Treating cancer with selective CDK4/6 inhibitors

Ben O'Leary[1], Richard S Finn[2], Nicholas C. Turner[1,3*]

1 The Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, Fulham Road, London, SW3 6JB, UK.

2 University of California Los Angeles, Los Angeles, CA, USA

3 Breast Unit, Royal Marsden Hospital, Fulham Road, London, SW3 6JJ, UK

*To whom correspondence should be addressed: nicholas.turner@icr.ac.uk

Abstract

At the heart of cancer as a pathological process lies uncontrolled cellular proliferation, mediated by dysregulation of the cell cycle machinery and activation of cyclin dependent kinase (CDKs) to promote of cell cycle progression. First generation non-selective CDK inhibitors were hampered by toxicity and lack of efficacy. A new generation of selective CDK4/6 inhibitors, including ribociclib, abemaciclib and palbociclib, has allowed targeting of tumour types where CDK4/6 plays a pivotal role in the G1-S cell cycle transition with an improved therapeutic window between cancerous and normal cells. Pivotal phase III trials with palbociclib in advanced oestrogen receptor (ER) positive breast cancer have demonstrated 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 may identify rational post-CDK4/6 therapeutic strategies. Selective CDK4/6 inhibitors face challenges in extending beyond ER positive breast cancer, and it will likely be necessary to identify both biomarkers predictive of response and combination therapies to optimise CDK4/6 targeting.

Introduction

Aberrant proliferation and deregulated cell division is one of the key hallmarks of cancer, and identifying therapeutic targets to block cell division has been a common approach to cancer treatment. For a cell to divide it must progress through a pre-determined number of stages regulated by a complex regulatory network termed the cell cycle, a process highly conserved between eukaryotes[1]. Each stage of the cell cycle must be passed through in turn with strict control exercised by signalling checkpoints, for example precluding progression in the presence of genetic damage[2]. Transition from one stage in the cell cycle to the next is controlled by the cyclin dependent kinases, activated by their partner cyclins. CDKs have therefore been long 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 as non-selective pan-CDK inhibition was toxic[5].

These issues appear to have been overcome by more selective targeting of CDK4 and 6, a pair of kinases similar in structure and function that mediate transition from G0/1 to S phase. Three of these new CDK4/6 inhibitors – abemaciclib, palbociclib and ribociclib - have emerged through early phase trials as agents with promising anti-cancer activity and manageable toxicity, each with phase III trials in progress. Palbociclib is the agent furthest

through development, having received accelerated approval from the US FDA in February 2015 and also reporting recent pivotal phase III data; both of these in the setting of hormone receptor-positive advanced breast cancer, a disease in which the cyclin D/CDK4 axis is known to be critical[6, 7]. 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 to date for the agents most advanced in development, and discuss the challenges facing scientists and clinicians with regards optimising their use.

Targeting the cell cycle through CDK4/6 in cancer

CDK4/6 and the classical view of G1/S phase transition

The cell cycle is orchestrated by the interaction of cyclins with their partner serine/threonine cyclin-dependent kinases (CDKs). The importance of CDKs to the cell cycle was first elucidated in cdc28/cdc2 (the homologs to CDK1 in humans) in budding and fission yeast respectively[8, 9], with the interacting cyclins described a decade later[10, 11]. It would take a further ten years for the homologs to be confirmed in mammalian systems and for the cyclin-CDK nomenclature to be adopted[12, 13]. To enter the cell cycle a cell must progress from G1 to S phase via the restriction point, a transition in part governed by the retinoblastoma protein (RB) and usually regulated through perturbations in a delicate balance between pro- and anti-mitotic signals. Although mitogenic signalling is critical for entry into the normal cell cycle, its importance is greatly reduced once the cell has entered S phase[14].

The classical view of the initiation of the cell cycle has the D-type cyclins, cyclins D1, D2 and D3, as the key drivers [15-18] (Figure 1A, 1B). The expression level of the D type cyclins is controlled by growth factor signalling, with transcription, turnover and nuclear transport all dependent on mitogenic signalling[19-21]. In early G1, a pro-mitotic signalling balance results in increased expression of the D-type cyclins, which complex with and activate CDK4/6. This complex subsequently phosphorylates RB, and the other RB-like,'pocket proteins' p130 and p107, at a number of positions [22-24]. In its hypophosphorylated state, RB represses transcription of genes necessary for cell cycle progression through binding to the transactivation domain of the E2F transcription factor family of proteins[25-28]. Increasing phosphorylation of RB by the cyclin D/CDK4 complex reduces inhibitory control of RB on the E2F transcription factor family. This initiates a positive feedback loop, as the E2Fs promote transcription of the E type cyclins, activating CDK2 and other proteins important for initiation of S phase and DNA synthesis[29, 30] (Figure 1B). CDK2-cyclin E further phosphorylates RB,

reducing E2F inhibition and promoting S phase entry. During S phase, CDK2 complexes with cyclin A and mediates transcriptional control of DNA synthesis[31-33]. Throughout the progression through S phase and G2, RB remains hyperphosphorylated, returning to its hypophosphorylated state only following mitosis[34-36].

Although regulation of the E2F family of transcription factors remains the best described mechanism through which RB exerts control over the cell cycle, there are likely to be others as RB interacts with over 100 other proteins, most of which are poorly described[37]. Furthermore, there is evidence that RB exerts transcriptional control through chromatin remodelling; phosphorylation of RB leads to a weakening of its interaction with histone deacetylase and modulation of cyclin E and cyclin A transcription through its forming of regulatory complexes with SWI/SNF [38, 39].

The INK4 and CIP/KIP proteins also regulate and control cyclin D-CDK4/6 activity, known collectively as the cyclin dependent kinase inhibitors (CKI) [19]. The INK4 group consists of 4 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 [40-43]. Of the INK4 group, p16 is the best described and is induced by a number of cellular mechanisms such oncogenic signalling, senescence, TGFβ and contact inhibition[44-46] (Figure 1A). Increased expression of p16 is a hallmark of tumours where functional RB protein has been lost. The CIP/KIP family is comprised of 3 proteins, the ubiquitously expressed p27 and p21, and a third member, p57, which is expressed in a limited number of tissues[47-52]. In contrast to the INK4 family, the CIP/KIP proteins are able to bind to all the CDKs involved in the cell cycle to varying degrees and have both a positive and negative regulatory role. The control exerted through these two groups of proteins on the G1-S transition is complex and interlinked, incorporating a number of feedback loops. The best known inhibitor of cyclin D/CDK4 is p16, which contributes to G1 arrest in two ways. Firstly, to become functional, CDK4 requires cytoplasmic, post-translational folding in a complex involving HSP90, an interaction disrupted by p16[53-55]. In addition, p16 can bind to CDK4 directly and inhibit its catalytic activity[40, 55]. The combination of these two mechanisms results in G1 arrest in cells with functional RB, but not RB-deficient cells[56]. In contrast, the CIP/KIP proteins p21 and p27 can stabilise the formation of cyclin D/CDK4 complexes, sequestering these proteins facilitating activation of CDK2[57-61] (Figures 1A, 1B).

Non-classical G1/S phase transition and CDK4/6 inhibitor efficacy

The classical view of G1/S phase transition has cyclin D and CDK4/6 as the key initiators of G1/S transition with CDK2 activity dependent on prior activation of CDK4/6 (Figure 1A, 1B). However, doubts over this classical view of G1-S phase transition were raised by *cdk4* and

cdk6 knockout mice. Cdk4-deficient mouse models were viable but small in size with reproductive and endocrine dysfunction[62-64]. Similarly Cdk6-deficient models were also viable, but with hypocellularity in the thymus and spleen, and with a small reduction in peripheral blood cells,[65]. The lack of a severe phenotype in these single knockout mice was assumed to be due to compensation between cdk4 and cdk6. Surprisingly, although double knockouts for *cdk4* and *cdk6* succumbed to anaemia in the late stages of embryonic development, many non-haematological cell types showed normal proliferation[65]. In addition, embryonic fibroblasts without cdk4 and cdk6 still entered S phase, although at a reduced efficiency, with evidence that D-type cyclins interacted with cdk2[65]. Although murine models may be limited in predicting CDK dependency in human cells, the phenotype of the *cdk4/6* knockout mouse predicted with high accuracy the toxicity profile seen with selective CDK4/6 inhibitors. The architecture of the classical view of the cell cycle, with the restriction point at G1/S, has also been challenged by the demonstration that CDK2 activity may persist directly after mitosis, with pre-mitosis levels of CDK2 and p21 activity predicting the fate of whether post-mitosis daughter cells continue to cycle or become quiescent [66].

Despite caveats in interpreting murine and *in vitro* models, it seems the classical view of cell cycle entry, with the necessary role for CDK4/6, is likely overly simple in many cell types. As well as CDK4/6, other CDKs can initate cell cycle entry due to redundancy between CDKs [67, 68], and as such CDK4/6 is potentially redundant in these cells (Figure 1C). The exact mechanisms that underlie redundancy have been incompletely described, although binding of cyclin D1 to CDK2 [65, 69] and dysregulation of cyclin E expression may contribute (Figure 1C). CDK3 can also contribute to cell cycle entry, phosphorylating RB at the G0/G1 transition[70].

Leveraging cell cycle biology to find a therapeutic window

The ideal CDK-targeted therapy would block the CDK-mediated signalling in malignant cells but spare the aspects of CDK activity critical to normal cell function to avoid toxicity.. Murine embryos lacking cdk1 fail to develop beyond the blastocyst stage[68], suggesting that inhibition of CDK1 by non-specific inhibitors could affect most or all cell types and result in toxicity. In addition, non-specific targeting of CDKs would inhibit CDKs 7, 8 and 9 whose functions are less well-described but include regulation of basal transcription, with CDK 7 also contributing to the cell cycle through its role as a CDK-activating kinase (CAK)[71-76]. This challenge in finding a therapeutic window with CDK inhibitors was reflected in the early clinical experience of pan-CDK inhibitors such as flavopiridol and roscovitine. Flavopiridol is a semi-synthetic flavone with activity against CDKs 1, 2, 4, 6, 7 and 9 and was extensively investigated in early phase trials. Responses were seen in phase II studies in

haematological malignancies, notably chronic lymphoid leukaemia, but dosing was limited by toxicity[77-82]. Roscovitine, a purine-based compound active against CDKs 1, 2, 5, 7 and 9, failed to demonstrate convincing clinical activity in two phase I studies[83, 84]. The toxicity profile of roscovitine included nausea, vomiting and fatigue in addition to hepatic dysfunction and electrolyte abnormalities. Flavopiridol caused fatigue, but also diarrhoea and a degree of myelosuppression[78, 79]. It is difficult to delineate to what degree these toxicities were the result of on-target effects. Roscovitine, with less activity at CDK4/6 (IC_{50} > 10μm), caused less myelosuppression, seen with both flavopiridol and the selective CDK4/6 inhibitors, both of which inhibit CDK4 at nanomolar concentrations (flavopiridol CDK 4 IC_{50} 100nm, palbociclib CDK 4 IC_{50} 11nm)[5].

More selective targeting of CDK4/6 has a number of potential advantages over less selective inhibitors. Many normal cell types in the body may be capable of initiating the cell cycle despite CDK4/6 inhibition[65]. Additionally, in contrast to the cytotoxic effects of less selective CDK inhibitors, CDK4/6 inhibitors are usually observed to be cytostatic, which may further limit their potential for clinical toxicity, although CDK4/6 inhibition-induced cell death has been noted in T cell leukaemia cell lines and xenografts[85, 86].

Selecting target groups - the CDK4/6 axis deranged in cancer

Selection of target groups for CDK4/6 inhibitors relies on identification of tumour types where CDK4/6 drives G1/S transition, and where the effects of CDK4/6 inhibition cannot be rescued by alternative CDKs. Aberrations in the cyclin D-CDK4/6 axis are frequent in cancer. Cyclin D activity is increased in a number of malignancies, a notable example being mantle cell lymphoma. This is characterised by the t(11;14)(q13;q32) translocation that juxtaposes *CCND1* with the *IGH* immunoglobulin heavy chain locus, resulting in the over expression of cyclin D1[87-90]. Amplification and over expression of cyclin D has been described in head and neck cancers[91-94], breast cancers[95-99], non-small cell lung cancers[100, 101], oesophageal cancers[102, 103], melanoma [104-106], and glioblastoma[107, 108].

A further potential activating mechanism in the cyclin D1/CDK4/6 axis is over-expression of the kinases, 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, though this also features *MDM2* and *HMGA2* and there is uncertainty over which genes are the key drivers[109-111]. Somatic amplifications in *CDK4* have been noted in melanoma and glioblastoma [105, 112, 113] and CDK6 in squamous cell oesophageal carcinoma[114] and a small number of B-cell lymphoproliferative disorders which have

undergone translocations involving 7q21[115-117]. The relationship between amplification of *CDK4*, CDK4 activity, and CDK4/6 inhibition is unclear, with reports that both increased expression and amplification is associated with resistance to selective CDK4/6 inhibition[112, 118]. Germline CDK4 mutations in the p16-binding domain have been reported in a small number of families with predisposition to melanoma[119-121].

Loss of p16 function is common in cancer and implies absence of the primary inhibitory brake on CDK4/6-driven signalling. Homozygous deletions of p16 are seen in pancreas, bladder, breast and prostate cancers and glioblastoma[122-124]. An important role for p16 is also implied in melanoma by the common deletion of *CDKN2A* in melanoma-prone kindreds[125]. Conversely, loss of RB results in constitutive activation of E2F, cyclin E1 and CDK2 expression, and therefore loss of reliance on CDK4/6 to initiate G1-S phase transition[126, 127].

Breast cancer subtype dependency on cyclin D1

In luminal oestrogen receptor (ER) positive breast cancer, representing approximately 75% of breast cancer, ER signalling activates the cyclin D promoter, and in many ER positive breast cancers cyclin D1 is expressed at a high level with or without *CCND1* gene amplification [95, 97]. Cyclin D1 is also known to have a number of CDK-independent functions that likely contribute to breast cancer pathogenesis [128]. Cyclin D1 binds to and facilitates ER transcription activity [128], likely reinforcing the dependence of ER positive luminal breast cancer on cyclin D1. In contrast, expression of cyclin E1 is low in ER-positive breast cancer [129], and RB1 is rarely inactivated by mutation[130].

Therefore ER-positive, luminal breast cancer presents the archetypal model for CDK4/6 inhibitors, reflecting the particular dependence of luminal breast cancer on cyclin D1 to initiate G1-S phase transition. In addition, as breast cancers become resistant to endocrine therapy they remain dependent on cyclin D1 and CDK4 to drive proliferation[131]. In contrast to luminal breast cancer, basal-like triple negative breast cancer is characterised by loss of RB [132-134] and by high expression of cyclin E1[129]. Consequently basal-like breast cancer cell lines are resistant to CDK4/6 inhibition[126]. High expression of cyclin E2 has been found in luminal B breast cancers and is correlated with shorter time to distant progression[135], although the role of Cyclin E2 in CDK4 inhibitor sensitivity remains to be determined [135].

Preclinical development of the selective CDK4/6 inhibitors

Three CDK4/6 inhibitors have currently reached early phase trials, abemaciclib (LY-2835219, Eli Lilly), palbociclib (PD-0332991, Pfizer), and ribociclib (LEE011, Novartis), with phase III data now available for palbociclib. These orally-administered compounds of similar structure (figure 2) bind in the ATP-binding pocket of CDK4 and CDK6[5, 136] (figure 3A), and all show a high degree of selectivity over CDK1 and CDK2. Preclinical work in cell lines and xenografts has focused on malignancies with established derangements in the cyclin D/CDK4/p16 axis and has revealed the predominant effect of CDK4/6 inhibitors to be cytostatic rather than inducing cell death and apoptosis.

Abemaciclib inhibits CDK4/6 at low nanomolar concentrations and has been shown to to reduce the phosphorylation of RB in colorectal and melanoma xenografts, inducing G1 arrest[137, 138]. In addition to CDK4 and 6, abemaciclib also reported activity at CDK9 although it is unclear whether this translates into inhibition of CDK9 in cellular activity[138]. Abemaciclib was also able to effect growth regression in vemurafenib-resistant melanoma models, where cyclin D1 was noted to be elevated in conjunction with MAPK pathway reactivation[139].

Palbociclib is also active at low nanomolar concentration at CDK4 and 6, but with limited activity against other CDKs or tyrosine kinases[140, 141]. Palbociclib was active in mantle cell lymphoma xenografts [142], and in glioblastoma cell lines, where in addition to functional RB co-deletion of CDKN2A was found to predict sensitivity[112, 143, 144]. In ovarian cell lines response was found to be most marked in cancers with low p16 expression, with deletions in *CDKN2A* associated with response and amplification of *CCNE1* associated with resistance[127]. Work in renal cell carcinoma identified low E2F1 expression as another potential marker for sensitivity in addition to p16 loss[145]. Additionally, palbociclib has demonstrated activity in acute myeloid leukaemia and myeloma, combined with bortezomib, in both cell line and xenograft models, although particular biomarkers for sensitivity were less clear in these experiments[146-148]. It has also shown activity in RB-replete prostate cancer[149] and in hepatocellular carcinoma, where curiously some activity in RB-deficient cells was observed, potentially through compensation via other pocket proteins[150].

In breast cancer models, palbociclib shows synergy with trastuzumab and tamoxifen treatment in HER2-amplified and ER-positive cells respectively, which are both luminal cancer types and therefore reliant on cyclin D1 to activate CDK4/6 [99, 126, 151, 152]. Synergy with endocrine therapy in ER-positive breast cancer at least in part reflects the simultaneous effects of endocrine therapy suppressing cyclin D1, and palbocicilb inhibiting CDK4/6. In the

presence of CDK4/6 inhibition alone, persistent cyclin E2 continues to allow a low level of S phase entry[153], and synergy is seen with endocrine therapy through suppressing residual cyclins. Treatment with palbociclib also results in growth arrest in breast cancer cell lines with *in vitro* derived resistance to endocrine therapy, but which remain dependent on CDK4/6[154].

Ribociclib inhibits CDK4/6 at nanomolar concentrations[155] and as a single agent ribociclib has demonstrated growth inhibition in neuroblastoma and liposarcoma cell lines, resulting in G1 arrest, a reduction in the phosphorylation of RB at Ser780 and Ser807/811 and significantly reduced tumour burden seen in xenografts[156, 157].

Efficacy and toxicity in early phase trials

Early phase studies into the selective CDK4/6 inhibitors showed a manageable toxicity profile with indications of promising clinical activity. Single agent efficacy appeared to manifest as predominantly as stable disease, hypothesised to be as a result of the cytostatic nature of these agents, although responses were demonstrated in particular in combination with endocrine therapy in breast cancer. Toxicity profiles vary between the inhibitors for reasons that are not understood, but which may have ramifications for optimising their clinical use and in combination with other therapies.

Abemaciclib

The initial phase I study for abemaciclib recruited a cohort of 55 patients of multiple tumour types, 52% experienced diarrhoea, 5% at grade 3[158]. Neutropaenia was far less prevalent than in the trials of ribociclib and palbociclib, allowing for continuous dosing. One patient with a homozygous deletion of *CDKN2A* had a partial response. In a further phase I trial in non-small cell lung cancer, 51% achieved at least stable disease with 41% of patients on treatment for at least 4 cycles[159]. In the metastatic breast cancer cohort of the phase I study, 33% had a partial response, despite relatively heavy pre-treatment, with a progression free survival for 9.1 months in 36 ER-positive patients[160].

Palbociclib

Two of the phase I studies of single agent palbociclib were conducted in RB-expressing cancers, with efficacy manifesting predominantly as stable disease[161, 162]. The third study involving 17 patients with mantle cell lymphoma resulted in 5 of the 17 patients experiencing a progression-free survival of over 12 months[163]. Similar dose-limiting toxicities were seen across the studies with grade 3 or 4 neutropaenia the most common. This required intermittent therapy for recovery of neutropaenia, establishing the dose of 125mg daily with 3

weeks on treatment and the fourth week off [161, 162]. Three of the phase I patients enrolled with teratoma syndrome, refractory to surgery and with confirmed strong expression of RB, achieved at least stable disease and remained on treatment for between 18 and 24 months[164]. A further similar case in a paediatric patient has also been reported[165]. A phase II study looking at this further treated 30 patients with relapsed, RB-proficient germ cell tumours and observed 8 patients with a progression free survival greater than 24 weeks[166].

Thirty seven patients with RB-proficient breast cancer were included in a phase II study of palbociclib as a single agent, with two partial responses and a further 5 patients achieving stable disease for at least 6 months despite heavy pre-treatment[167]. A phase II trial recruiting exclusively patients with liposarcoma found that 66% of 29 evaluable patients had not progressed at 12 weeks, with one patient having a partial response[168].

Ribociclib

Ribociclib has been tested as a single agent in phase I with two dosing schedules, either continuously or 3 weeks on, 1 week off. In a cohort of 132 advanced solid tumours and lymphomas the predominant dose-limiting toxicity was cytopaenias, particularly neutropaenia and leukopaenia, with the most common side effects of all grades otherwise being nausea and fatigue [169]. Two patients experienced a partial response, one with melanoma and one with breast cancer, both of them with amplification of *CCND1*. In a trial of 14 patients with *NRAS*-mutated melanoma who received ribociclib in conjunction with binimetinib, a MEK inhibitor, 6 had a partial response[170]. There are ongoing phase Ib/II studies examining ribociclib in combination with BYL719, a PIK3CA inhibitor, or everolimus in conjunction with an aromatase inhibitor in post-menopausal breast cancer. Although limited data have been reported, no safety concerns have been raised[171, 172].

Differences between CDK4/6 inhibitors

Whilst the early stage efficacy and toxicity of palbociclib and ribociclib are very comparable, abemaciclib shows differences. Abemaciclib has a different toxicity profile with less bone marrow suppression and increased diarrhoea. In terms of efficacy it possibly has a higher response rate as a single agent in pre-treated breast cancer. Of the three inhibitors, abemaciclib is the more potent against CDK4 as opposed to CDK6 on *in vitro* kinase assays. However, it is unclear whether this could explain possible increased activity or the more marked diarrhoea, and the potential role of CDK9 inhibition by abemaciclib is unknown.

There appear to be differences in absorption across the blood-brain barrier between the inhibitors although the evidence is partially conflicting. Abemaciclib appears better absorbed

across the blood brain barrier than palbociclib[173, 174], an observation potentially relevant for the treatment of patients with brain metastases or CNS tumours. Nonetheless, there are case studies involving effective treatment of patients with palbociclib for intracranial teratoma[165].

Randomised studies of CDK4/6 inhibitors in breast cancer

Although later stage randomised studies are recruiting with CDK4/6 inhibitors in multiple cancer types, the only published evidence to date comprises data from breast cancer, where our discussion will focus.

Two randomised studies have reported with palbociclib in hormone receptor positive advanced breast cancer. The first study to report was the randomised open-label phase II study, PALOMA-1/TRIO-18, conducted in patients with advanced ER-positive, HER2-negative breast cancer untreated for advanced disease. Patients had either no prior adjuvant aromatase inhibitor (AI) or had stopped adjuvant AI therapy at least a year prior to relapse[6]. One hundred and sixty five patients were randomised between letrozole alone or in combination with palbociclib, with the study recruiting two consecutively accrued cohorts. The first cohort recruited all ER-positive HER2-negative, the second cohort further restricted based on either amplification of *CCND1* or loss of p16. The intention was for the first cohort to be exploratory, and second the primary cohort for PFS analysis. However, after an unplanned interim analysis demonstrated significantly improved PFS and a low probability of a difference with selection, the study was amended to stop accrual to the *CCND1* and p16 selection and to analyse both cohorts together. At the final PFS analysis and a median follow up 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 (HR 0.488, 95%CI 0.319 – 0.748, p = 0.0004, figure 4). Consistent with prior studies the principal toxicity was neutropaenia, although no cases of febrile neutropaenia were reported. Low grade (1-2) fatigue and nausea were also more prevalent with the addition of palbociclib (36% v 22% and 23% versus 12% respectively), along with slightly higher levels of the side-effects typically seen with aromatase inhibitors such as hot flushes and arthralgia.

The PALOMA 1/ TRIO 18 study served as the basis for accelerated approval of palbociclib by the FDA on February 3, 2015.

Phase III registration studies

The first phase III study to report with a CDK4/6 inhibitor was the PALOMA-3 study, a double blind, randomised controlled trial of 521 patients with advanced, hormone receptor positive, *HER2*-negative breast cancer that had progressed on prior endocrine therapy. Patients were randomised in a 2:1 ratio between palbociclib and fulvestrant versus placebo and fulvestrant[7], fulvestrant being a selective oestrogen receptor degrader with activity in breast cancer after progression on prior endocrine therapies[175]. The study was positive at the pre-planned interim analysis, with revealed a median PFS 9.2 months for the palbociclib/fulvestrant arm compared with 3.8 months with placebo/fulvestrant, (hazard ratio of 0.42, 95%CI 0.32 – 0.56, $p < 0.001$, figure 4). The majority of enrolled women were post-menopausal, although 21% were pre-menopausal and treated with a GnRH agonist to induce ovarian suppression.

Consistent with PALOMA-1/TRIO18 the toxicity profile included frequent haematological adverse events, but also a small increase in mostly grade 1/2 fatigue, alopecia and stomatitis. Although a relatively large proportion of the palbociclib arm experienced grade 3 or 4 neutropaenia (62%) and 31% required a dose reduction, the palbocicilb dose intensity was 91.7% and only 2.6% patients stopped palbociclib due to adverse effects. As in PALOMA-1/TRIO18, despite the high rate of neutropaenia the rate of febrile neutropaenia was minimal at 0.6% in both arms. Infections, mainly of grade 1 or 2 severity, were seen more frequently with palbociclib (32.4% versus 24.4%). Global quality of life was significantly improved on palbociclib compared to placebo, as measured using the QLQ-C30. The PALOMA3 study will lead to registration of palbocicilb in many territories.

In terms of ongoing phase III trials the confirmatory PALOMA-2/TRIO-22 study, testing the combination of palbociclib/letrozole versus placebo/letrozole in first-line treatment of advanced ER positive breast cancer, has completed accrual but is yet to report. Both abemaciclib and ribociclib are also currently in phase III trials. MONARCH-2 (NCT02107703), with a similar design to PALOMA-3 but testing abemaciclib is currently recruiting, and the MONALEESA-7 trial (NCT02278120) is examining the combination of ribociclib with endocrine therapy in pre-menopausal women with advanced hormone receptor positive breast cancer[176, 177].

Patient selection, anticipating resistance and future challenges

Though there are a number of plausible biomarkers for CDK4/6 inhibition, for example cyclin D, *CDKN2A* and RB (figure 3B), the only selection marker currently confirmed in the clinical setting is ER positivity in breast cancer. It is anticipated that further positive selection

markers may be difficult to identify for ER positive breast cancer, 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* status offered no further selection advantage in the phase II PALOMA-1 study [6], although this data is very limited and requires further confirmation.

Further work remains to identify the potential biomarkers of resistance to CDK4/6 inhibitors in ER positive breast cancer. RB loss is an obvious candidate, but loss of RB is rare in ER positive breast cancer[130], although there are few data on whether RB loss changes in frequency with resistance to prior therapy. Amplification of E2F or loss of p21, commonly observed in cancers and linked to tamoxifen resistance[178], are two plausible markers of resistance that have been proposed (figure 3B). Identification of the potential of cyclin E-CDK2 to rescue CDK4/6 inhibition, potentially through assessment of cyclin E levels, or through gene expression predictors of RB1/E2F proficiency could be interesting future approaches. In terms of resistance, breast cancer cell lines with derived resistance to palbociclib select loss of RB and amplification of cyclin E1[153], favouring the non-classical G1-S transition phenotype. Cell lines with acquired cyclin E1 amplification show sensitivity to CDK4/6 and CDK2 combination inhibition, potentially identifying a therapeutic strategy for cell lines with acquired resistance[153].

Other tumour types likely show subtype sensitivity to CDK4/6 inhibitors such as mantle cell lymphoma. However, in many other tumour types biomarkers are likely to be important in identifying selective dependence on cyclin D1-CDK4/6. The phase II/III Lung-MAP trial has an experimental arm where patients with recurrent squamous cell carcinoma are allocated to palbociclib on the basis of aberrations in *CDK4* and *CCND1-3* (NCT02154490). The SIGNATURE trial includes patients treated with ribociclib on the basis of cyclin D/p16/CDK4 aberrations. More information regarding the efficacy of various biomarkers will become available with ongoing biopsy-driven studies examining CDK 4/6 inhibitors in the neo-adjuvant setting and at progression on CDK 4/6 therapies.

Combination therapy with CDK4/6 inhibitors

Which endocrine therapy in ER positive breast cancer?

CDK4/6 inhibitors have been developed almost exclusively in combination with endocrine therapies in ER positive breast cancer, based on sound preclinical evidence of combination efficacy. The selection of the most active endocrine therapy for an individual patient is likely important for combination, though also dictated by the licensed indications. For endocrine-naïve patients, combination with an aromatase inhibitor is likely advantageous, as per

PALOMA1, whereas in patients with endocrine pre-treated breast cancer fulvestrant is suitable, as per PALOMA3. There are no data at this time for continuing the endocrine therapy beyond resistance whilst adding in CDK4/6 inhibitor, and therefore it is uncertain if this approach would be efficacious.

In breast cancer particularly there appears to be a strong case for combining PI3K inhibitors and mTOR inhibition with CDK4/6 inhibitors (figure 5). If, as has been shown in breast cancer cell lines, endocrine resistance is in part mediated through ligand-independent ER interacting with CDK4 and with PI3K hyper-activation[131], and CDK4/6 inhibition can overcome resistance to both PI3K inhibition[179] and endocrine therapy[126], then combination could prevent the emergence of resistance (table 1). Similarly, CDK4/6 inhibition could also offer a means to address the activity of ligand-independence conferred by activating mutations in *ESR1*[180-182]. There is also a strong rationale for the use of CDK4/6 in combination with HER2-directed therapy in *HER2*-amplified breast cancers. Increased cyclin D1 is found in cellular and mouse models of *HER2* over expression and in transgenic mice with activating mutations in *HER2*[151], with evidence that cyclin D1 and CDK4 is required for tumorigenesis in these cancers[183]. Consistent with this, palbociclib was observed to be synergistic with trastuzumab in *HER2*-amplified cells[126]. This combination is being taken forward in a number of early phase trials (NCT01976169, NCT02448420).

Combination strategies in other malignancies

A number of combination strategies with CDK4/6 inhibitors are also being pursued in haematological malignancies, including with bortezomib in myeloma[184], with preclinical evidence to support the combination of CDK4 inhibition with ibrutinib or PI3K inhibition in mantle cell lymphoma [185, 186] (table 1). There is also evidence for CDK4/6 inhibition in combination with MAPK pathway inhibition with MEK or BRAF inhibitors in melanoma[187] and colorectal cancer[188] (figure 5). CDK4/6 inhibition can also re-sensitise melanoma cell lines with *BRAF V600E* mutation to vemurafenib once resistance has developed[139]. The mechanism of all these combinations in part reflects suppression of cyclin D/E levels to limit the ability of alternative CDKs to bypass CDK4/6 inhibition. RAS signalling has also been shown to promote cycling by reducing levels of p27[189].

In lung cancer cell lines and xenografts, knock down of CDK4 was seen to produce a greater degree of inhibition in *KRAS*-mutant cells than those with *KRAS* wild type[190], in keeping with previous work which had suggested a degree of synthetic lethality between *Cdk4* ablation and KRAS activity[191]. In addition, the potential of using CDK4/6 inhibitors to prevent repopulation between cycles of chemotherapy has been raised for cancers dependent on CDK4/6, but this presents substantial scheduling challenges in the clinic. A

large number of early stage clinical trials examining combinations of 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 one that has proved challenging without appropriate selection. 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 ER positive breast cancer. Extending the benefit outside ER positive breast cancer will require identification of cancer subtypes that show dependence on the cyclin D/CDK4/6/RB pathway, the identification of effective clinical biomarkers to expand indications, and effective drug combinations to mitigate resistance.

References

1. Hartwell, L.H., Culotti, J., Pringle, J.R. & Reid, B.J. Genetic control of the cell division cycle in yeast. *Science* **183**, 46-51 (1974).
2. Kastan, M.B. & Bartek, J. Cell-cycle checkpoints and cancer. *Nature* **432**, 316-323 (2004).
3. Malumbres, M. & Barbacid, M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* **9**, 153-166 (2009).
4. Lapenna, S. & Giordano, A. Cell cycle kinases as therapeutic targets for cancer. *Nat Rev Drug Discov* **8**, 547-566 (2009).
5. Asghar, U., Witkiewicz, A.K., Turner, N.C. & Knudsen, E.S. The history and future of targeting cyclin-dependent kinases in cancer therapy. *Nat Rev Drug Discov* **14**, 130-146 (2015).
6. Finn, R.S. et al. The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. *The Lancet Oncology* **16**, 25-35 (2015).
7. Turner, N.C. et al. Palbociclib in Hormone-Receptor-Positive Advanced Breast Cancer. *N Engl J Med* (2015).
8. Hartwell, L.H. Saccharomyces cerevisiae cell cycle. *Bacteriological reviews* **38**, 164 (1974).
9. Nurse, P.M. Nobel Lecture: Cyclin dependent kinases and cell cycle control. *Bioscience reports* **22**, 487-499 (2002).
10. Dorée, M. & Hunt, T. From Cdc2 to Cdk1: when did the cell cycle kinase join its cyclin partner? *Journal of Cell Science* **115**, 2461-2464 (2002).
11. Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D. & Hunt, T. Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33**, 389-396 (1983).
12. Pines, J. & Hunter, T. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature* **346**, 760-763 (1990).

13. Tsai, L.-H., Harlow, E. & Meyerson, M. Isolation of the human cdk2 gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase. *Nature* **353**, 174-177 (1991).
14. Blagosklonny, M.V. & Pardee, A.B. The Restriction Point of the Cell Cycle. *Cell Cycle* **1**, 102-109 (2002).
15. Lew, D.J., Dulić, V. & Reed, S.I. Isolation of three novel human cyclins by rescue of G1 cyclin (cln) function in yeast. *Cell* **66**, 1197-1206 (1991).
16. Matsushime, H., Roussel, M.F., Ashmun, R.A. & Sherr, C.J. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* **65**, 701-713 (1991).
17. Xiong, Y., Connolly, T., Futcher, B. & Beach, D. Human D-type cyclin. *Cell* **65**, 691-699 (1991).
18. Baldin, V., Lukas, J., Marcote, M.J., Pagano, M. & Draetta, G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes & Development* **7**, 812-821 (1993).
19. Sherr, C.J. & Roberts, J.M. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & Development* **13**, 1501-1512 (1999).
20. Aktas, H., Cai, H. & Cooper, G.M. Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27KIP1. *Molecular and cellular biology* **17**, 3850-3857 (1997).
21. Peeper, D.S. et al. Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. *Nature* **386**, 177-181 (1997).
22. Matsushime, H. et al. Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. *Cell* **71**, 323-334 (1992).
23. Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E. & Sherr, C.J. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes and Development* **7**, 331-331 (1993).
24. Meyerson, M. & Harlow, E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Molecular and Cellular Biology* **14**, 2077-2086 (1994).
25. Weintraub, S.J., Prater, C.A. & Dean, D.C. Retinoblastoma protein switches the E2F site from positive to negative element. *Nature* **358**, 259-261 (1992).
26. Hiebert, S.W., Chellappan, S.P., Horowitz, J.M. & Nevins, J.R. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes Dev* **6**, 177-85 (1992).
27. Sellers, W.R., Rodgers, J.W. & Kaelin, W.G., Jr. A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. *Proc Natl Acad Sci U S A* **92**, 11544-8 (1995).
28. Weintraub, S.J. et al. Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature* **375**, 812-816 (1995).
29. Goodrich, D.W., Wang, N.P., Qian, Y.-W., Lee, E.Y.H.P. & Lee, W.-H. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell* **67**, 293-302 (1991).
30. Harbour, J.W., Luo, R.X., Santi, A.D., Postigo, A.A. & Dean, D.C. Cdk Phosphorylation Triggers Sequential Intramolecular Interactions that Progressively Block Rb Functions as Cells Move through G1. *Cell* **98**, 859-869 (1999).
31. Pagano, M., Draetta, G. & Jansen-Durr, P. Association of cdk2 kinase with the transcription factor E2F during S phase. *Science* **255**, 1144-7 (1992).
32. Devoto, S.H., Mudryj, M., Pines, J., Hunter, T. & Nevins, J.R. A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. *Cell* **68**, 167-176 (1992).
33. Lees, E., Faha, B., Dulic, V., Reed, S. & Harlow, E. Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. *Genes & Development* **6**, 1874-1885 (1992).
34. DeCaprio, J.A. et al. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell* **58**, 1085-1095 (1989).

35. Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J.Y.J. & Lee, W.-H. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* **58**, 1193-1198 (1989).
36. Buchkovich, K., Duffy, L.A. & Harlow, E. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell* **58**, 1097-1105 (1989).
37. Classon, M. & Harlow, E. The retinoblastoma tumour suppressor in development and cancer. *Nat Rev Cancer* **2**, 910-917 (2002).
38. Zhang, H.S. et al. Exit from G1 and S Phase of the Cell Cycle Is Regulated by Repressor Complexes Containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell* **101**, 79-89 (2000).
39. Luo, R.X., Postigo, A.A. & Dean, D.C. Rb Interacts with Histone Deacetylase to Repress Transcription. *Cell* **92**, 463-473 (1998).
40. Serrano, M., Hannon, G.J. & Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *nature* **366**, 704-707 (1993).
41. Hannon, G.J. & Beach, D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371**, 257-61 (1994).
42. Hirai, H., Roussel, M.F., Kato, J., Ashmun, R.A. & Sherr, C.J. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Molecular and cellular biology* **15**, 2672-2681 (1995).
43. Chan, F., Zhang, J., Cheng, L., Shapiro, D.N. & Winoto, A. Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. *Molecular and cellular biology* **15**, 2682-2688 (1995).
44. Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D. & Lowe, S.W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**, 593-602 (1997).
45. Zhang, H.S., Postigo, A.A. & Dean, D.C. Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. *Cell* **97**, 53-61 (1999).
46. Wieser, R.J., Faust, D., Dietrich, C. & Oesch, F. p16INK4 mediates contact-inhibition of growth. *Oncogene* **18**, 277-81 (1999).
47. Zerfass-Thome, K. et al. p27KIP1 blocks cyclin E-dependent transactivation of cyclin A gene expression. *Molecular and Cellular Biology* **17**, 407-15 (1997).
48. Wade Harper, J., Adami, G.R., Wei, N., Keyomarsi, K. & Elledge, S.J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**, 805-816 (1993).
49. Toyoshima, H. & Hunter, T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78**, 67-74 (1994).
50. Polyak, K. et al. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78**, 59-66 (1994).
51. Lee, M.H., Reynisdóttir, I. & Massagué, J. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes & Development* **9**, 639-649 (1995).
52. Matsuoka, S. et al. p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes & Development* **9**, 650-662 (1995).
53. Lamphere, L. et al. Interaction between Cdc37 and Cdk4 in human cells. *Oncogene* **14**, 1999-2004 (1997).
54. Zhao, Q., Boschelli, F., Caplan, A.J. & Arndt, K.T. Identification of a conserved sequence motif that promotes Cdc37 and cyclin D1 binding to Cdk4. *J Biol Chem* **279**, 12560-4 (2004).
55. Stepanova, L., Leng, X., Parker, S.B. & Harper, J.W. Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. *Genes Dev* **10**, 1491-502 (1996).
56. Medema, R.H., Herrera, R.E., Lam, F. & Weinberg, R.A. Growth suppression by p16ink4 requires functional retinoblastoma protein. *Proc Natl Acad Sci U S A* **92**, 6289-93 (1995).

57. Harper, J.W. et al. Inhibition of cyclin-dependent kinases by p21. *Molecular Biology of the Cell* **6**, 387-400 (1995).
58. Blain, S.W., Montalvo, E. & Massagué, J. Differential Interaction of the Cyclin-dependent Kinase (Cdk) Inhibitor p27Kip1 with Cyclin A-Cdk2 and Cyclin D2-Cdk4. *Journal of Biological Chemistry* **272**, 25863-25872 (1997).
59. McConnell, B.B., Gregory, F.J., Stott, F.J., Hara, E. & Peters, G. Induced expression of p16(INK4a) inhibits both CDK4- and CDK2-associated kinase activity by reassortment of cyclin-CDK-inhibitor complexes. *Mol Cell Biol* **19**, 1981-9 (1999).
60. Parry, D., Mahony, D., Wills, K. & Lees, E. Cyclin D-CDK subunit arrangement is dependent on the availability of competing INK4 and p21 class inhibitors. *Mol Cell Biol* **19**, 1775-83 (1999).
61. LaBaer, J. et al. New functional activities for the p21 family of CDK inhibitors. *Genes & Development* **11**, 847-862 (1997).
62. Rane, S.G. et al. Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. *Nat Genet* **22**, 44-52 (1999).
63. Tsutsui, T. et al. Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. *Mol Cell Biol* **19**, 7011-9 (1999).
64. Martin, J. et al. Genetic rescue of Cdk4 null mice restores pancreatic beta-cell proliferation but not homeostatic cell number. *Oncogene* **22**, 5261-9 (2003).
65. Malumbres, M. et al. Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. *Cell* **118**, 493-504 (2004).
66. Spencer, Sabrina L. et al. The Proliferation-Quiescence Decision Is Controlled by a Bifurcation in CDK2 Activity at Mitotic Exit. *Cell* **155**, 369-383 (2013).
67. Tetsu, O. & McCormick, F. Proliferation of cancer cells despite CDK2 inhibition. *Cancer Cell* **3**, 233-45 (2003).
68. Santamaria, D. et al. Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* **448**, 811-5 (2007).
69. Xiong, Y., Zhang, H. & Beach, D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* **71**, 505-14 (1992).
70. Ren, S. & Rollins, B.J. Cyclin C/cdk3 promotes Rb-dependent G0 exit. *Cell* **117**, 239-51 (2004).
71. Rochette-Egly, C., Adam, S., Rossignol, M., Egly, J.-M. & Chambon, P. Stimulation of RARα activation function AF-1 through binding to the general transcription factor TFIIH and phosphorylation by CDK7. *Cell* **90**, 97-107 (1997).
72. Tirode, F., Busso, D., Coin, F. & Egly, J.-M. Reconstitution of the transcription factor TFIIH: assignment of functions for the three enzymatic subunits, XPB, XPD, and cdk7. *Molecular cell* **3**, 87-95 (1999).
73. Wallenfang, M.R. & Seydoux, G. cdk-7 is required for mRNA transcription and cell cycle progression in Caenorhabditis elegans embryos. *Proceedings of the National Academy of Sciences* **99**, 5527-5532 (2002).
74. Firestein, R. et al. CDK8 is a colorectal cancer oncogene that regulates [bgr]-catenin activity. *Nature* **455**, 547-551 (2008).
75. Nguyen, V.T., Kiss, T., Michels, A.A. & Bensaude, O. 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. *Nature* **414**, 322-325 (2001).
76. Yang, Z., Zhu, Q., Luo, K. & Zhou, Q. The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. *Nature* **414**, 317-22 (2001).
77. Rathkopf, D. et al. Phase I Study of Flavopiridol with Oxaliplatin and Fluorouracil/Leucovorin in Advanced Solid Tumors. *Clinical Cancer Research* **15**, 7405-7411 (2009).
78. Byrd, J.C. et al. Treatment of Relapsed Chronic Lymphocytic Leukemia by 72-Hour Continuous Infusion or 1-Hour Bolus Infusion of Flavopiridol: Results from Cancer and Leukemia Group B Study 19805. *Clinical Cancer Research* **11**, 4176-4181 (2005).

79. Byrd, J.C. et al. Flavopiridol administered using a pharmacologically derived schedule is associated with marked clinical efficacy in refractory, genetically high-risk chronic lymphocytic leukemia. *Blood* **109**, 399-404 (2006).
80. Schwartz, G.K. et al. Phase I Study of the Cyclin-Dependent Kinase Inhibitor Flavopiridol in Combination With Paclitaxel in Patients With Advanced Solid Tumors. *Journal of Clinical Oncology* **20**, 2157-2170 (2002).
81. Luke, J.J. et al. The Cyclin-Dependent Kinase Inhibitor Flavopiridol Potentiates Doxorubicin Efficacy in Advanced Sarcomas: Preclinical Investigations and Results of a Phase I Dose-Escalation Clinical Trial. *Clinical Cancer Research* **18**, 2638-2647 (2012).
82. Shah, M.A. et al. A Phase I Clinical Trial of the Sequential Combination of Irinotecan Followed by Flavopiridol. *Clinical Cancer Research* **11**, 3836-3845 (2005).
83. Benson, C. et al. A phase I trial of the selective oral cyclin-dependent kinase inhibitor seliciclib (CYC202; R-Roscovitine), administered twice daily for 7 days every 21 days. *Br J Cancer* **96**, 29-37 (2007).
84. Le Tourneau, C. et al. Phase I evaluation of seliciclib (R-roscovitine), a novel oral cyclin-dependent kinase inhibitor, in patients with advanced malignancies. *Eur J Cancer* **46**, 3243-50 (2010).
85. Choi, Y.J. et al. The requirement for cyclin D function in tumor maintenance. *Cancer Cell* **22**, 438-51 (2012).
86. Sawai, C.M. et al. Therapeutic Targeting of the Cyclin D3:CDK4/6 Complex in T Cell Leukemia. *Cancer Cell* **22**, 452-465 (2012).
87. Erikson, J., Finan, J., Tsujimoto, Y., Nowell, P.C. & Croce, C.M. The chromosome 14 breakpoint in neoplastic B cells with the t(11;14) translocation involves the immunoglobulin heavy chain locus. *Proc Natl Acad Sci U S A* **81**, 4144-8 (1984).
88. Bosch, F. et al. PRAD-1/cyclin D1 gene overexpression in chronic lymphoproliferative disorders: a highly specific marker of mantle cell lymphoma. *Blood* **84**, 2726-32 (1994).
89. Rosenberg, C.L. et al. PRAD1, a candidate BCL1 oncogene: mapping and expression in centrocytic lymphoma. *Proc Natl Acad Sci U S A* **88**, 9638-42 (1991).
90. Tsujimoto, Y. et al. Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukemias with the t(11;14) chromosome translocation. *Science* **224**, 1403-6 (1984).
91. Akervall, J.A. et al. Amplification of cyclin D1 in squamous cell carcinoma of the head and neck and the prognostic value of chromosomal abnormalities and cyclin D1 overexpression. *Cancer* **79**, 380-9 (1997).
92. Michalides, R. et al. Overexpression of cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck. *Cancer Res* **55**, 975-8 (1995).
93. Jares, P. et al. PRAD-1/cyclin D1 gene amplification correlates with messenger RNA overexpression and tumor progression in human laryngeal carcinomas. *Cancer research* **54**, 4813-4817 (1994).
94. Bova, R.J. et al. Cyclin D1 and p16INK4A expression predict reduced survival in carcinoma of the anterior tongue. *Clin Cancer Res* **5**, 2810-9 (1999).
95. Gillett, C. et al. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res* **54**, 1812-7 (1994).
96. Weinstat-Saslow, D. et al. Overexpression of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions. *Nat Med* **1**, 1257-60 (1995).
97. Kenny, F.S. et al. Overexpression of cyclin D1 messenger RNA predicts for poor prognosis in estrogen receptor-positive breast cancer. *Clin Cancer Res* **5**, 2069-76 (1999).
98. McIntosh, G.G. et al. Determination of the prognostic value of cyclin D1 overexpression in breast cancer. *Oncogene* **11**, 885-91 (1995).
99. Yu, Q. et al. Requirement for CDK4 kinase function in breast cancer. *Cancer Cell* **9**, 23-32 (2006).

100. Betticher, D.C. et al. Prognostic significance of CCND1 (cyclin D1) overexpression in primary resected non-small-cell lung cancer. *British journal of cancer* **73**, 294 (1996).
101. Gautschi, O., Ratschiller, D., Gugger, M., Betticher, D.C. & Heighway, J. Cyclin D1 in non-small cell lung cancer: a key driver of malignant transformation. *Lung Cancer* **55**, 1-14 (2007).
102. Jiang, W. et al. Altered expression of the cyclin D1 and retinoblastoma genes in human esophageal cancer. *Proc Natl Acad Sci U S A* **90**, 9026-30 (1993).
103. Jiang, W. et al. Amplification and expression of the human cyclin D gene in esophageal cancer. *Cancer Res* **52**, 2980-3 (1992).
104. Smalley, K.S. et al. Increased cyclin D1 expression can mediate BRAF inhibitor resistance in BRAF V600E-mutated melanomas. *Mol Cancer Ther* **7**, 2876-83 (2008).
105. Curtin, J.A. et al. Distinct sets of genetic alterations in melanoma. *N Engl J Med* **353**, 2135-47 (2005).
106. Chraybi, M. et al. Oncogene abnormalities in a series of primary melanomas of the sinonasal tract: NRAS mutations and cyclin D1 amplification are more frequent than KIT or BRAF mutations. *Hum Pathol* **44**, 1902-11 (2013).
107. Brennan, Cameron W. et al. The Somatic Genomic Landscape of Glioblastoma. *Cell* **155**, 462-477 (2013).
108. Sottoriva, A. et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proceedings of the National Academy of Sciences* **110**, 4009-4014 (2013).
109. Barretina, J. et al. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. *Nat Genet* **42**, 715-721 (2010).
110. Italiano, A. et al. HMGA2 is the partner of MDM2 in well-differentiated and dedifferentiated liposarcomas whereas CDK4 belongs to a distinct inconsistent amplicon. *International Journal of Cancer* **122**, 2233-2241 (2008).
111. Italiano, A. et al. Clinical and Biological Significance of CDK4 Amplification in Well-Differentiated and Dedifferentiated Liposarcomas. *Clinical Cancer Research* **15**, 5696-5703 (2009).
112. Cen, L. et al. p16-Cdk4-Rb axis controls sensitivity to a cyclin-dependent kinase inhibitor PD0332991 in glioblastoma xenograft cells. *Neuro Oncol* **14**, 870-81 (2012).
113. Young, R.J. et al. Loss of CDKN2A expression is a frequent event in primary invasive melanoma and correlates with sensitivity to the CDK4/6 inhibitor PD0332991 in melanoma cell lines. *Pigment Cell Melanoma Res* **27**, 590-600 (2014).
114. Baba, Y. et al. LINE-1 hypomethylation, DNA copy number alterations, and CDK6 amplification in esophageal squamous cell carcinoma. *Clin Cancer Res* **20**, 1114-24 (2014).
115. Parker, E.P.K. et al. Sequencing of t(2;7) Translocations Reveals a Consistent Breakpoint Linking CDK6 to the IGK Locus in Indolent B-Cell Neoplasia. *The Journal of Molecular Diagnostics* **15**, 101-109 (2013).
116. Parker, E., MacDonald, J.R. & Wang, C. Molecular characterization of a t(2;7) translocation linking CDK6 to the IGK locus in CD5− monoclonal B-cell lymphocytosis. *Cancer Genetics* **204**, 260-264 (2011).
117. Douet-Guilbert, N. et al. Translocation t(2;7)(p11;q21) associated with the CDK6/IGK rearrangement is a rare but recurrent abnormality in B-cell lymphoproliferative malignancies. *Cancer Genet* **207**, 83-6 (2014).
118. Olanich, M.E. et al. CDK4 amplification reduces sensitivity to CDK4/6 inhibition in fusion-positive rhabdomyosarcoma. *Clin Cancer Res* (2015).
119. Zuo, L. et al. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. *Nature genetics* **12**, 97-99 (1996).
120. FitzGerald, M.G. et al. Prevalence of germ-line mutations in p16, p19ARF, and CDK4 in familial melanoma: analysis of a clinic-based population. *Proceedings of the National Academy of Sciences* **93**, 8541-8545 (1996).

121. Soufir, N. et al. Individuals with presumably hereditary uveal melanoma do not harbour germline mutations in the coding regions of either the P16INK4A, P14ARF or cdk4 genes. *Br J Cancer* **82**, 818-822 (2000).
122. Cairns, P. et al. Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. *Nature genetics* **11**, 210-212 (1995).
123. Parsons, D.W. et al. An Integrated Genomic Analysis of Human Glioblastoma Multiforme. *Science* **321**, 1807-1812 (2008).
124. Caldas, C. et al. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat Genet* **8**, 27-32 (1994).
125. Hussussian, C.J. et al. Germline p16 mutations in familial melanoma. *Nat Genet* **8**, 15-21 (1994).
126. Finn, R. et al. PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. *Breast Cancer Research* **11**, R77 (2009).
127. Konecny, G.E. et al. Expression of p16 and retinoblastoma determines response to CDK4/6 inhibition in ovarian cancer. *Clin Cancer Res* **17**, 1591-602 (2011).
128. Musgrove, E.A., Caldon, C.E., Barraclough, J., Stone, A. & Sutherland, R.L. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer* **11**, 558-72 (2011).
129. Sørlie, T. et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proceedings of the National Academy of Sciences* **100**, 8418-8423 (2003).
130. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61-70 (2012).
131. Miller, T.W. et al. ERalpha-dependent E2F transcription can mediate resistance to estrogen deprivation in human breast cancer. *Cancer Discov* **1**, 338-51 (2011).
132. Bosco, E.E. & Knudsen, E.S. RB in Breast Cancer: The Crossroads of Tumorigenesis and Treatment. *Cell Cycle* **6**, 667-671 (2007).
133. Ertel, A. et al. RB-pathway disruption in breast cancer: differential association with disease subtypes, disease-specific prognosis and therapeutic response. *Cell Cycle* **9**, 4153-63 (2010).
134. Herschkowitz, J.I., He, X., Fan, C. & Perou, C.M. The functional loss of the retinoblastoma tumour suppressor is a common event in basal-like and luminal B breast carcinomas. *Breast Cancer Res* **10**, R75 (2008).
135. Caldon, C.E. et al. Cyclin E2 Overexpression Is Associated with Endocrine Resistance but not Insensitivity to CDK2 Inhibition in Human Breast Cancer Cells. *Molecular Cancer Therapeutics* **11**, 1488-1499 (2012).
136. Mariaule, G. & Belmont, P. Cyclin-Dependent Kinase Inhibitors as Marketed Anticancer Drugs: Where Are We Now? A Short Survey. *Molecules* **19**, 14366-14382 (2014).
137. Tate, S.C. et al. Semi-Mechanistic Pharmacokinetic/Pharmacodynamic Modeling of the Antitumor Activity of LY2835219, a New Cyclin-Dependent Kinase 4/6 Inhibitor, in Mice Bearing Human Tumor Xenografts. *Clinical Cancer Research* **20**, 3763-3774 (2014).
138. Gelbert, L. et al. Preclinical characterization of the CDK4/6 inhibitor LY2835219: in-vivo cell cycle-dependent/independent anti-tumor activities alone/in combination with gemcitabine. *Investigational New Drugs* **32**, 825-837 (2014).
139. Yadav, V. et al. The CDK4/6 Inhibitor LY2835219 Overcomes Vemurafenib Resistance Resulting from MAPK Reactivation and Cyclin D1 Upregulation. *Molecular Cancer Therapeutics* **13**, 2253-2263 (2014).
140. Fry, D.W. et al. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. *Molecular Cancer Therapeutics* **3**, 1427-1438 (2004).
141. Toogood, P.L. et al. Discovery of a potent and selective inhibitor of cyclin-dependent kinase 4/6. *J Med Chem* **48**, 2388-406 (2005).

142. Marzec, M. et al. Mantle cell lymphoma cells express predominantly cyclin D1a isoform and are highly sensitive to selective inhibition of CDK4 kinase activity. *Blood* **108**, 1744-50 (2006).
143. Wiedemeyer, W.R. et al. Pattern of retinoblastoma pathway inactivation dictates response to CDK4/6 inhibition in GBM. *Proc Natl Acad Sci U S A* **107**, 11501-6 (2010).
144. Michaud, K. et al. Pharmacologic inhibition of cyclin-dependent kinases 4 and 6 arrests the growth of glioblastoma multiforme intracranial xenografts. *Cancer Res* **70**, 3228-38 (2010).
145. Logan, J.E. et al. PD-0332991, a potent and selective inhibitor of cyclin-dependent kinase 4/6, demonstrates inhibition of proliferation in renal cell carcinoma at nanomolar concentrations and molecular markers predict for sensitivity. *Anticancer Res* **33**, 2997-3004 (2013).
146. Baughn, L.B. et al. A novel orally active small molecule potently induces G1 arrest in primary myeloma cells and prevents tumor growth by specific inhibition of cyclin-dependent kinase 4/6. *Cancer Res* **66**, 7661-7 (2006).
147. Menu, E. et al. A novel therapeutic combination using PD 0332991 and bortezomib: study in the 5T33MM myeloma model. *Cancer Res* **68**, 5519-23 (2008).
148. Wang, L. et al. Pharmacologic inhibition of CDK4/6: mechanistic evidence for selective activity or acquired resistance in acute myeloid leukemia. *Blood* **110**, 2075-83 (2007).
149. Comstock, C.E. et al. Targeting cell cycle and hormone receptor pathways in cancer. *Oncogene* **32**, 5481-91 (2013).
150. Rivadeneira, D.B. et al. Proliferative suppression by CDK4/6 inhibition: complex function of the retinoblastoma pathway in liver tissue and hepatoma cells. *Gastroenterology* **138**, 1920-30 (2010).
151. Lee, R.J. et al. Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. *Mol Cell Biol* **20**, 672-83 (2000).
152. Yu, Q., Geng, Y. & Sicinski, P. Specific protection against breast cancers by cyclin D1 ablation. *Nature* **411**, 1017-21 (2001).
153. Herrera-Abreu, M.T. et al. 86OPI3 kinase/mTOR inhibition increases sensitivity of ER positive breast cancers to CDK4/6 inhibition by blocking cell cycle re-entry driven by cyclinD1 and inducing apoptosis. *Annals of Oncology* **26**, iii29 (2015).
154. Thangavel, C. et al. Therapeutically activating RB: reestablishing cell cycle control in endocrine therapy-resistant breast cancer. *Endocr Relat Cancer* **18**, 333-45 (2011).
155. Kim, S. et al. Abstract PR02: LEE011: An orally bioavailable, selective small molecule inhibitor of CDK4/6– Reactivating Rb in cancer. *Molecular Cancer Therapeutics* **12**, PR02 (2013).
156. Rader, J. et al. Dual CDK4/CDK6 inhibition induces cell-cycle arrest and senescence in neuroblastoma. *Clin Cancer Res* **19**, 6173-82 (2013).
157. Zhang, Y.X. et al. Antiproliferative effects of CDK4/6 inhibition in CDK4-amplified human liposarcoma in vitro and in vivo. *Mol Cancer Ther* **13**, 2184-93 (2014).
158. Shapiro, G. et al. A first-in-human phase I study of the CDK4/6 inhibitor, LY2835219, for patients with advanced cancer. *J Clin Oncol* **31**, (suppl; abstr 2500). (2013).
159. Goldman, J.W. et al. Clinical activity of LY2835219, a novel cell cycle inhibitor selective for CDK4 and CDK6, in patients with non-small cell lung cancer. *J Clin Oncol* **32** (2014).
160. Patnaik, A. et al. in Proceedings of the 105th Annual Meeting of the American Association for Cancer Research 5-9 (2014).
161. Flaherty, K.T. et al. Phase I, Dose-Escalation Trial of the Oral Cyclin-Dependent Kinase 4/6 Inhibitor PD 0332991, Administered Using a 21-Day Schedule in Patients with Advanced Cancer. *Clinical Cancer Research* **18**, 568-576 (2012).
162. Schwartz, G.K. et al. Phase I study of PD 0332991, a cyclin-dependent kinase inhibitor, administered in 3-week cycles (Schedule 2/1). *Br J Cancer* **104**, 1862-1868 (2011).
163. Leonard, J.P. et al. Selective CDK4/6 inhibition with tumor responses by PD0332991 in patients with mantle cell lymphoma. *Blood* **119**, 4597-4607 (2012).

164. Vaughn, D.J. et al. Treatment of Growing Teratoma Syndrome. *New England Journal of Medicine* **360**, 423-424 (2009).
165. Schultz, K.A.P., Petronio, J., Bendel, A., Patterson, R. & Vaughn, D.J. PD0332991 (Palbociclib) for treatment of pediatric intracranial growing teratoma syndrome. *Pediatric Blood & Cancer* **62**, 1072-1074 (2015).
166. Vaughn, D.J. et al. Phase 2 trial of the cyclin-dependent kinase 4/6 inhibitor palbociclib in patients with retinoblastoma protein-expressing germ cell tumors. *Cancer* **121**, 1463-8 (2015).
167. DeMichele, A. et al. CDK 4/6 Inhibitor Palbociclib (PD0332991) in Rb+ Advanced Breast Cancer: Phase II Activity, Safety, and Predictive Biomarker Assessment. *Clinical Cancer Research* **21**, 995-1001 (2015).
168. Dickson, M.A. et al. Phase II trial of the CDK4 inhibitor PD0332991 in patients with advanced CDK4-amplified well-differentiated or dedifferentiated liposarcoma. *J Clin Oncol* **31**, 2024-8 (2013).
169. Infante, J.R. et al. in ASCO Annual Meeting Proceedings 2528 (2014).
170. Sosman, J.A. et al. in ASCO Annual Meeting Proceedings 9009 (2014).
171. Munster, P.N. et al. in ASCO Annual Meeting Proceedings 533 (2014).
172. Juric, D. et al. Abstract P5-19-24: Phase Ib/II study of LEE011 and BYL719 and letrozole in ER+, HER2– breast cancer: Safety, preliminary efficacy and molecular analysis. *Cancer Research* **75**, P5-19-24 (2015).
173. Parrish, K.E. et al. Abstract C81: BBB efflux pump activity limits brain penetration of palbociclib (PD0332991) in glioblastoma. *Molecular Cancer Therapeutics* **12**, C81 (2013).
174. Sanchez-Martinez, C. et al. Abstract B234: LY2835219, a potent oral inhibitor of the cyclin-dependent kinases 4 and 6 (CDK4/6) that crosses the blood-brain barrier and demonstrates in vivo activity against intracranial human brain tumor xenografts. *Molecular Cancer Therapeutics* **10**, B234-B234 (2011).
175. Leo, A.D. et al. Final Overall Survival: Fulvestrant 500mg vs 250mg in the Randomized CONFIRM Trial. *Journal of the National Cancer Institute* **106** (2014).
176. Debu Tripathy et al. Phase III, randomized, double-blind, placebo-controlled study of ribociclib (LEE011) in combination with either tamoxifen and goserelin or a non-steroidal aromatase inhibitor (NSAI) and goserelin for the treatment of premenopausal women with HR+, HER2– advanced breast cancer (aBC): MONALEESA-7. *J Clin Oncol* **33**, (suppl; abstr TPS625) (2015).
177. Llombart, A. et al. Abstract OT1-1-07: A phase III study of abemaciclib (LY2835219) combined with fulvestrant in women with hormone receptor positive (HR+), human epidermal growth factor receptor 2 negative (HER2-) breast cancer (MONARCH 2). *Cancer Research* **75**, OT1-1-07-OT1-1-07 (2015).
178. Abukhdeir, A.M. et al. Tamoxifen-stimulated growth of breast cancer due to p21 loss. *Proceedings of the National Academy of Sciences* **105**, 288-293 (2008).
179. Vora, Sadhna R. et al. CDK 4/6 Inhibitors Sensitize PIK3CA Mutant Breast Cancer to PI3K Inhibitors. *Cancer Cell* **26**, 136-149 (2014).
180. Toy, W. et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet* **45**, 1439-1445 (2013).
181. Robinson, D.R. et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nat Genet* **45**, 1446-1451 (2013).
182. Wardell, S.E. et al. Efficacy of SERD/SERM Hybrid-CDK4/6 inhibitor combinations in models of endocrine therapy resistant breast cancer. *Clinical Cancer Research* (2015).
183. Yu, Q. et al. Requirement for CDK4 kinase function in breast cancer. *Cancer Cell* **9**, 23-32 (2006).

184. Niesvizky, R. et al. Phase 1/2 study of cyclin-dependent kinase (CDK)4/6 inhibitor palbociclib (PD-0332991) with bortezomib and dexamethasone in relapsed/refractory multiple myeloma. *Leuk Lymphoma*, 1-9 (2015).
185. Chiron, D. et al. Cell-Cycle Reprogramming for PI3K Inhibition Overrides a Relapse-Specific C481S BTK Mutation Revealed by Longitudinal Functional Genomics in Mantle Cell Lymphoma. *Cancer Discovery* **4**, 1022-1035 (2014).
186. Chiron, D. et al. Induction of prolonged early G1 arrest by CDK4/CDK6 inhibition reprograms lymphoma cells for durable PI3Kdelta inhibition through PIK3IP1. *Cell Cycle* **12**, 1892-900 (2013).
187. Kwong, L.N. et al. Oncogenic NRAS signaling differentially regulates survival and proliferation in melanoma. *Nat Med* **18**, 1503-10 (2012).
188. Ziemke, E.K. et al. Sensitivity of KRAS Mutant Colorectal Cancers to Combination Therapy that Co-Targets MEK and CDK4/6. *Clin Cancer Res* (2015).
189. Olson, M.F., Paterson, H.F. & Marshall, C.J. Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1. *Nature* **394**, 295-299 (1998).
190. Mao, C.Q. et al. Synthetic lethal therapy for KRAS mutant non-small-cell lung carcinoma with nanoparticle-mediated CDK4 siRNA delivery. *Mol Ther* **22**, 964-73 (2014).
191. Puyol, M. et al. A synthetic lethal interaction between K-Ras oncogenes and Cdk4 unveils a therapeutic strategy for non-small cell lung carcinoma. *Cancer Cell* **18**, 63-73 (2010).
192. Bardia, A. et al. Phase Ib/II study of LEE011, everolimus, and exemestane in postmenopausal women with ER+/HER2-metastatic breast cancer. *Journal of Clinical Oncology* **32** (2014).
193. Li, C. et al. AMG 925 Is a Dual FLT3/CDK4 Inhibitor with the Potential to Overcome FLT3 Inhibitor Resistance in Acute Myeloid Leukemia. *Molecular Cancer Therapeutics* **14**, 375-383 (2015).
194. Barton, K.L. et al. PD-0332991, a CDK4/6 inhibitor, significantly prolongs survival in a genetically engineered mouse model of brainstem glioma. *PLoS One* **8**, e77639 (2013).
195. Ismail, A. et al. Early G1 Cyclin-Dependent Kinases as Prognostic Markers and Potential Therapeutic Targets in Esophageal Adenocarcinoma. *Clinical Cancer Research* **17**, 4513-4522 (2011).
196. Liu, F. & Korc, M. Cdk4/6 inhibition induces epithelial-mesenchymal transition and enhances invasiveness in pancreatic cancer cells. *Mol Cancer Ther* **11**, 2138-48 (2012).
197. Heilmann, A.M. et al. CDK4/6 and IGF1 receptor inhibitors synergize to suppress the growth of p16INK4A-deficient pancreatic cancers. *Cancer Res* **74**, 3947-58 (2014).

Acknowledgments

We acknowledge NHS funding to the Royal Marsden NIHR Biomedical Research Centre.

Figure legends

Figure 1. Classical and non-classical models of the cell cycle in RB-proficient cells.

A. Resting cells in G0/early G1. The retinoblastoma protein RB 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 CDK4/6.

B. The classical model of G1/S transition. Mitogenic and oestrogen receptor signalling upregulates the transcription of the D type cyclins. The D-type cyclins complex with CDK4/6 to phosphorylate RB, activating the E2F family of proteins that results in transcription of cyclins E, A and CDK2. The phosphorylation of RB also effects chromatin remodelling in favour of transcription (not shown). CDK4/6/cyclin D complexes sequester the CIP/KIP proteins, reducing their inhibitory effect on CDK2, and reducing the threshold for activation of CDK2 by E-type cyclins. As cyclin E rises, it complexes with CDK2 to hyperphosphorylate RB, forming a positive feedback loop via E2F to push the cell from G1 to S phase.

C. The non-classical model of G1/S transition. CDK2 is active in early G1 complexing with cyclins E and potentially cyclin D directly. Both CDK4/6 and CDK2 phosphorylate RB, and drive G1/S transition. The mechanisms through which CDK2 becomes active in G1 without requiring prior CDK4/6 activation are poorly understood, although in some rapidly proliferative cells CDK2 remains active immediately after mitosis.

D – D-type cyclins, E – E-type cyclins, A – cyclin A, CDK2 – cyclin dependent kinase 2, CDK4/6 – cyclin dependent kinase 4 or 6, RB – retinoblastoma protein, P = phosphate group

Figure 2. The structure of selective CDK4/6 inhibitors with the half-maximal inhibitory concentrations (IC50) for a number of cyclin-dependent kinases.

Figure 3. The cell cycle and the role of CDK4/6 inhibition.

A. G1 arrest caused by CDK4/6 inhibition. CDK4/6 inhibitors interact with CDK4 and 6 to prevent their kinase activity via ATP-competitive binding. The cyclin D/CDK4/RB/p16 axis is commonly deranged in cancer, for example through over expression of cyclin D or under expression of p16. In these cases CDK4/6 inhibitors can block the disinhibited phosphorylation of RB, leading to G1 arrest in the absence of an escape mechanism.

B. Potential mechanisms of resistance to CDK4/6 inhibition. In cancer cells deficient in RB, the E2F transcription family in constitutively active and CDK4/6 is redundant. In RB-replete cells, overexpression of cyclin E or loss of the CIP/KIP proteins may bypass CDK4/6

inhibition by activating CDK2. . E2F amplification is another posited mechanism for bypassing RB,

D – D-type cyclins, E – E-type cyclins, A – cyclin A, CDK2 – cyclin dependent kinase 2, CDK4/6 – cyclin dependent kinase 4 or 6, RB – retinoblastoma protein, P = phosphate group, ciclib = CDK4/6 inhibitor

Figure 4. Activity of palbocicilb in advanced ER positive breast cancer

A. Kaplan-Meier plot showing progression free survival for women with advanced, hormone receptor positive, HER2 negative breast cancer treated with either palbociclib and letrozole or letrozole alone in the PALOMA-1/TRIO-18 phase II study, taken from Finn et al 2015 Lancet Oncology.

B. Kaplan-Meier plot showing progression free survival for women with advanced, hormone receptor positive, HER2 negative breast cancer treated with either palbociclib and fulvestrant or fulvestrant and placebo in the PALOMA-3 study, taken from Turner et al 2015 NEJM.

Figure 5. Combination therapy approached with CDK4/6 inhibitors.

A. The CDKs and cyclins act both in parallel and downstream of cellular signal transduction pathways and oestrogen signalling to promote cell cycle progression. Activation of the MAPK and PI3K pathways by receptor tyrosine kinases promotes cell cycle progression through upregulation of D and E type cyclins. RTK signalling therefore both activates CDK4/6 but may also promote CDK4/6 inhibitor bypass, potentially through promotion of cyclin E or through inhibition of p21/p27. Similarly oestrogen recepotor signalling in ER positive breast cancer may promote bypass of CDK4/6 inhibtion, with ER signalling in part facilitated by cyclin D1 binding.

B. Promising strategies for combinatorial efficacy with CDK4/6 inhibition based on preclinical models include blockade of oestrogen receptor signalling with tamoxifen, aromatase inhibitors or SERDs, PI3K pathway blockade with PI3-kinase inhibitors and mTOR inhibition with rapalogs and MAPK pathway blockade with BRAF and MEK inhibitiors.

RTK - receptor tyrosine kinase, PI3K – phosphoinositide-3 kinase, MAPK – mitogen-activated protein kinase, mTOR – mammalian target of rapamycin, D – D-type cyclins, E – E-type cyclins, A – cyclin A, CDK2 – cyclin dependent kinase 2, CDK4/6 – cyclin dependent

kinase 4 or 6, RB – retinoblastoma protein, ER – oestrogen receptor, P = phosphate group, AI – aromatase inhibitor, SERD – selective oestrogen receptor degrader.

List of figures

Figure 1.

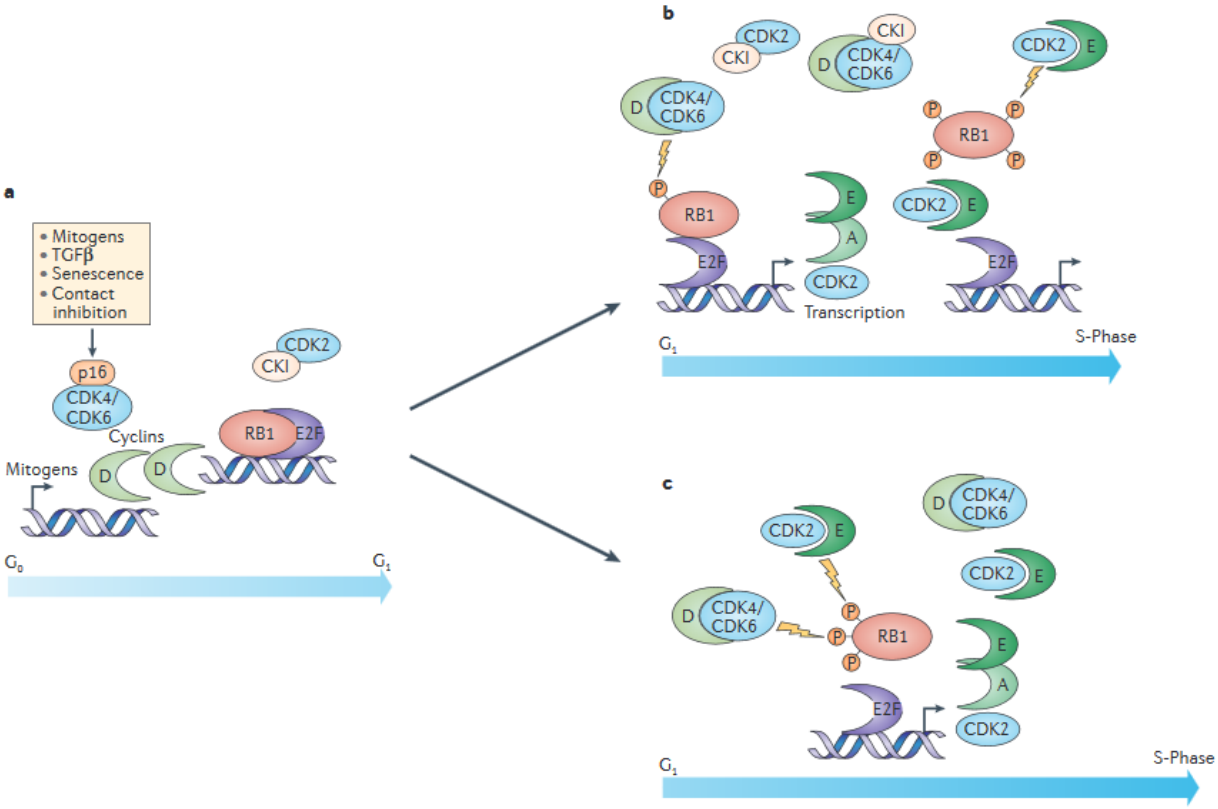
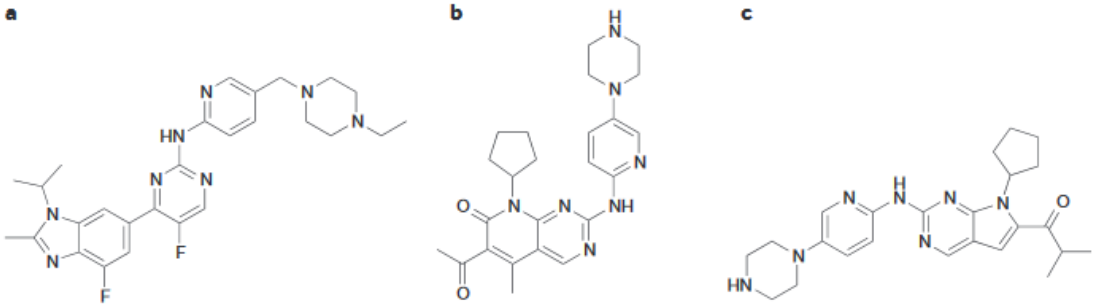


Figure 2.



	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 3.

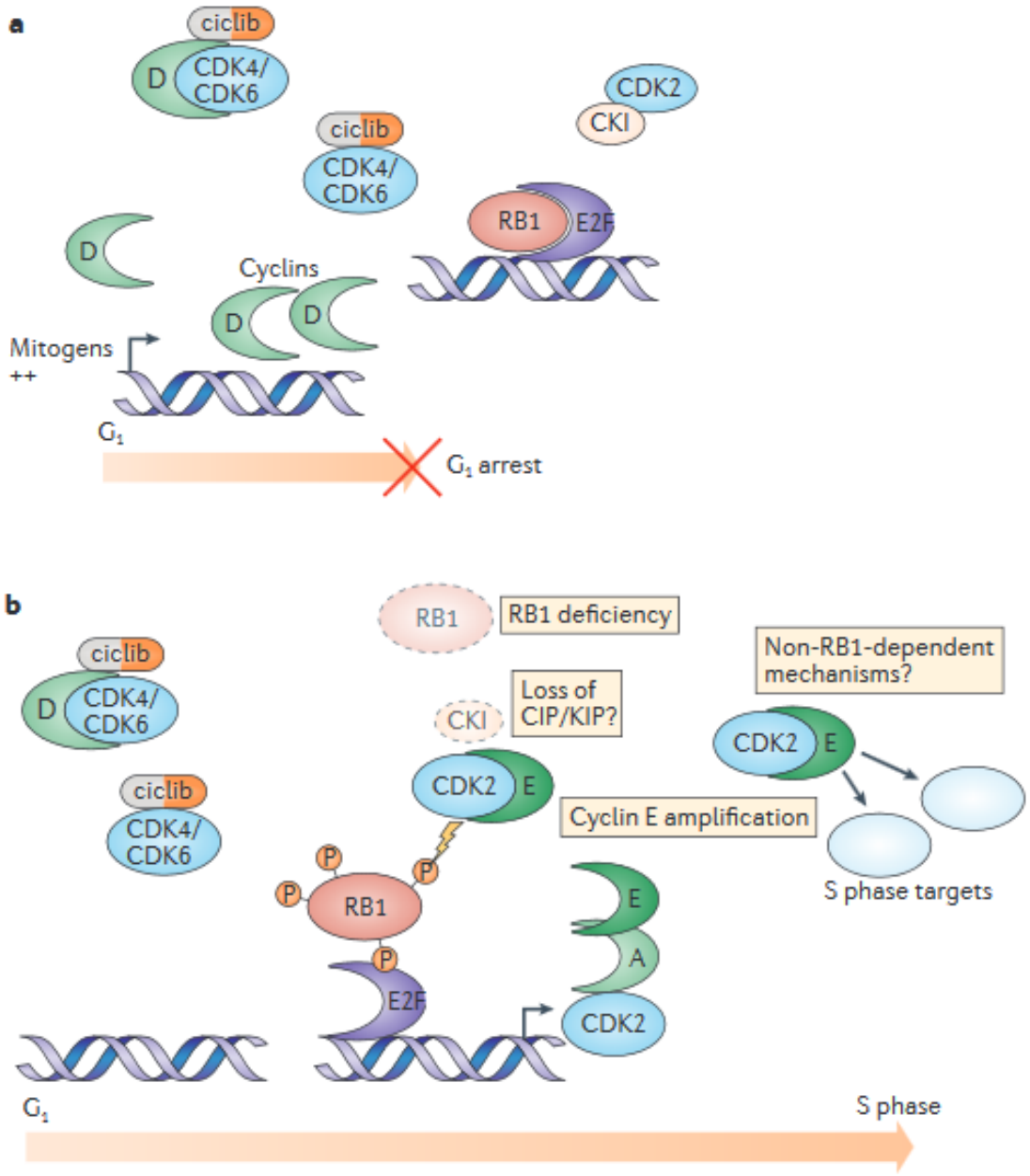

a
ciclib
D
CDK4/
CDK6
CDK2
CKI
ciclib
CDK4/
CDK6
RB1
E2F
D
Cyclins
D
D
Mitogens
++
G1
G1 arrest
b
RB1
RB1 deficiency
Non-RB1-dependent
mechanisms?
ciclib
D
CDK4/
CDK6
Loss of
CIP/KIP?
CKI
CDK2
E
ciclib
CDK4/
CDK6
Cyclin E amplification
P
P
P
RB1
E
A
P
E2F
CDK2
S phase targets
G1
S phase

Figure 4.

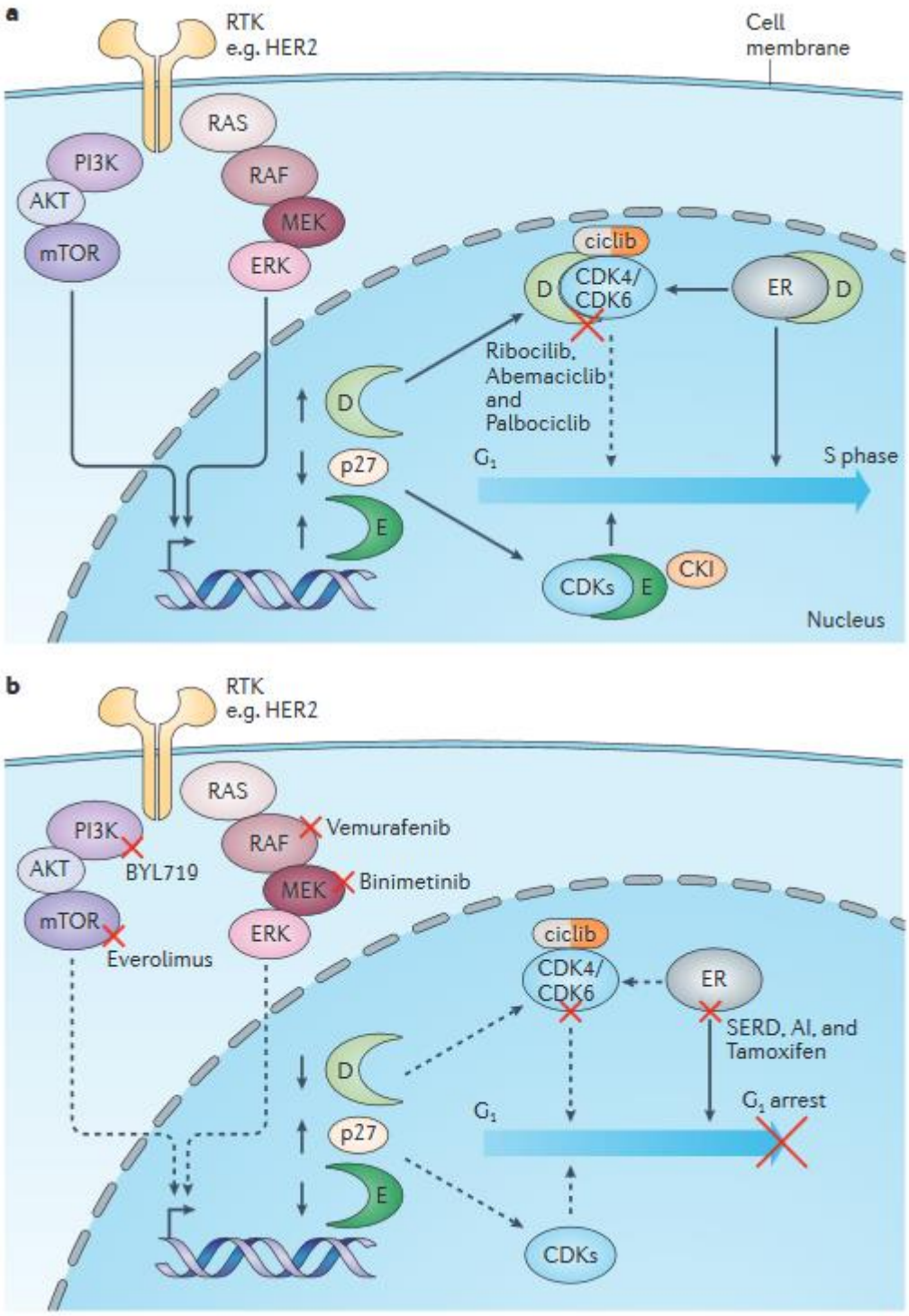

a
RTK
e.g. HER2
Cell
membrane
RAS
PI3K
AKT
mTOR
RAF
MEK
ERK
ciclib
D
CDK4/
CDK6
ER
D
Ribocilib,
Abemaciclib
and
Palbociclib
D
p27
E
G1
S phase
CDKs
E
CKI
Nucleus
b
RTK
e.g. HER2
RAS
PI3K
BYL719
AKT
mTOR
Everolimus
RAF
Vemurafenib
MEK
Binimetinib
ERK
ciclib
CDK4/
CDK6
ER
SERD, AI, and
Tamoxifen
D
p27
E
G1
G1 arrest
CDKs

Table 1. Current clinical strategies using CDK4/6 inhibition, alone or in combination by cancer type and potential biomarker if available.

Therapy	Cancer	Biomarker	Evidence
CDK4/6i plus aromatase inhibitor or SERD	Hormone receptor positive advanced breast cancer	ER positive	Phase I, II and III[6, 7, 161, 167] Preclinical[126]
CDK4/6i plus endocrine therapy, plus PIK3CA/mTOR inhibiton	Hormone receptor positive advanced breast cancer	ER positive	Phase I[172, 192] Preclinical[126, 131, 179]
CDK4/6i plus HER2-directed therapy	HER2+ve breast cancer	HER2-amplification	Preclinical[99, 126]
CDK4/6i plus bortezomib or dexamethasone	Myeloma	None	Phase I/II[184] Preclinical[146, 147]
CDK4/6i alone or in combination with ibrutinib and PI3K inhibition	Mantle cell lymphoma	t(11:14) deregulating *CCND1* Mutated Bruton tyrosine kinase	Phase I[163] Preclinical[142, 185, 186]
CDK4/6i alone	Acute lymphoblastic leukaemia	None	Preclinical[85, 86]
Combined CDK4/6i and FLT3 inhibition	Acute myeloid leukaemia	FLT3	Preclinical[148, 193]
CDK4/6i alone	Liposarcoma	Not clear, *CDK4* amplification highly prevalent	Phase II[168] Preclinical[157, 168]
CDK4/6i alone	Fusion positive rhabdomyosarcoma	Absence of CDK4 amplification	Preclinical[118]
CDK4/6i alone	Teratoma	RB replete	Phase I and II[162, 164-166]
CDK4/6i alone	Glioma	P16-deficient RB replete	Preclinical[112, 143, 144, 194]
CDK4/6i plus MEK inhibitor or BRAF inhibitor	Melanoma	*NRAS* mutation	Phase I[158, 170] Preclinical[139, 187]
CDK4/6i alone	Oesophageal adenocarcinoma	RB-replete	Preclinical[195]

CDK4/6i alone	Neuroblastoma	Amplification of *MYCN*	Preclinical[156]
CDK4/6i alone	NSCLC	*KRAS* mutation	Preclinical[190, 191]
CDK4/6i alone or in combination with MAPK inhibition	Colorectal cancer	*KRAS* mutation	Preclinical[140]
CDK4/6i with TGF-β receptor inhibitors or IGF1R inhibitors	Pancreatic cancer	*CDKN2A* mutation	Preclinical[196, 197]
CDK4/6i alone	Ovarian cancer	RB replete P16 deficient	Preclinical[127]
CDK4/6i alone	Renal cell carcinoma	Low expression/loss p15, p16 and E2F1	Preclinical[145]
CDK4/6i alone	Hepatocellular carcinoma	None	Preclinical[150]
CDK4/6i alone	Prostate cancer	RB replete	Preclinical[149]