequestering these proteins and facilitating the activation of CDK2 (REFS 73–77) (FIG. 1a,b).

Non-classical CDK4/6 and G_1–S transition

According to the classic view of G_1-to-S-phase transition, cyclin D and CDK4/6 are the key initiators, with the activity of CDK2 depending on prior activation of CDK4/6 (FIG. 1a,b). However, doubts over this view of G_1-to-S-phase transition were raised by the

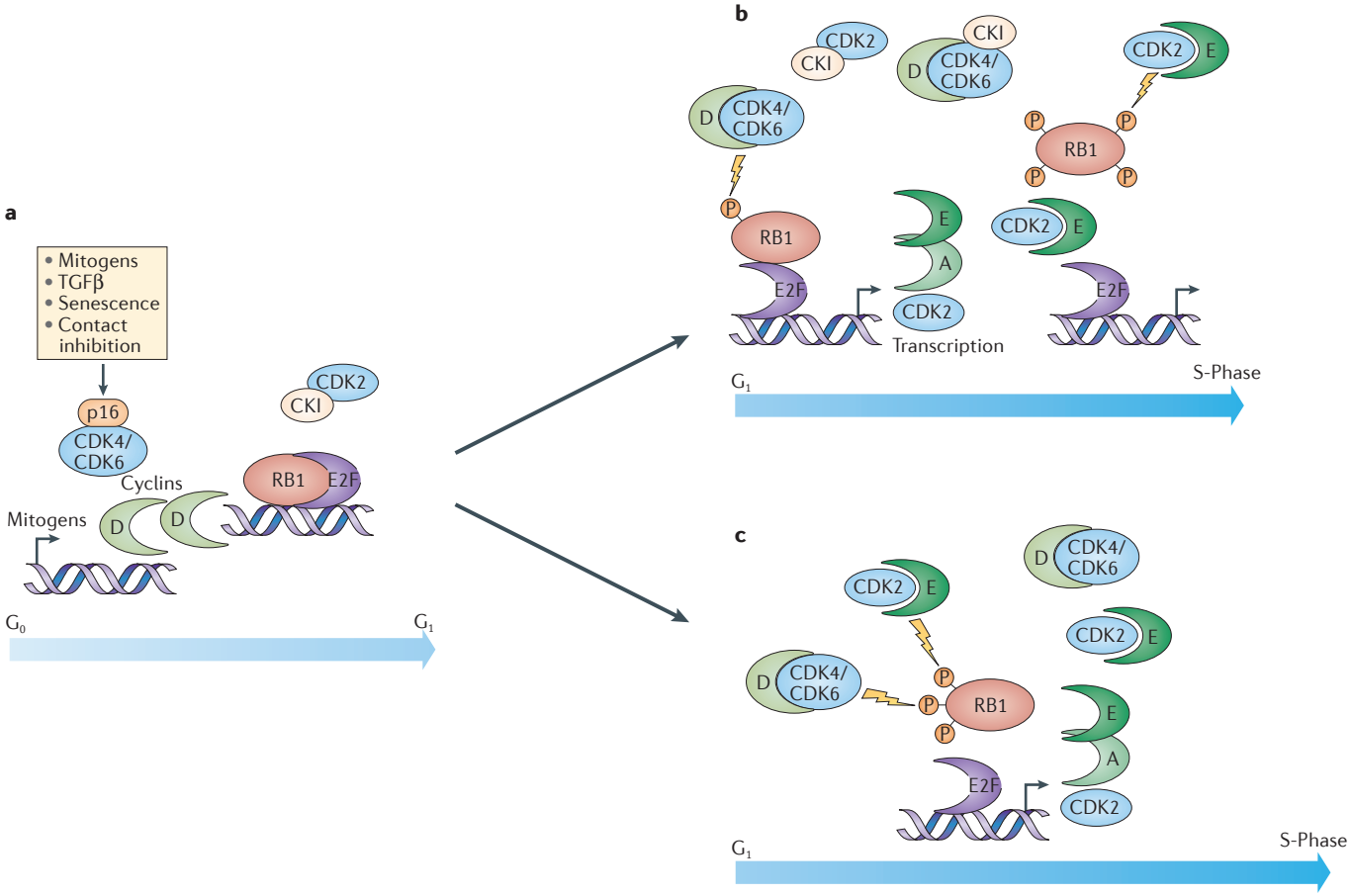


Figure 1 | **Classical and non-classical models of the cell cycle in RB1-proficient cells. a** | Resting cells in the G_0 or early G_1 phase. The retinoblastoma protein, RB1, is hypophosphorylated and inhibits the transcriptional activity of the E2F family of proteins. The INK4 protein p16, acts as a brake on the activation of cyclin-dependant kinase (CDK) 4 and/or CDK6. **b** | The classical model of G_1–S-phase transition. Mitogenic and oestrogen receptor signalling upregulates the transcription of the D-type cyclins. These D-type cyclins form a complex with CDK4/6 to phosphorylate RB1, thus partially activating the E2F-family proteins, which results in transcription of cyclins A and E, and CDK2. The phosphorylation of RB1 also induces chromatin remodelling that favours transcription (not shown). CDK4/6–cyclin D complexes sequester CDK inhibitor 1/kinase inhibitory protein (CIP/KIP) proteins, reducing their inhibitory effect on CDK2, and reducing the threshold for activation of CDK2 by E-type cyclins. As cyclin E levels rise, cyclin E complexes with CDK2 to hyperphosphorylate RB1, forming a positive feedback loop via E2F, releasing and fully activating E2F, to push the cell from G_1 to S phase. **c** | The non-classical model of G_1–S-phase transition. CDK2 is active in early G_1, by forming complexes with cyclins E and potentially cyclin D directly. Both CDK4/6 and CDK2 phosphorylate RB1, and drive G_1–S-phase transition. The mechanisms through which CDK2 becomes active in G_1 without requiring prior CDK4/6 activation are poorly understood, although in some rapidly proliferative cells CDK2 remains active immediately after mitosis. CKI; cyclin-dependent kinase inhibitor.

findings of experiments conducted using *cdk4* and *cdk6* knockout mouse models. *cdk4*-deficient mice were viable, but small in size, with reproductive and endocrine dysfunction[78–80]. Similarly, *cdk6*-deficient mice were also viable, but with hypocellularity in the thymus and spleen, and with a small reduction in the abundance of peripheral blood cells[81]. The lack of phenotypes with more-severe consequences for survival in these single-knockout mice was assumed to reflect functional compensation between *cdk4* and *cdk6*. Surprisingly, although *cdk4*/*cdk6* double-knockout mice succumbed to anaemia in the late stages of embryonic development, many non-haematological cell types from these mice were able to proliferate normally[81]. In addition, embryonic fibroblasts without *cdk4* and *cdk6* could enter S phase, although with reduced efficiency, with evidence indicating that D-type cyclins can interact with cdk2 to drive cell-cycle transition[81]. Experimental data from mouse models might be limited in predicting CDK dependency in human cells; however, the phenotype of the *cdk4/6* knockout mouse predicted, with a high level of accuracy, the toxicity profile seen with first-generation selective CDK4/6 inhibitors in human patients[6,10–13]. The architecture of the classical view of the cell cycle, with the restriction point at the G_1–S transition, has also been challenged by the demonstration that CDK2 activity might persist directly after mitosis, with

premitotic levels of CDK2 and p21^{CIP1} activity predicting whether postmitotic daughter cells continue to progress through the cycle or become quiescent[82].

Despite caveats in extrapolating experimental data from murine and *in vitro* models to human patients, data indicate that the classical view of cell-cycle entry, with the necessary role of CDK4/6, is probably overly simple in many cell types. As well as CDK4/6, other CDKs can also initiate entry to the cell cycle, owing to redundancy in function between different CDKs[83,84], and as such, CDK4/6 is potentially redundant in some cells (FIG. 1c). The exact mechanisms that underlie this redundancy among the CDKs have been incompletely described, although binding of cyclin D1 to CDK2 (REFS 81,85), and dysregulation of *CCNE1* (the gene that encodes cyclin E) expression might all contribute (FIG. 1c). CDK3 can also contribute to cell-cycle entry by phosphorylating RB1 during the G_0-to-G_1 transition[86].

Identifying a therapeutic window

The ideal CDK-targeted therapy would block CDK-mediated signalling in malignant cells, but spare the aspects of CDK activity that are critical to normal cell function, thus avoiding toxicity. Mouse embryos lacking *cdk1* fail to develop beyond the blastocyst stage[84], suggesting that inhibition of CDK1 by nonspecific inhibitors could affect most or all cell types and result in toxicity. In addition, nonspecific targeting of CDKs might also result in inhibition of CDKs 7, 8 and 9, the exact functions of which are less well-established, but include regulation of basal transcription; CDK 7 also contributes to the cell cycle as a CDK-activating kinase[87–92]. The challenge in finding a therapeutic window wherein CDK inhibition is both safe and effective was reflected in the early clinical experience with pan-CDK inhibitors, such as alvociclib and seliciclib. Alvociclib is a semi-synthetic flavone that inhibits CDKs 1, 2, 4, 6, 7 and 9, and was extensively investigated in early phase trials. Responses to alvociclib were seen in phase II studies in patients with haematological malignancies, notably chronic lymphoid leukaemia, but dosing was limited by toxicity[93–98]. Seliciclib, a purine-based compound that is active against CDKs 1, 2, 5, 7 and 9, failed to demonstrate any convincing clinical activity in two phase I studies[99,100]. The toxicity profile of seliciclib included nausea, vomiting and fatigue, in addition to hepatic dysfunction and abnormalities in electrolyte levels; alvociclib caused fatigue, diarrhoea and a degree of myelosuppression[94,95]. Delineating to what degree these toxicities were the result of on-target effects remains difficult. Of note, seliciclib has less inhibitory activity on CDK4/6 (IC_{50} >10 μM) than alvociclib or the selective CDK4/6 inhibitors, both of which inhibit CDK4 at nanomolar concentrations (reported IC_{50} of 100 nM and 11 nM for alvociclib and palbociclib, respectively), and seliciclib treatment resulted in less myelosuppression than that seen following treatment with other CDK inhibitors[5].

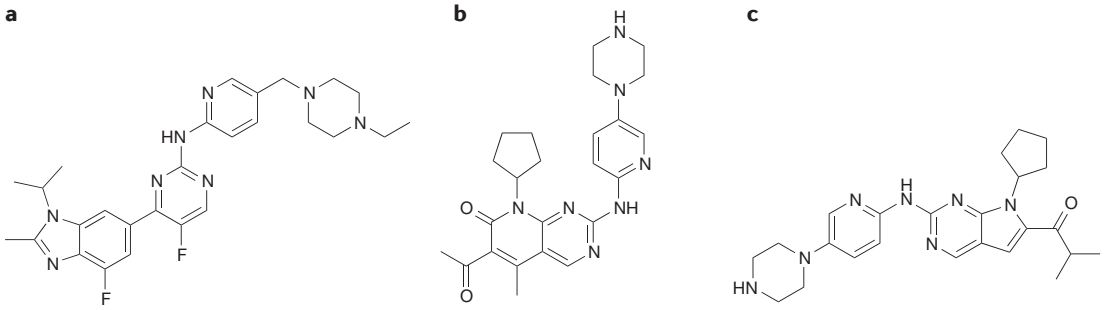
More-selective targeting of CDK4/6 has a number of potential advantages over the use of less-selective inhibitors. Many types of somatic cells might be capable of initiating the cell cycle despite CDK4/6 inhibition[81]. Additionally, in contrast to the cytotoxic effects of less selective CDK inhibitors, CDK4/6 inhibitors are usually found to have cytostatic effects, which might further limit the potential of these agents to cause clinical toxicity, although CDK4/6 inhibition-induced cell death has been noted *in vitro* in cell lines and in xenografts derived from patients with T-cell leukaemia[101,102].

The CDK4/6 axis deranged in cancer

Selection of appropriate target groups for CDK4/6 inhibition relies on successful identification of the tumour types in which CDK4/6 drives G_1-to-S transition, and in which the effects of CDK4/6 inhibition cannot be rescued by the activity of other CDKs. Aberrations in the cyclin D–CDK4/6 axis are frequent occurrences in patients with cancer. A notable example is provided by mantle-cell lymphoma; this form of lymphoma is characterized by the t(11;14) (q13;q32) translocation that juxtaposes *CCND1* with the *IGH* immunoglobulin heavy chain locus, resulting in the overexpression of cyclin D1 (REFS 103–106). Furthermore, amplification and overexpression of cyclin D has been described in patients with head and neck cancer[107–110], breast cancer[111–115], non-small-cell lung cancer (NSCLC)[116,117], oesophageal cancers[118,119], melanoma[120–122] and glioblastoma[123,124].

Overexpression of the CDKs is a further potential mechanism of activation of the cyclin D–CDK4/6 signalling axis, although activating somatic mutations are very rare. Amplifications of *CDK4* are seen in well-differentiated and de-differentiated liposarcomas, as part of a 12q14.15 amplicon, although this amplified section of chromosome 12 also features *MDM2*, which encodes E3 ubiquitin-protein ligase MDM2, and *HMGA2,* which encodes high-mobility group protein HMGI-C, meaning that uncertainty exists regarding the identity of the key driver[125–127]. Somatic amplifications of *CDK4* have been observed in patients with melanoma and in those with glioblastoma[121,128,129], and amplifications of *CDK6* have been detected in patients with squamous-cell oesophageal carcinoma[130] and in a small number of patients with B-cell lymphoproliferative disorders with translocations involving chromosome 7q21 (REFS 131–133). The relationship between amplification of *CDK4*, CDK4 activity, and CDK4/6 inhibition is unclear, with reports suggesting that both increased expression and amplification of *CDK4* is associated with resistance to selective CDK4/6 inhibition[128,134]. Germline *CDK4* mutations in the p16^{INK4A} binding domain have been reported in a small number of families with a genetic predisposition to melanoma[135–137].

Loss of p16^{INK4A} function is a common occurrence in cancer and implies an absence of the primary inhibitory brake on CDK4/6-driven signalling. Homozygous deletions of *CDKN2A*, (the gene that encodes p16^{INK4A}) are seen in tumours of the pancreas, bladder, breast, prostate and in glioblastoma[138–140]. An important role of p16^{INK4A} is implied in melanoma by the finding of a common deletion of *CDKN2A* in kindreds with a high risk of melanoma[141]. Conversely, loss of RB1 function results in constitutive activation of E2F, cyclin E1 and CDK2 expression, and therefore loss of reliance on CDK4/6 to initiate G_1-to-S-phase transition[142,143].



	Abemaciclib (LY-2835219)	Palbociclib (PD-0332991)	Ribociclib (LEE011)
IC_{50}	CDK1: >1 μM	CDK1: >10 μM	CDK1: >100 μM
	CDK2: >500 nM	CDK2: >10 μM	CDK2: >50 μM
	CDK4: 2 nM	CDK4: 9–11 nM	CDK4: 10 nM
	CDK5: ND	CDK5: >10 μM	CDK5: ND
	CDK6: 5 nM	CDK6: 15 nM	CDK6: 39 nM
	CDK7: 300 nM	CDK7: ND	CDK7: ND
	CDK9: 57 nM	CDK9: ND	CDK9: ND

Figure 2 | **Chemical structure of selective CDK4/6 inhibitors. a** | Abemaciclib. **b** | Palbociclib. **c** | Ribociclib. The half maximal inhibitory concentrations (IC_{50}) of these agents for a number of cyclin-dependent kinases (CDKs) are also shown.

Breast cancer subtype dependency of cyclin D1. In patients with luminal oestrogen receptor (ER)-positive breast cancer, which represents approximately 75% of all breast cancers, ER signalling activates the *CCND1* promoter, and in many ER-positive breast cancers, cyclin D1 is expressed at a high level, with or without *CCND1* gene amplification[111,113]. Cyclin D1 is also known to have a number of CDK-independent functions that probably contribute to the pathogenesis of breast cancer[144]. Cyclin D1 binds to, and facilitates ER transcriptional activity[144], likely reinforcing the dependence of ER-positive luminal breast cancer on cyclin D1. By contrast, expression levels of cyclin E1 are low in patients with ER-positive breast cancer[145], and RB1 is rarely inactivated by mutation[146].

Thus, ER-positive, luminal breast cancer is the archetypal model for investigating the effectiveness of CDK4/6 inhibitors, reflecting the particular dependence of these cancers on cyclin D1 to initiate G_1-to-S-phase transition. In addition, even as breast cancers become resistant to endocrine therapy, they remain dependent on cyclin D1 and CDK4 to drive cell proliferation[147]. In contrast with luminal breast cancer, basal-like triple-negative breast cancer is characterized by the loss of RB1 activity[148–150] and by increased expression of cyclin E1 (REF. 145). Consequently, basal-like breast cancer cell lines are resistant to CDK4/6 inhibition *in vitro*[142]. High expression levels of cyclin E2 have been found in luminal B breast cancers and are correlated with a shorter time to distant progression, although the role of cyclin E2 in CDK4-inhibitor sensitivity remains to be determined[151].

Preclinical development

Three CDK4/6 inhibitors have currently reached early phase trials, abemaciclib, palbociclib and ribociclib[6–17], with phase III data now available for palbociclib[18,19]. These orally-administered compounds are of similar structure (FIG. 2), bind within the ATP-binding pocket of CDK4 and CDK6 (REFS 5,152) (FIG. 3a), and all have a high degree of selectivity for CDK4 and CDK6, compared with CDK1 and CDK2. Preclinical research in cell lines and xenografts has focused on malignancies with established derangements of the cyclin D–CDK4–$p16^{INK4A}$ axis and has revealed the predominant effect of CDK4/6 inhibitors to be cytostatic rather than cytotoxic[153–161].

Abemaciclib inhibits CDK4/6 at low nanomolar concentrations and has been shown to reduce the phosphorylation of RB1 in colorectal cancer and melanoma xenografts, thus inducing G_1 arrest[153,154]. In addition to CDK4 and CDK6, abemaciclib is also reported to reduce the activity of CDK9; although, whether this translates into inhibition of the cellular activity of CDK9 is currently unclear[154]. Abemaciclib has also been demonstrated to induce growth regression in vemurafenib-resistant melanoma models, in which expression of cyclin D1 was noted to be elevated in conjunction with MAPK-pathway reactivation *in vitro*[162].

Palbociclib is an inhibitor of CDKs 4 and 6 at low nanomolar concentrations, but has limited inhibitory effects on other CDKs or tyrosine kinases[155,163]. Palbociclib has been shown to be active in mantle-cell lymphoma xenografts[156], and in glioblastoma cell lines wherein, in addition to the presence of functional RB1, codeletion of *CDKN2A* and *CDKN2C* (which encodes $p15^{INK4B}$) was found to predict sensitivity to this agent[128,157,158]. In ovarian cancer cell lines, a response to palbociclib was found to be most marked in RB1-proficient cell lines with low $p16^{INK4A}$ expression, with deletions in *CDKN2A* associated with responsiveness, and amplification of *CCNE1* associated with resistance[143]. Research into the effects of palbociclib on renal-cell carcinoma cell lines identified low E2F1 expression as another potential marker of sensitivity, in addition to loss of $p16^{INK4A}$ expression[159]. Additionally, activity of palbociclib, in combination with bortezomib, has been demonstrated in both cell line and xenograft models of acute myeloid leukaemia and

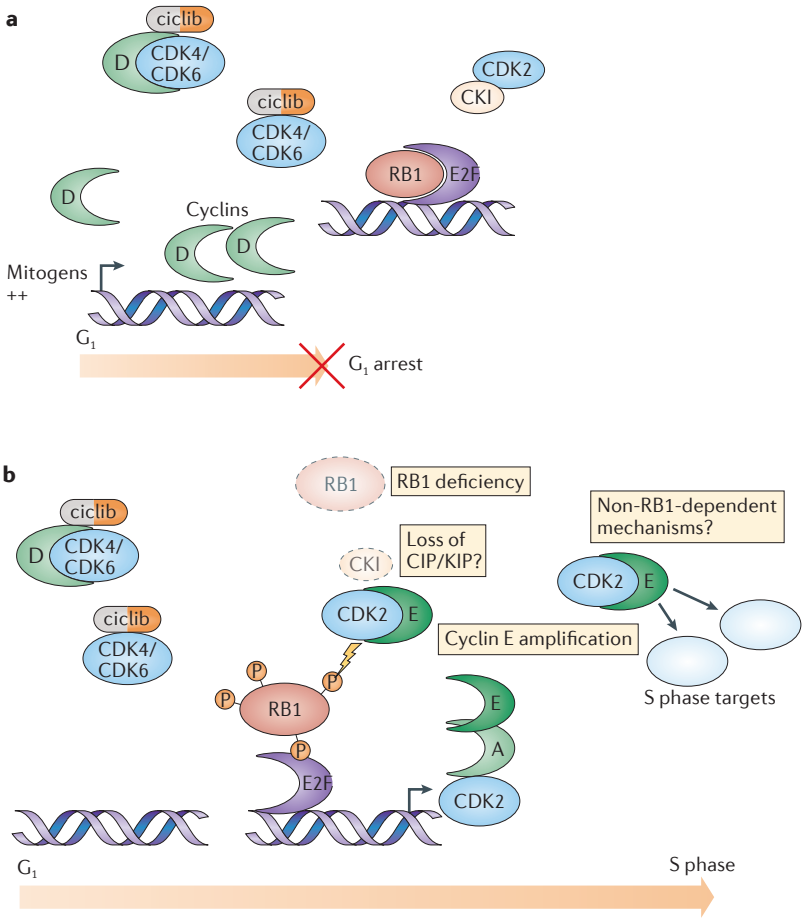


Figure 3 | **The cell cycle and the role of CDK4/6 inhibition. a** | G_1 arrest caused by cyclin-dependent kinase (CDK) 4/6 inhibition. CDK4/6 inhibitors bind to the ATP-binding domain of CDK4/6, thus competitively inhibiting the kinase activity of these proteins. The cyclin D–CDK4–retinoblastoma protein (RB1)–CDKN2A axis is commonly disrupted in cancer, for example, owing to overexpression of cyclin D or underexpression of *CDKN2A*. Under such conditions, and in the absence of an escape mechanism, CDK4/6 inhibitors can block the disinhibited phosphorylation of RB1, resulting in G_1 arrest. **b** | Potential mechanisms of resistance to CDK4/6 inhibition. In cancer cells that lack RB1 function, the E2F transcription factor family members are constitutively active and CDK4/6 signalling is redundant. In RB1-replete cells, overexpression of cyclin E or loss of the inhibitor 1/kinase inhibitory protein (CIP/KIP) proteins might bypass CDK4/6 inhibition by activating CDK2. E2F amplification is posited as another mechanism for bypassing RB1. CKI; cyclin-dependent kinase inhibitor.

myeloma, although specific biomarkers of palbociclib sensitivity were not identified in these experiments[164–166]. Palbociclib also has proven activity in RB1-replete prostate cancer cells[167] and hepatocellular carcinoma cells *in vitro*, in which, curiously, some activity was observed in RB1-deficient cells, potentially owing to compensation via other pocket proteins, such as p107 (REF. 168).

When investigated in models of breast cancer, palbociclib combined with trastuzumab or tamoxifen had a synergistic inhibitory effect on the proliferation of HER2-amplified and ER-positive cells, respectively, which are both luminal cancer types and are, therefore, reliant on cyclin D1 to activate CDK4/6 (REFS 115,142,169,170). Synergy between palbociclib and endocrine therapy in ER-positive breast cancer at least in part reflects the simultaneous effects of endocrine therapy in suppressing cyclin D1, and palbociclib in inhibiting CDK4/6. In the presence of CDK4/6 inhibition alone, persistent cyclin E2 expression continues to allow a low level of S-phase entry[171], and synergy is seen with endocrine therapy through suppression of residual cyclins. Treatment with palbociclib also results in growth arrest in breast cancer cell lines with acquired resistance to endocrine therapy, as these cells remain dependent on CDK4/6 (REF. 172).

Ribociclib inhibits CDK4/6 at nanomolar concentrations[173] and, as a single agent, has been demonstrated to inhibit the growth of neuroblastoma and liposarcoma cell lines, resulting in G_1 arrest, a reduction in the phosphorylation of RB1 at Ser780 and Ser807/811, and a significantly reduced tumour burden in neuroblastoma and liposarcoma xenografts[160,161].

Results of early phase trials

Findings from early phase trials, primarily designed to investigate the tolerability of selective CDK4/6 inhibitors, showed a manageable toxicity profile with indications of promising clinical activity[6–17]. The efficacy of selective CDK4/6 inhibitors as single agents in these trials was manifested predominantly as stable disease, which hypothetically might reflect the cytostatic nature of these agents; however, the best responses were demonstrated when tested in combination with endocrine therapy in patients with breast cancer[6].Toxicity profiles seem to vary between the different selective CDK4/6 inhibitors. The reasons for these variations are currently not understood, but might have ramifications for optimizing their clinical use and combination with other therapies.

Abemaciclib

The initial phase I study designed to investigate the effects of abemaciclib[7] comprised a cohort of 55 patients, with a variety of different tumour types; 52% of these patients experienced diarrhoea, 5% at grade 3, as a treatment-related adverse event[7]. Neutropenia was a far less prevalent adverse event in this study[7] than in the trials investigating ribociclib and palbociclib[6–17], thus enabling the use of continuous dosing schedules. One patient with *CDKN2A*$^{-/-}$ *NRAS*-mutant melanoma had a partial response to abemaciclib. In an expansion cohort of this trial in patients with NSCLC, 51% achieved at least stable disease, with 41% of patients receiving at least four cycles of treatment[8]. In the metastatic breast cancer cohort within the phase I study, 33% had a partial response, despite many patients being heavily pretreated with several other therapies; a median progression-free survival (PFS) of 9.1 months was noted in 36 patients with ER-positive breast cancer[9].

Palbociclib

Two of the phase I studies investigating palbociclib as a single agent were conducted in patients with RB1-expressing cancers, with signs of efficacy manifesting predominantly as stable disease[10,11]. In a third study comprising 17 patients with mantle-cell lymphoma, five patients had a PFS duration of >12 months[12]. Similar dose-limiting toxicities were seen across these

three phase I studies[10–12], and among grade 3–4 events neutropenia was the most common. This adverse event required intermittent therapy, and a dosing schedule of 125 mg daily for 3 weeks with the fourth week off therapy in two of these trials[10,11]. Three patients participated in a phase I trial that enrolled those with growing teratoma syndrome, which was refractory to surgery and had confirmed strong expression of RB1 (REF. 174). These patients achieved at least stable disease and remained on treatment for 18–24 months[174]; a similar case in a paediatric patient with inoperable growing teratoma syndrome showed disease stabilization in response to palbociclib[175]. The efficacy of palbociclib has been investigated further in a phase II study of 30 patients with relapsed, RB1-proficient germ-cell tumours, in which eight patients had a PFS duration of >24 weeks[176].

In a phase II study of palbociclib as a single agent, 37 patients with RB-proficient breast cancer were included; two patients had partial responses to treatment, and a further five patients achieved stable disease for at least 6 months despite having been heavily pretreated[13]. In a phase II trial with a cohort of patients with liposarcoma, 66% of the 29 evaluable patients in this cohort had no disease progression after 12 weeks of pabociclib treatment, with one patient having a partial response[177].

Ribociclib

Ribociclib has been tested as a single agent in phase I trials using two different dosing schedules: either continuously, or 3 weeks on, 1 week off. In a cohort of 132 patients with RB1-positive advanced-stage solid tumours or lymphomas, cytopenias were the predominant dose-limiting toxicities, particularly neutropenia and leukopenia, with the most common adverse effects of any grade being nausea and fatigue[14]. Two patients had a partial response to treatment; of these, one with melanoma and one with breast cancer, and both had amplifications of *CCND1*. In a trial comprising a cohort of 14 patients with *NRAS*-mutated melanoma who received ribociclib in combination with the MEK inhibitor binimetinib, six patients had a partial response[15]. Phase Ib/II trials examining the efficacy of ribociclib in combination with the PIK3CA inhibitor BYL719 or the mTOR inhibitor everolimus, in conjunction with an aromatase inhibitor in patients with postmenopausal breast cancer are currently ongoing. Only limited data from these combination trials have been reported, although, to date, no safety concerns have been raised[16,17].

Differences between CDK4/6 inhibitors

The efficacy and toxicity of palbociclib and ribociclib from early phase clinical data are very similar; however, the current experience with abemaciclib has revealed differences for this agent. Specifically, treatment with abemaciclib generally results in less bone marrow suppression and increased incidence and severity of diarrhoea[7]. In terms of efficacy, patients with pretreated breast cancer possibly have a higher response rate to abemaciclib as a single agent than to other selective CDK4/6 inhibitors[178]. Of the three inhibitors with early phase clinical evidence available, abemaciclib is the more-potent inhibitor of CDK4 as opposed to CDK6, according to data from *in vitro* kinase assays. Whether or not this factor could explain the possible increased antitumour activity of abemaciclib or the more marked diarrhoea is unclear; furthermore, the potential role of CDK9 inhibition by abemaciclib is unknown.

Differences in absorption across the blood–brain barrier exist between palbociclib, ribociclib and abemaciclib, although the evidence on this aspect is partially conflicting. Abemaciclib seems to be better absorbed across the blood–brain barrier than palbociclib[179,180], an observation that is potentially relevant for the treatment of patients with brain metastases or CNS tumours. Nonetheless, case reports describing effective treatment of patients with intracranial teratoma with palbociclib are available, suggesting that this agent can also cross the blood–brain barrier[175].

Trials in patients with breast cancer

Later-phase randomized studies aimed at investigating the therapeutic efficacy of specific CDK4/6 inhibitors are currently recruiting patients with a range of cancer types[181–186], the only published evidence to date comprises data from patients with breast cancer.

Two randomized trials have investigated the efficacy of palbociclib in patients with HR-positive advanced-stage breast cancer. The randomized, open-label, phase II PALOMA-1/TRIO-18 study[6] was conducted in patients with previously untreated, advanced-stage, ER-positive HER2-negative breast cancer. Patients had received either no prior adjuvant aromatase inhibitor or had stopped adjuvant aromatase inhibitor therapy at least 1 year before relapse[6]. 165 patients were randomly assigned to receive either letrozole alone, or in combination with palbociclib, with two consecutively accrued cohorts recruited to the study[6]. The first cohort of patients all had ER-positive HER2-negative breast cancer, whereas the second cohort was restricted on the basis of either *CCND1* amplification or loss of *CDKN2A*. During the study design, the intention was for the first cohort of patients to be exploratory, and the second to be the primary cohort for analysis of PFS. After the findings of an unplanned interim analysis demonstrated significantly improved PFS in the first cohort and a low probability of a difference with patient selection based on *CCND1* amplification or loss of *CDKN2A*, the study protocol was amended to stop accrual to a separate second cohort and to analyse both cohorts together. At the final PFS analysis, after a median follow-up duration of 30 months, this analysis demonstrated an improvement in median PFS from 10.2 months to 20.2 months with the addition of palbociclib to letrozole[6] (TABLE 1). Consistent with the findings of earlier studies, the principal toxicity associated with palbociclib was neutropenia; although, no incidences of febrile neutropenia were reported. Low-grade (grades 1–2) fatigue and nausea were also more prevalent with the addition of palbociclib to letrozole (36% versus 22%, and 23% versus 12%, respectively), along with a slightly increased incidence of the adverse effects typically seen with use of aromatase inhibitors, such as hot flushes and arthralgia. Evidence

Table 1 | **Palbociclib in women with advanced, HR-positive breast cancer**

Trial	*n*	Treatment	Outcomes
PALOMA-1/TRIO 18 (REF. 6)	165	Letrozole versus Letrozole + palbociclib	PFS: 10.2 months (5.7–12.6) versus 20.2 (13.8–27.5) months, HR 0.49; *P* = 0.0004*
PALOMA-3 (REFS 18,208)	521	Fulvestrant + placebo versus Fulvestrant + palbociclib	PFS: 4.6 months (3.5–5.6) versus 9.5 (9.2–11.0) months, HR 0.46; *P* <0.0001‡

CI, confidence interval; HR, hazard ratio; PFS, progression-free survival; *One-sided *P*-value, ‡Two-sided *P*-value.

obtained from the PALOMA-1/TRIO 18 study[6] served as the basis for accelerated approval of palbociclib by the FDA for postmenopausal, ER-positive, HER2-negative breast cancer on 3 February 2015 (REF. 187).

Phase III registration studies

The PALOMA-3 phase III[18,19] was the first to provide data on the efficacy of a CDK4/6 inhibitor. This double-blind, randomized controlled trial had a cohort of 521 patients with advanced-stage, HR-positive, HER2-negative breast cancer that had progressed on prior endocrine therapy. Patients were randomized in a 2:1 ratio to receive either palbociclib and fulvestrant or placebo and fulvestrant[18,19] — fulvestrant being a selective ER-degrading agent that has activity in patients with breast cancer[188] — after disease progression on prior endocrine therapies. The PALOMA-3 study was positive for its primary end point, with a median PFS of 9.5 months in the palbociclib plus fulvestrant arm compared with 4.6 months in the placebo plus fulvestrant arm (TABLE 1). The majority of enrolled women were postmenopausal: 21% of the women were premenopausal and were additionally treated with gonadotropin-releasing hormone (GnRH) agonists to induce ovarian suppression.

Consistent with findings from the PALOMA-1/TRIO18 study[6], the toxicity profile observed in patients in the PALOMA-3 study[18,19] included frequent haematological adverse events, but also a small increase in the incidence of mostly grade 1 or 2 fatigue, alopecia, and stomatitis. A relatively large proportion of patients in the palbociclib arm experienced grade 3–4 neutropenia (65%), and 34% required a dose reduction. Only 4% of patients stopped treatment in the palbociclib plus fulvestrant arm owing to adverse events, compared with 2% in the fulvestrant plus placebo arm. Similar to the PALOMA-1/TRIO18 trial[6], despite the high rate of neutropenia, the rate of febrile neutropenia was minimal at 1% in both arms. Infections, mainly of grade 1–2 severity, were seen more frequently with palbociclib (40% versus 27%). Global quality of life, as measured using the quality of life questionnaire C30, was significantly improved in patients who received palbociclib plus fulvestrant compared with those who received placebo plus fulvestrant. Data from the PALOMA-3 study[18,19] will likely lead to approval of palbocicilb for clinical use in many countries.

In terms of ongoing phase III trials, the confirmatory PALOMA-2/TRIO-22 study, which is designed to investigate the combination of palbociclib plus letrozole versus placebo plus letrozole in first-line treatment of patients with advanced-stage ER-positive breast cancer, has completed accrual, but the investigators are yet to report results[184]. Both abemaciclib and ribociclib are also currently the subject of investigation in ongoing phase III trials: MONARCH-2 (REF. 183), which has a similar design to PALOMA-3 (REFS 18,19) but incorporating abemaciclib instead of palbociclib, is currently recruiting; and MONALEESA-7 (REF. 181), in which researchers are examining the combination of ribociclib with endocrine therapy in premenopausal women with advanced-stage, HR-positive breast cancer.

Future challenges

A number of biologically plausible biomarkers of sensitivity to CDK4/6 inhibition are available, for example cyclin D, *CDKN2A* and/or RB1 status (FIG. 3b); however, ER-positivity in patients with breast cancer is the only selection marker currently confirmed for use in the clinical setting. Identification of further biomarkers for treatment selection in patients with ER-positive breast cancer might be difficult, as this subtype of breast cancer is often dependent on cyclin D1 and, therefore, CDK4/6 to drive proliferation. Of note, amplification of *CCND1* and/or loss of *CDKN2A* offered no further selection advantage in the phase II PALOMA-1 study[6]; although, these data are limited given the early closure of the *CCND1/CDKN2A* selected cohort and these findings would require further confirmation.

Further research is required to identify biomarkers of resistance to CDK4/6 inhibitors in patients with ER-positive breast cancer. Loss of RB1 function is an obvious candidate; loss of RB1 function is rare in patients with ER-positive breast cancer[146], although limited data are available on whether the frequency of RB1 loss changes with development of resistance to prior therapies. Amplification of *E2F* or loss of *CDKN1A*, which are both commonly observed in a variety of cancers and linked to tamoxifen resistance[189], have been proposed as two plausible markers of resistance to treatment (FIG. 3b). Identification of the potential of cyclin E–CDK2 complexes to rescue CDK4/6 inhibition, potentially through assessment of cyclin E levels, or through gene expression-based predictors of RB1/E2F proficiency could also be interesting to assess. In terms of resistance, breast cancer cell lines with derived resistance to palbociclib often acquire selective loss of RB1 and amplification of *CCNE1* (REF. 171), thus favouring the nonclassic phenotype of G_1–S-phase transition. Cell lines with acquired *CCNE1* amplification are sensitive to combined inhibition of CDK4/6 and CDK2, potentially suggesting a therapeutic strategy for treatment of tumours with acquired resistance[171].

Other tumour types, such as mantle-cell lymphoma, probably have subtype-specific sensitivity to CDK4/6 inhibitors. In many other tumour types, however, biomarkers are likely to be important in identifying selective dependence on cyclin D1–CDK4/6 signalling. The phase II/III Lung–MAP trial[185] has an experimental arm in which patients with recurrent squamous-cell carcinoma are being allocated to receive

palbociclib on the basis of aberrations in *CDK4* and *CCND1–3*. In the SIGNATURE trial[186], patients are being allocated to treatment with ribociclib on the basis of *CCND*/*CDKN2A*/*CDK4* aberrations. More information regarding the validity of various biomarkers will become available with the completion of ongoing biopsy-driven studies examining the efficacy of CDK4/6 inhibitors in the neoadjuvant setting, and the extent of progression of patients receiving CDK4/6 inhibitors.

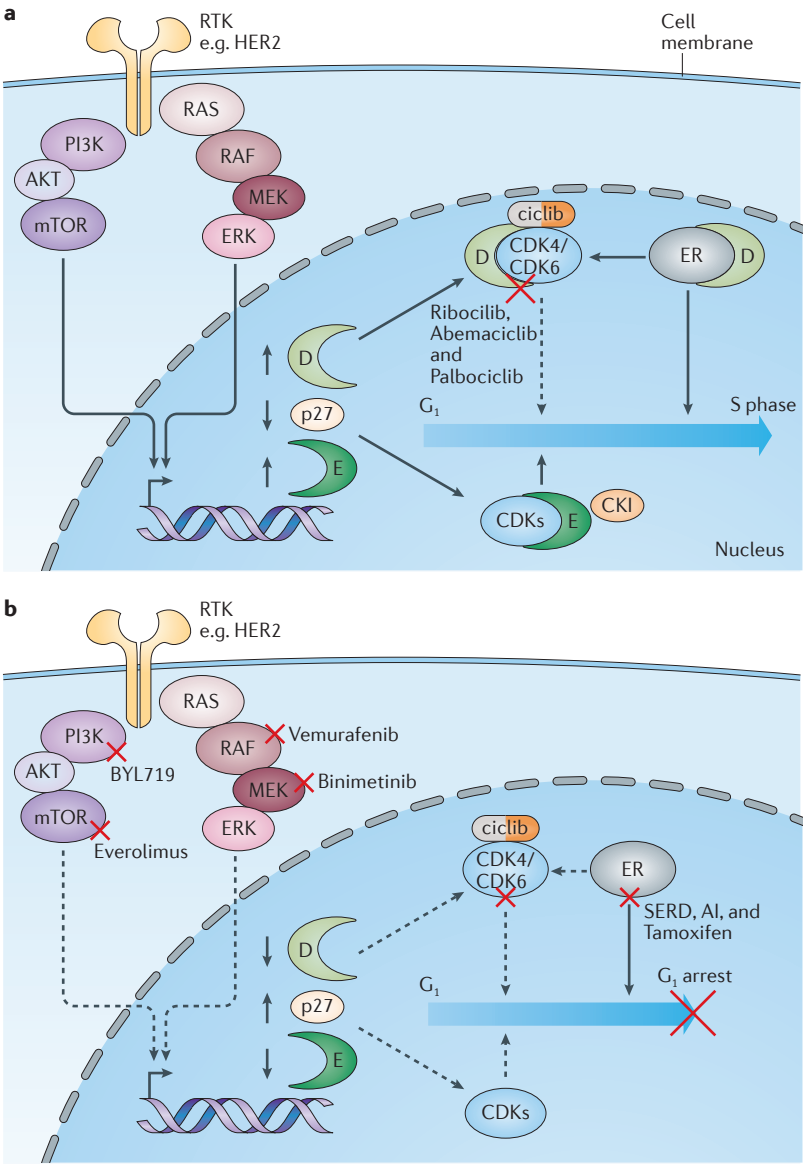


Figure 4 | **Possible combination therapies CDK4/6 inhibitors. a** | The cyclin-dependent kinases (CDKs) and cyclins act both in parallel with and downstream of cellular signal-transduction pathways and oestrogen-signalling pathways to promote cell-cycle progression. Activation of the MAPK and PI3K pathways by receptor tyrosine kinases (RTKs) promotes cell-cycle progression through upregulation of D-type and E-type cyclins. RTK signalling activates CDK4/6 signalling, but might also promote CDK4/6 inhibitor resistance, potentially through promotion of cyclin E or through inhibition of CDK inhibitor 1/CDK inhibitor 1B. Similarly oestrogen receptor (ER) signalling in ER-positive breast cancer might promote bypass of CDK4/6 inhibition, in part facilitated by cyclin D1 binding. **b** | Promising strategies for combinations of CDK4/6 inhibition with other antitumour agents, based on data from preclinical models, including blockade of ER signalling with tamoxifen, aromatase inhibitors or selective oestrogen-receptor degraders (SERDs), PI3K-pathway blockade with PI3K inhibitors and mTOR inhibition with rapamycin analogues, and MAPK pathway blockade with BRAF and MEK inhibitors. CKI; Cyclin-dependent kinase inhibitor.

Combination therapy

Which endocrine therapy in ER-positive breast cancer? CDK4/6 inhibitors have been developed almost exclusively in combination with endocrine therapies in patients with ER-positive breast cancer, based on sound preclinical evidence of the efficacy of such approaches[142]. Selection of the most-appropriate endocrine therapy for an individual patient will probably be important for the success of the combination, although this decision is also dictated by licensed indications. For endocrine-therapy-naive patients, the combination of CDK4/6 inhibition with an aromatase inhibitor is likely to be effective, as demonstrated in the PALOMA-1/TRIO-18 (REF. 6) trial, whereas in patients with endocrine-therapy-pretreated breast cancer, fulvestrant is a more-suitable combination partner, as demonstrated in the PALOMA-3 (REFS 18,19) trial. No data are available to support continued use of endocrine therapy beyond the emergence of resistance while adding a CDK4/6 inhibitor; therefore, uncertainty remains as to whether or not this approach would be effective.

A strong case, particularly in the treatment of breast cancer, exists for combining PI3K inhibitors and mTOR inhibitors with CDK4/6 inhibitors (FIG. 4). If, as has been shown in breast cancer-derived cell lines, endocrine resistance is mediated in part through ligand-independent interactions of the ER with CDK4, resulting in PI3K hyperactivation[147], and CDK4/6 inhibition can overcome resistance to both PI3K inhibition[190] and endocrine therapy[142], then this combination of targeted inhibition might prevent the emergence of resistance (TABLE 2). Of note, patients in the PALOMA3 study identified as having activating *PIK3CA* mutations in circulating tumour DNA derived a benefit from palbociclib comparable to those without such mutations. Similarly, CDK4/6 inhibition could also offer a means to address ligand-independent ER signalling conferred by activating mutations in *ESR1* and resulting in hormone-independent breast cancer[191–193]. A strong rationale also exists for the use of CDK4/6 inhibitors in combination with HER2-directed therapy in patients with *HER2*-amplified breast cancers. Increased cyclin D1 expression is observed in cellular and mouse models of HER2 overexpression and in transgenic mice with activating mutations in HER2 (REF. 169), with evidence suggesting that cyclin D1 and CDK4 are required for tumorigenesis in these cancers[194]. Consistent with this evidence, palbociclib, combined with trastuzumab had a synergistic effect in inhibiting the growth of *HER2*-amplified cells[142]. This combination is being taken forward in a number of early phase clinical trials.

Combination strategies in other malignancies. A number of combination strategies with CDK4/6 inhibitors are also being pursued as treatments for patients with haematological malignancies, including combination with bortezomib in patients with myeloma[195]; furthermore, preclinical evidence supports the combination of CDK4

inhibition with ibrutinib or PI3K inhibition in the treatment of mantle-cell lymphoma[196,197] (TABLE 2). Preclinical evidence of effectiveness also exists for CDK4/6 inhibition in combination with MAPK-pathway inhibition with MEK or BRAF inhibitors in melanoma[198] and colorectal cancer[199] (FIG. 4). CDK4/6 inhibition can also resensitize melanoma cell lines with *BRAF*^{V600E} mutations to vemurafenib once resistance has developed[162]. The mechanisms of all these combinations in part reflect suppression of cyclin D and/or cyclin E levels, thus limiting the ability of alternative CDKs to bypass CDK4/6 inhibition. RAS signalling has also been shown to promote cell cycling by reducing levels of p27^{KIP1} (REFS 200).

In lung cancer cell lines and xenografts, knockdown of CDK4 produces a greater degree of growth inhibition in *KRAS*-mutant cells than in those with wild-type *KRAS*[201]; this finding is in keeping with previous work, which suggests a degree of synthetic lethality between *cdk4* ablation and KRAS activity[202]. In addition, the potential for using CDK4/6 inhibitors to prevent tumour repopulation

Table 2 | **Current clinical strategies using CDK4/6 inhibition, alone or in combination**

Therapy	Cancer type	Biomarker	Level of evidence
CDK4/6 inhibitor plus aromatase inhibitor or SERD	HR-positive advanced-stage breast cancer	ER-positive cancer	Preclinical[142], phase I, II and III trials[6,10,13,18,19]
CDK4/6 inhibitor plus endocrine therapy, plus PIK3CA/mTOR inhibition	HR-positive advanced-stage breast cancer	ER-positive cancer	Preclinical[142,147,190], phase I trials[17,203]
CDK4/6 inhibitor plus HER2-directed therapy	HER2-positive breast cancer	*HER2*-amplification	Preclinical[115,142]
CDK4/6 inhibitor plus bortezomib or dexamethasone	Myeloma	None	Preclinical[164,165], phase I/II trial[195]
CDK4/6 inhibitor alone or in combination with ibrutinib and PI3K inhibition	Mantle-cell lymphoma	t(11:14) translocation, deregulating *CCND1*, mutated Bruton tyrosine kinase	Preclinical[156,196,197], phase I trial[12]
CDK4/6 inhibitor alone	Acute lymphoblastic leukaemia	None	Preclinical[101,102]
Combined CDK4/6 inhibitor and FLT3 inhibition	Acute myeloid leukaemia	FLT3	Preclinical[166,204]
CDK4/6 inhibitor alone	Liposarcoma	Not reported, *CDK4* amplification highly prevalent	Preclinical[161,177], phase II trial[177]
CDK4/6 inhibitor alone	Fusion-protein-positive rhabdomyosarcoma	Absence of *CDK4* amplification	Preclinical[134]
CDK4/6 inhibitor alone	Teratoma	*RB1* replete	Phase I and II trials[11,174–176]
CDK4/6 inhibitor alone	Glioma	p16 deficient, *RB1* replete	Preclinical[128,157,158,205]
CDK4/6 inhibitor plus MEK inhibitor or BRAF inhibitor	Melanoma	*NRAS* mutations	Preclinical[155,198], phase I trials[7,15]
CDK4/6 inhibitor alone	Oesophageal adenocarcinoma	*RB1* replete	Preclinical[206]
CDK4/6 inhibitor alone	Neuroblastoma	Amplification of *MYCN*	Preclinical[160]
CDK4/6 inhibitor alone	Non-small-cell lung cancer	*KRAS* mutation	Preclinical[201,202]
CDK4/6 inhibitor alone or in combination with MAPK inhibition	Colorectal cancer	*KRAS* mutation	Preclinical[155,199]
CDK4/6 inhibitor with TGF-β receptor inhibitors or IGF1R inhibitors	Pancreatic cancer	*CDKN2A* mutation	Preclinical[207,208]
CDK4/6 inhibitor alone	Ovarian cancer	*RB1* replete, p16 deficient	Preclinical[143]
CDK4/6 inhibitor alone	Renal-cell carcinoma	Low expression/loss of p15, p16 and E2F1	Preclinical[159]
CDK4/6 inhibitor alone	Hepatocellular carcinoma	None	Preclinical[168]
CDK4/6 inhibitor alone	Prostate cancer	*RB1* replete	Preclinical[167]

CDK4/6, cyclin-dependent kinase 4/6; CDK4/6i, CDK4/6 inhibition; ER, oestrogen receptor; HR, hormone receptor; RB1, retinoblastoma protein; SERD, selective oestrogen-receptor degrader.

between cycles of chemotherapy has been raised for cancers that are dependent on CDK4/6 signalling, although this approach presents substantial treatment scheduling challenges in the clinic. A large number of early stage clinical trials examining combinations of various other therapies with CDK4/6 inhibitors are currently under way.

Conclusions

Targeting the cell-cycle machinery directly in cancer treatment is a logical therapeutic approach, but also one that has proved challenging without appropriate target selection. Clinical use of selective CDK4/6 inhibitors, combined with appropriate selection of the target population now has proven efficacy, and will change the standard of care for patients with advanced-stage ER-positive breast cancer. Extending the benefit of CDK4/6 inhibition outside of patients with ER-positive breast cancer will require identification of cancer subtypes that are dependent on the cyclin D–CDK4/6–RB1 pathway, the identification of effective clinical biomarkers to expand indications, and effective drug combinations to mitigate against resistance.

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