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Targeting cyclin-dependent kinases 4 and 6 in cancer

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Abstract

Cyclin-dependent kinase (CDK) 4 and CDK6 (CDK4/6) are critical mediators of cellular transition into S phase, and are important for the initiation, growth, and survival of many cancer types. Pharmacological inhibitors of CDK4/6 have rapidly become a new standard of care for patients with advanced hormone receptor-positive breast cancer. As expected, CDK4/6 inhibitors arrest sensitive tumour cells in the G1 phase of the cell cycle. However, the effects of CDK4/6 inhibition are far more wide-reaching. New insights into their mechanisms of action have triggered identification of new therapeutic opportunities including the development of novel combination regimens, expanded application to a broader range of cancers, and use as supportive care to ameliorate the toxicity of other therapies. Exploring these new opportunities in the clinic is an urgent priority, which in many cases has not been adequately addressed. Here, we provide a framework for conceptualising the activity of CDK4/6 inhibitors in cancer and explain how this framework might shape the future clinical development of these agents. We also discuss the biologic underpinnings of CDK4/6 inhibitor resistance, an increasingly common challenge in clinical oncology.

Introduction

For a mammalian cell to divide, it must pass through the series of well-orchestrated phases known collectively as the cell cycle. Progression through the cell cycle in normal cells is tightly regulated by both proliferative and anti-proliferative forces. Cellular division is triggered by mitogenic signals, increasing the levels of cyclin proteins that bind and activate cyclin-dependent kinases (CDKs). This activity is balanced by inhibitory proliferative checkpoints, which prevent inappropriate cell division. Cancer is classically envisaged as a disease in which this balance is disturbed to favour cell division driven by excessive mitogenic signalling, a failure of inhibitory checkpoints, or both. Given this, the

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development of inhibitors of the CDKs that regulate the cell cycle (particularly CDKs 1, 2, 4 and 6) has been a longstanding aspiration of cancer researchers[1].

The development of a safe and effective small molecule CDK inhibitor proved difficult for many years. The first compounds tested (exemplified by flavopiridol) were potent inhibitors of numerous CDKs, rendering them prohibitively toxic and suggesting the need for more selective agents[1,2]. Unfortunately, creation of these selective inhibitors has, until recently, presented a medicinal chemistry challenge[3]. Notwithstanding this technical obstacle, the biological complexities of cell cycle regulation in cancer also raise the concern that inhibition of a single CDK might be ineffective in many cases. First, the human cyclin–CDK network is diverse, exhibiting redundancy and plasticity[4]. This means that inhibition of one or more cyclins or CDKs can be compensated by an increase in the activity of others[5–8]. Second, cancer cells can harbour genetic aberrations – often in key cell cycle genes – that alter their dependencies on specific CDKs[9]. Third, tumours originating from distinct cellular lineages use different interphase CDKs as their primary driver of proliferation[10–12].

Considering these obstacles, the success story of selective CDK4/6 inhibitors in modern oncology is remarkable. In 2004, the chemical structure of the first CDK4/6 inhibitor – palbociclib – was published, and within eleven years, this agent received accelerated approval from the United States Food and Drug Administration (FDA) as a treatment for breast cancer[13,14]. Since then, two other agents (ribociclib and abemaciclib) have also entered mainstream breast oncology practice, and all three agents have proven to be well tolerated by patients[14]. This success has triggered widespread preclinical, translational and clinical research efforts exploring the CDK4/6 pathway across many cancer types, and the research community is now at a crossroads. Behind us lies the clinical success of CDK4/6 inhibitors as treatment for hormone receptor (HR)-positive breast cancer. Ahead of us lies a plethora of preclinical data suggesting other indications where clinical testing of these drugs is warranted. Given this, a detailed Review of CDK4/6 inhibition in cancer is timely and may help focus research efforts as we strive to fully exploit the promise of these drugs. Here, we focus our discussion on emerging concepts in the CDK4/6 field, including a detailed discussion of the biological mechanisms by which CDK4/6 inhibitors exert their effects in cancer. We then suggest how knowledge of these mechanisms will inform the rational development of CDK4/6 inhibitor-based therapeutic approaches in years to come.

The CDK4/6 pathway in cancer

Regulation of S phase entry by CDK4/6

The human cyclin–CDK network comprises over 20 CDKs and up to 30 distinct cyclin proteins[15]. CDK4 and CDK6 bind to the D-type cyclins (cyclin D1, cyclin D2 and cyclin D3) and are specifically implicated in driving cellular transition from the G1 phase to the S phase of the cell cycle, when DNA synthesis occurs[16–18]. According to the classical model, the G1 to S transition is driven by mitogenic signalling from extracellular growth factors, which increase cyclin D levels[19–22]. Cyclin D-bound CDK4/6 then phosphorylates the tumour suppressor protein RB, as well as the RB-like ‘pocket proteins’ p107 and p130[23–25]. The canonical function of hypo-phosphorylated RB is to bind to and thus sequester activity of the E2F transcription factors, thereby restraining cellular entry into S phase. RB enacts

this repression by (i) binding to and blocking the E2F transactivation domain[26–29]; and (ii) recruiting chromatin modifiers to E2F-bound promoters, which enforce a repressive chromatin environment at these sites[30–32]. RB phosphorylation by CDK4/6 leads to the release of E2F transcription factors, increasing transcription of the E2F target genes cyclin E1 (*CCNE1*) and cyclin E2 (*CCNE2*). E-type cyclins then bind and activate CDK2, leading to RB hyperphosphorylation and phosphorylation of numerous other proteins, which collectively drive an irreversible commitment to S phase[33,34] (Figure 1a).

In addition to the various mechanisms that control the expression, nuclear export, and degradation of D-type cyclins (discussed throughout this Review), CDK4/6 activity is also regulated by the INK4 (INK4B (p15), INK4A (p16), INK4C (p18) and INK4D (p19)) and WAF1 and KIP (p21 (WAF1), p27 (KIP1) and p57 (KIP2)) cyclin-dependent kinase inhibitor protein families. INK4 proteins bind to and inhibit monomeric CDK4 or CDK6 by (i) displacing CDK4 or CDK6 from the heat shock protein 90 (HSP90) co-chaperone CDC37, disrupting their correct folding and assembly; and (ii) inducing a conformational change in CDK4 or CDK6 that weakens associations with cyclin D[35–38]. WAF1 and KIP proteins play a more nuanced role. On one hand, they bind to and inhibit a broad range of fully assembled cyclin–CDK complexes (including cyclin D-CDK4) to restrain cell cycle progression[39–41]. However, WAF1 and KIP proteins (particularly p21 and p27) have also been reported to promote S phase progression by forming stable complexes with CDK4/6 and cyclin D, which (i) stimulate CDK4/6 enzymatic function[42,43]; and (ii) act as reservoirs that sequester p21 and/or p27 and thus allow unrestrained activity of CDK2[44]. Importantly, several *in vitro* and transgenic animal studies have called into question the essentiality of p21 and/or p27 for CDK4/6 enzymatic activity[45–48].

The role of cyclin E–CDK2 in S phase regulation

Although the classical model described above suggests that CDK4/6-mediated RB phosphorylation is a prerequisite for CDK2 activity and thus S phase entry, this linear concept is overly simplistic. Indeed, either CDK4/6 or CDK2 alone could drive proliferation in certain circumstances. For example, certain RB-proficient cancer cells can proliferate in the absence of CDK2[49]. Similarly, mammalian cells lacking both CDK4 and CDK6 proteins can proliferate due to the formation of atypical cyclin D–CDK2 complexes that retain the capacity to phosphorylate RB[5]. As is discussed throughout this Review, the redundancy between CDK4/6 and CDK2 is critical when considering the variable responses of RB-proficient cancers to inhibition of CDK4/6.

Hyperactivity of CDK4/6 kinases in cancer

Alterations predicted to drive hyperactivity of the cyclin D–CDK4/6 axis are common in human cancers (Figure 1b and Supplementary Table S1). Amplifications of *CCND1*, *CDK4*, and *CDK6* are reported in a variety of tumour types, as is homozygous deletion of *CDKN2A* (which encodes INK4A). In addition, a series of rarer genetic events – described as ‘D-cyclin activating features’ (DCAF)[50] – can markedly enhance cancer cell dependence on CDK4/6. These include the classical t11:14 translocation of mantle cell lymphoma (juxtaposing *CCND1* with the immunoglobulin heavy chain (*IGH*) locus)[51], focal amplifications of *CCND2* and *CCND3*[50], loss of and/or mutations in the 3’-untranslated

region (3'-UTR) of cyclin D genes[52], and expression of the Kaposi's sarcoma-associated herpesvirus v-cyclin[53]. Finally, mutations in cyclin D genes that prevent either ubiquitination and degradation or nuclear export of cyclin D have been reported in a variety of tumour types[50,54–58].

Equally important drivers of CDK4/6 hyperactivity in cancer are the ubiquitous mechanisms underlying cyclin D upregulation in the absence of genetic alterations in cyclin D–CDK4/6 pathway genes themselves. The majority of these are underpinned by inappropriately high levels of mitogenic signalling, which disrupts the finely tuned rise and fall of cyclin D levels typically seen during G1 and S phase[59,60]. Increased MAPK pathway signalling (through mutation or other means) directly drives *CCND1* transcription[21], whereas PI3K–AKT signalling increases cyclin D through various mechanisms including derepression of cyclin D gene transcription[61], increased mRNA translation[62], and reduced nuclear export and protein degradation[22]. Furthermore, mTOR complex 1 (mTORC1) (downstream of both PI3K–AKT and MAPK pathways) can specifically increase cyclin D mRNA translation[63,64]. Numerous other signalling pathways can also increase CDK4/6 activity in cancer cells, through diverse mechanisms. For example, (i) steroid hormone signalling through the estrogen receptor (ER) increases *CCND1* transcription in estrogen-sensitive cancers such as breast carcinoma[65], and signalling through the androgen receptor (AR) in prostate cancer drives increased cyclin D mRNA translation via mTORC1[66]; (ii) β-catenin activates transcription from the *CCND1* promoter[67]; (iii) *CCND2* is a direct target gene of MYC[68]; (iv) signalling through the Notch intracellular domain can drive *CCND1* and *CCND3* transcription[69,70]; and (v) Hippo pathway members can increase transcription of both *CCND1* and *CDK6*[71–73].

Insights from transgenic mouse models

Transgenic animal models have offered fundamental insights into the biological processes regulated by the cyclin D–CDK4/6 axis within tumours. Many of these come from studies of mammary and lymphoid malignancies, stemming from the critical role of cyclin D1–CDK4 and cyclin D3–CDK6 in the development of these organs, respectively[5,70,74–76]. In the mammary gland, cyclin D1 is a weak oncogene and its overexpression is sufficient to induce mammary carcinogenesis[77], a phenomenon underpinned by factors including CDK4/6-dependent stimulation of mammary epithelial cell proliferation[78], suppression of oncogene-induced senescence[79], and a number of less well-characterised kinase-independent functions of cyclin D1[80]. Cyclin D1–CDK4 is required for the development of mammary carcinomas driven by upstream oncogenes such as *Neu* (also known as *Erbb2*) and *HRAS*, but not others such as *Myc* or *Wnt1*[11,81]. Analogous to this, cyclin D3 is required for the development of *Notch1* and *Lck*-driven T cell malignancies[70].

Separate to their role in tumorigenesis, CDK4 and CDK6 can promote the sustained growth of established tumours in seemingly distinct and tissue-specific ways. Genetic ablation of cyclin D1 in established mammary tumours leads to cell cycle arrest and cellular senescence, consistent with the role of hypophosphorylated RB as a mediator of senescence[12,82]. In contrast, cyclin D3 ablation triggers leukaemia cell apoptosis in T cell acute lymphoblastic leukaemia, mediated by accumulation of reactive oxygen species

(ROS) as a consequence of inhibited CDK6-mediated phosphorylation of key metabolic enzymes (i.e., an RB-independent process)[12,76,83]. These examples demonstrate that the precise mechanism by which cyclin D–CDK4/6 complexes sustain tumour growth can differ between tumours depending on which cyclin D–CDK pairing they employ, and that this in turn is often linked to tissue of origin. Collectively, this incontrovertible *in vivo* genetic evidence coupled with the frequency of cyclin D–CDK4/6 axis activation in cancer have provided a strong rationale for the development of pharmacological inhibitors of CDK4 and CDK6.

Selective CDK4/6 inhibitors

Generation of currently approved CDK4/6 inhibitors

First- and second-generation CDK inhibitors developed over the last three decades have almost universally failed clinical development. In most instances, these compounds inhibited both interphase CDKs as well as CDKs regulating RNA polymerase II-mediated transcriptional activity (CDK7 and CDK9), and this lack of selectivity resulted in significant clinical toxicity. Furthermore, patient selection was not driven by specific tumour types or molecular biomarkers.

Interest in inhibitors of cell cycle CDKs was revitalised by the development of highly specific, potent ATP-competitive inhibitors of CDK4 and CDK6. A critical step in this development was optimisation of the pyrido[2,3-d]pyrimidine scaffold by including a methyl substituent at the C-5 position, which was sufficient to confer excellent selectivity for CDK4 and CDK6[84]. This breakthrough led the biopharmaceutical company Pfizer to develop palbociclib (PD-0332991)[13], and Novartis scientists to develop ribociclib using a similar scaffold[85]. Meanwhile, medicinal chemists at Lilly utilised the 6-pyrimidine benzimidazole core to develop abemaciclib[86,87]. All three of these agents are administered orally and are currently approved as therapy for advanced HR-positive breast cancer. A fourth recently approved inhibitor – trilaciclib – is based on a tricyclic lactam scaffold, given via intravenous injection, and approved for the reduction of chemotherapy-induced bone marrow suppression in patients with small-cell lung cancer (SCLC)[88,89]. At the time of writing, many other selective CDK4/6 inhibitors are in various stages of clinical development, and whilst a comprehensive discussion of these molecules lies outside the scope of our Review, they are listed in Table 1.

Mechanism of action and inhibitory spectra

Remarkably, the precise molecular mechanisms by which currently approved CDK4/6 inhibitors suppress cellular proliferation remains an open question, with recent provocative studies positing that we might not fully understand whether these drugs directly inhibit CDK4/6 in cells, and if they do, to what extent. Each compound interacts with the ATP-binding pocket of CDK4 and CDK6, which has been presumed to result in competitive inhibition of active CDK4/6 kinases. However, whilst active CDK4/6 in living cells often exists in a trimer with cyclin D and p21 and/or p27[42], the *in vitro* potency of each compound was determined using cyclin D–CDK4/6 dimers[13,86,88]. Intriguingly, palbociclib, ribociclib, and abemaciclib do not appear to inhibit active trimeric complexes *in vitro*,

calling into question whether they directly inhibit active CDK4/6 in cells. One alternate model is that CDK4/6 inhibitors bind and sequester monomeric (inactive) CDK4/6, thereby preventing assembly of holoenzyme trimers, which in turn frees p21 to inhibit CDK2[43]. The concept that CDK4/6 inhibitors exert their effects primarily through indirect CDK2 inhibition is intriguing, remains a somewhat open question[48], and would be strengthened by demonstrating that CDK4/6 inhibitor-sensitive cancer cells are sensitive to genetic disruption of CDK2 kinase function, even more so than to disruption of CDK4 and CDK6 kinases. Another layer of complexity arises from the fact that CDK6 exists in both thermounstable (HSP90-CDC37 bound) and thermostable (HSP90-CDC37 unbound) forms, the latter exhibiting resistance to existing pharmacological CDK4/6 inhibitors[90]. Better understanding of the relevance of these findings to cancer therapy are urgently needed, as they have major implications for understanding drug activity and resistance mechanisms.

In vitro kinase and chemo-proteomic studies have suggested that the approved CDK4/6 inhibitors do have distinct non-CDK4/6 kinase targets[86,91,92]. This is particularly true for abemaciclib, which has been reported to inhibit CDK9, PIM1, homeodomain-interacting protein kinase 2 (HIPK2), dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2), glycogen synthase kinase-3β (GSK3β), and even CDK2 *in vitro*[86,93]. Although these targets have been proposed to explain the distinct toxicity and efficacy profiles of abemaciclib, the extent to which they are inhibited in tumour cells by physiological drug concentrations remains unclear[50,94–96]. Moreover, there is a strong correlation between sensitivity to palbociclib and abemaciclib across large panels of cancer cell lines, suggesting that they act by inhibiting the same pathway(s)[50]. Finally, and notwithstanding the question of whether CDK4/6 inhibitors directly inhibit CDK4/6, the relative potency of each compound for CDK4 versus CDK6 (Table 1) has been proposed as a determinant of its efficacy or toxicity. However, this notion remains speculative at present.

Clinical development in breast cancer

Clinical trial participants with a wide variety of tumour types have received oral CDK4/6 inhibitor therapy, and an exhaustive review of the related literature lies outside the scope of this Review. However, it is noteworthy that some of the earliest reported clinical data demonstrated anti-proliferative and clinical activity of palbociclib in a cohort of 17 patients with mantle cell lymphoma[97]. At present, these agents are only approved for the treatment of advanced HR-positive breast cancer. Early development of the compounds for breast cancer was triggered by seminal preclinical studies using breast cancer cell lines. Finn *et al.*[98] treated a panel of 47 human breast cancer or immortalised mammary cell lines and, consistent with findings from transgenic mice described above, demonstrated that luminal and human epidermal receptor 2 (HER2)-amplified cancer cells were the most sensitive to CDK4/6 inhibition. Synergy was also observed between CDK4/6 inhibitors and other common breast cancer therapies (anti-estrogen therapy in ER-positive cells and the monoclonal antibody trastuzumab targeting HER2 in HER2-amplified cells)[98].

The ensuing clinical trials have cemented a role for CDK4/6 inhibitors as a mainstay of therapy for advanced HR-positive disease and have been complemented by several neoadjuvant therapy trials, which have added mechanistic insights. Fundamental lessons

from these trials include: (i) CDK4/6 inhibitor monotherapy demonstrates anti-proliferative and clinical activity in HR-positive breast cancer[99–103]; (ii) CDK4/6 inhibition can overcome endocrine therapy (ET) resistance, and these two agents act synergistically to improve progression-free and overall survival when compared to ET alone[101,102,104–112]; and (iii) these benefits extend to both pre- and post-menopausal women[106,113,114]. Clinical trials also demonstrate that the distinct toxicity profiles of these agents influence their schedule of administration. Palbociclib and ribociclib induce higher rates of grade 3 or 4 haematological toxicity (presumably an on-target effect of CDK6 inhibition), which necessitate intermittent dose interruptions (typically given as a 21 days on, 7 days off cycle). Haematological toxicity is seen with abemaciclib as well, but it is less frequent, which allows for the drug to be dosed continuously. The extent to which these distinctions account for differences in clinical activity, most notably the higher response rate with abemaciclib monotherapy[103], remains a subject of conjecture.

A mechanistic framework for CDK4/6 inhibition

Although preclinical studies suggest a wide range of potential applications and indications for CDK4/6 inhibitors, they are currently only approved to treat two cancer types (Table 1). Successful prioritisation of areas for future clinical development will require a detailed understanding of the mechanisms by which CDK4/6 inhibitors exert their effects in cancer. Here, we provide a mechanistic framework to facilitate this, with six features enumerating the variety of cellular and molecular processes that ultimately dictate phenotypic responses to CDK4/6 inhibition.

Downregulation of E2F target gene expression

The best characterised consequence of CDK4/6 inhibitor-mediated inhibition of E2F transcriptional activity is RB-dependent proliferative arrest (Figure 2a), which has been documented in preclinical and clinical studies[12,13,86,97,98,100,104,115–119]. This cytostatic effect would be expected to stabilise tumour growth at best and cannot alone explain the objective clinical responses (i.e., tumour shrinkage) seen in CDK4/6 inhibitor monotherapy trials[97,99,103]. Importantly, E2F targets regulate processes other than S phase entry, including replication origin licensing, DNA repair, DNA methylation and chromatin condensation, cellular metabolism, cellular differentiation, and apoptosis[120–127], and CDK4/6 inhibition can also impact these processes in RB-proficient cells in a manner that influences phenotypic responses to treatment[128–132].

Cellular senescence

CDK4/6 inhibitors can induce a ‘senescence-like’ state in cancer cells (Figure 2b), evidenced by cellular enlargement and increased β-galactosidase activity (a recognized marker of cellular senescence)[12,133,134]. Early studies showed that this phenomenon is mediated by active RB[133,135], which is not surprising given the role of RB as a principal mediator of the cellular senescence program[82,136]. The senescent phenotype might also be augmented by reduced activity of the direct CDK4/6 substrates forkhead box protein M1 (FOXM1) and DNA methyltransferase 1 (DNMT1)[79,137]. However, *FOXM1* and *DNMT1* are also E2F targets, and there is currently no evidence that inhibition of the phosphorylation

of FOXM1 and DNMT1 without downregulation of other E2F targets (e.g., after CDK4/6 inhibitor treatment of RB-deficient cancer cells) is sufficient to induce senescence[137,138].

The phenotypic hallmarks of classical cellular senescence include cell cycle withdrawal, chromatin remodelling, metabolic dysregulation, apoptosis resistance, and secretion of growth factors and cytokines (known as the senescence-associated secretory phenotype (SASP))[139]. These phenomena were characterised in models of DNA damage-induced senescence in fibroblasts, and it cannot be assumed that they are recapitulated in cancer cells treated with CDK4/6 inhibitors. Unlike classical senescence in benign cells, CDK4/6 inhibitor-induced senescence is distinct in that (i) the initiating mechanism is RB activation rather than DNA damage, which activates both RB and p53[140,141]; (ii) it occurs in cancer cells that harbour DNA mutations and have therefore presumably acquired senescence escape mechanisms.

Notwithstanding this, certain aspects of classical senescence have been described in CDK4/6 inhibitor-treated cancer cells. Watt and colleagues[142] reported chromatin remodelling in cell-based models and clinical specimens of breast cancer treated with CDK4/6 inhibitors, characterised by widespread enhancer activation akin to that seen in senescent fibroblasts[143]. The transcriptional activity mediated by newly activated enhancers was directly implicated in mediating apoptotic evasion and enhanced cellular immunogenicity, both known features of classical senescence, and was principally driven by the AP-1 transcription factors, which have also been implicated as master regulators of the classical senescence phenotype[142,144]. Notably, these new enhancers also provoked a more differentiated cellular phenotype, supporting the mechanistic link between RB activation and cellular differentiation in cancer[142,145].

CDK4/6 inhibition, either alone or in combination with other agents that heighten the senescent phenotype, can also induce a SASP, presumably attributable to the enhancer remodelling described above[135,146,147]. The SASP factors released can include immunomodulatory proteins that mediate surveillance by components of the innate immune system[146], pro-angiogenic factors and matrix metalloproteinases that facilitate tumour angiogenesis[147], growth factors that can drive autocrine tumour cell proliferation[148], and classical SASP factors described in fibroblast models[135]. SASP factors secreted by CDK4/6 inhibitor-treated cells are likely to vary by tumour lineage, and a more comprehensive portrait of these is required given the major impact they can have on the tumour microenvironment.

Although p53 plays a critical role in classical senescence, its role in CDK4/6 inhibitor-mediated senescence remains unclear. On one hand, genetic and pharmacological CDK4/6 inhibition can induce phenotypic features of senescence in malignant cells that lack functional p53[133,135,149,150]. Conversely, transgenic mouse models of *KRAS*-driven lung carcinoma have shown that genetic inhibition of CDK4 kinase activity can only induce tumour cell senescence if p53 function is intact[151]. Furthermore, *TP53* mutations emerged as the strongest genomic predictor of CDK4/6 inhibitor resistance in a panel of 560 cancer cell lines, suggesting that p53 function contributes to drug-induced proliferative arrest[50]. RB and p53 have partially overlapping but distinct functions in classical senescence, and

it is conceivable that both p53 wild-type and mutant cancer cells can display features of senescence in response to CDK4/6 inhibition, but that the two senescent phenotypes are qualitatively different.

Cellular apoptosis and cytostasis

Pharmacological CDK4/6 inhibition can also directly induce tumour cell apoptosis. As described above, the evidence for this is strongest in haematological malignancies that are primarily driven by cyclin D3–CDK6 activity[12,76,83,152]. In contrast, the dominant response to CDK4/6 inhibitor monotherapy in solid tumours that are thought to be primarily driven by Cyclin D1–CDK4 activity (e.g. in breast cancer and non-small-cell lung cancer) is cytostasis[12,153] (Figure 2c).

Non-canonical functions of hypo-phosphorylated RB

Hypo-phosphorylated RB has non-canonical functions, including the recruitment of histone modifying enzymes to DNA, and the stimulation of transcriptional activity of factors such as JUN and the glucocorticoid receptor[154–156] (Figure 2d). It is conceivable that these functions could also be triggered by CDK4/6 inhibition in RB-proficient cells, as has been demonstrated for the case of JUN and other AP-1 factors[142].

Non-RB substrates

CDK4 and CDK6 bind and phosphorylate numerous non-RB substrates. In brief, they include a variety of transcription factors[79,157–159], proteins regulating oncogene expression[152], DNMTs[121], metabolic enzymes[83], ubiquitin ligase adaptor proteins and deubiquitinases[160,161], suppressors of oncogenic kinase signalling[153,162,163], and others[79] (Figure 2e). Inhibiting the phosphorylation of these proteins with CDK4/6 inhibitors can impact diverse biological processes including senescence and lysosome biogenesis[79,137,159], apoptosis[83], tumour cell metastasis[160], tumour cell immunogenicity[161], and T lymphocyte activation[158], but we lack the information needed to dissect the relative contributions of these effects in any given tumour context. Importantly, (i) hypo-phosphorylation of these substrates by CDK4/6 inhibitors is expected to impact both RB-proficient and RB-deficient cells; and (ii) although the network of CDK4/6 substrates is not fully characterized, several non-RB substrates are unique to either CDK4 or CDK6[79,83,152].

Effects on stromal cell types

Although most CDK4/6 inhibitor studies have focused on the treatment of cancer cell lines *in vitro*, other cell types, including lymphocytes[130,158,164], fibroblasts[140], and endothelial cells[165] can respond to CDK4/6 inhibitors in ways that can meaningfully impact response to therapy (Figure 2f). In particular, CDK4/6 inhibitors have been shown to directly stimulate $CD8^+$ T cell effector function while repressing T regulatory (T_{reg}) cell proliferation *in vitro* and *in vivo*, thereby promoting anti-tumour responses[130,158,164]. The reported impact of CDK4/6 inhibition on fibroblasts, and the consequences for tumour behaviour, are mixed. On one hand, treatment of normal mouse embryonic fibroblasts (MEFs) with palbociclib induced senescence *in vitro*, and co-injection of palbociclib-treated MEFs with tumour cells into immunocompetent mice enhanced tumour growth in certain models of melanoma with

WT *Braf* and *Nras*[140]. Conversely, a recent study showed that although abemaciclib-treated fibroblasts do exhibit as SASP, this is not sufficient to stimulate cancer cell proliferation[166]. Likewise, treatment of endothelial cells with palbociclib *in vitro* results in cell cycle arrest, but the consequences of this effect in cancer was not explored[165].

Designing rational combination therapies

The key to improving the utility of CDK4/6 inhibitor therapy for a broad range of cancer types will be the identification of effective and tolerable combination therapy regimens. By changing tumour cell and stromal cell phenotypes so profoundly, CDK4/6 inhibitors invoke new cancer phenotypes, dependencies, and vulnerabilities that can be exploited through the addition of additional therapeutic agents[167]. Currently, combination therapies with CDK4/6 inhibitors are being evaluated in a vast number of clinical trials encompassing numerous and varied pharmacological agents in multiple cancer types (Supplementary Table S2). In this section, we discuss novel combination therapy approaches and the biological rationale for their development.

Endocrine therapy in breast cancer

Preclinical and clinical studies clearly demonstrate synergy between ET and CDK4/6 inhibitors in luminal breast cancer, which underpins the remarkable efficacy of this combination[8,98,102,168–170]. The ER and cyclin D1–CDK4 interact at several levels: (i) *CCND1* is a direct ER target gene[65]; (ii) ER directs an estrogen-independent, E2F-driven transcriptional signature that facilitates tumour cell proliferation[171]; and (iii) cyclin D1 can directly bind ER and promote expression of ER target genes in a CDK-independent fashion[172]. How these interactions give rise to synergistic interactions between ET and CDK4/6 inhibitors is not clear, but likely relates to heightened inhibition of RB phosphorylation[170].

Combining oncogenic kinase and CDK4/6 inhibitors

A plethora of preclinical data demonstrates cooperativity between CDK4/6 inhibitors and inhibitors of mitogenic signaling pathways *in vitro* and *in vivo*, with studies having been performed in dozens of tumour types. Here, we outline the major principles that have emerged from this literature.

Synergy between CDK4/6 inhibition and inhibition of other oncogenic kinases (e.g. those in the PI3K–AKT signalling pathway, the MAPK pathway, and upstream receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), HER2, fibroblast growth factor receptors (FGFRs) and insulin-like growth factor 1 receptor (IGF1R)) is bi-directional. On one hand, persistent CDK4/6 enzymatic activity can mediate resistance to inhibition of oncogenic kinases[153,173–177], and in breast cancer specifically, this is related to a failure of upstream kinase inhibitors to reduce cellular levels of cyclin D1[153,173]. Conversely, uninhibited hyperactivity of mitogenic pathways can mediate resistance to CDK4/6 inhibitors through a variety of mechanisms discussed below[8,178–181]. These two phenomena often co-occur, underpinning potent synergy between CDK4/6 inhibitors and inhibitors of upstream kinases known to be active in a particular tumour.

CDK4/6 inhibition often induces adaptive rewiring of kinase circuits in cancer cells (Figure 3a), enhancing their dependence on other signalling pathways. This has been demonstrated in a variety of cell-based and animal models: for example, CDK4/6 inhibition increases (i) phosphorylation and activity of HER receptors in HER2-positive breast cancer[153,182]; (ii) PI3K–AKT pathway activity in numerous breast cancer models[8,170,173,180,182]; (iii) IGF1R pathway activity in models of Ewing's sarcoma and pancreatic carcinoma[177,181]; (iv) MAPK pathway activity in models of prostate carcinoma[183]; and (v) FGFR1 activity in models of *KRAS*-mutant lung cancer[148]. Several mechanisms underlie this adaptive rewiring. First, CDK4/6 can directly stimulate mTORC1 activity by binding and phosphorylating the tuberous sclerosis 2 (TSC2) tumour suppressor[162,163,184], and partial inhibition of mTORC1 by CDK4/6 inhibitors can relieve feedback inhibition on upstream receptor tyrosine kinases[153,185]. Second, hypo-phosphorylation of RB by CDK4/6 inhibitors directly facilitates access of AKT to SIN1, a member of the mTORC2 complex, ultimately increasing AKT phosphorylation by mTORC2[186]. Third, CDK4/6 inhibitors can induce secretion of growth factors from tumour cells (possibly as part of the SASP), which stimulate signaling through receptor tyrosine kinases in an autocrine manner[148,187].

In many instances, these adaptive responses are sufficient to drive acquired CDK4/6 inhibitor resistance, explaining the efficacy of co-targeting CDK4/6 and other mitogenic pathways and possibly the failure of CDK4/6 inhibitor monotherapy in some clinical trials[188]. A common theme is that upstream mitogenic signalling enhances the activity of CDK2, facilitating RB phosphorylation and S phase entry despite ongoing CDK4/6 blockade[8,145,148,189,190]. Several mechanisms for stimulation of mitogenic signaling-driven increase in CDK2 activity have been proposed, including (i) increased formation of atypical, enzymatically active cyclin D1–CDK2 complexes due to elevated cyclin D1 levels[8,148]; (ii) sequestration of p21 and/or p27 in cyclin D1–CDK4/6 complexes[190,191]; and (iii) direct downregulation of p27[189]. A second possibility is that increased upstream mitogenic signalling increases the activity of mTORC1, another regulator of S phase entry[192], which drives proliferation in the face of sustained CDK4/6 inhibition[193,194]. Consistent with this, several studies have demonstrated synergistic efficacy from combined CDK4/6 and mTOR inhibition[148,190,195,196]. Finally, these two mechanisms could be connected, given that mTORC1 can directly enhance cyclin D1 and/or cyclin E1 translation[190].

Cellular responses to combined inhibition of CDK4/6 and other oncogenic kinases can include cytostasis, apoptosis, or both. In cases where a combination therapy induces cytostasis, it is typically accompanied by greater RB hypo-phosphorylation than seen with either agent alone, accompanied by increased downregulation of E2F targets, and heightened senescence – all of which could be linked to suppression of CDK4/6 together with either CDK2 or mTORC1[135,153,173–175,177,197–200]. Frank apoptosis has also been observed in several *in vitro* and *in vivo* models[8,176,180,196,201,202]. However, the underlying molecular determinants of both of these two phenotypes are not well understood.

Immunological effects of CDK4/6 inhibition

Several preclinical studies have converged upon the notion that CDK4/6 inhibitors can stimulate anti-tumour immune responses through tumour cell-intrinsic and -extrinsic

mechanisms. Although this was initially considered counterintuitive given concerns that CDK6 inhibition would dampen immune function by preventing T lymphocyte expansion[130,203], it is now clear that these drugs induce an 'inflamed' tumour microenvironment, and that $CD8^+$ and/or $CD4^+$ T lymphocytes partially mediate therapeutic responses[130,158,164,204–206].

The association between CDK4/6 hyperactivity and impaired immunogenicity in tumour cells is supported by clinical data from multiple tumour types. Tumour cells harbouring amplifications in *CCND1* (multiple tumour types) or *CDK4* (melanoma) express lower levels of major histocompatibility complex (MHC) class I genes and demonstrate resistance to immune checkpoint inhibitors (ICIs)[130,207,208]. Similarly, a tumour cell-specific gene expression signature reflecting CDK4/6 activity is associated with worse ICI response in patients with melanoma[205]. Finally, pharmacological CDK4/6 inhibition upregulates interferon-driven gene expression programs in early-stage luminal breast cancers[101,130].

CDK4/6 inhibition enhances tumour cell immunogenicity through a variety of RB-dependent mechanisms (Figure 3b). First, these agents can induce a viral mimicry response in luminal breast cancer cells, characterised by activation of an interferon-driven gene expression program that fosters secretion of lymphokines and enhances antigen presentation to $CD8^+$ T cells via MHC class I[130,204]. Second, drug-induced chromatin remodelling activates a subset of enhancers specifically predicted to regulate interferon-driven genes[142]. Third, CDK4/6 inhibitors can induce metabolic stress in tumour cells, which drives secretion of immunostimulatory chemokines[206]. Each of these mechanisms is likely linked to underlying therapy-induced senescence, and similar findings have been reported in mouse models of lung and pancreatic carcinomas induced into a senescence-like state by combined CDK4/6 and MEK inhibition[146,209].

T lymphocyte activation is also a common feature of CDK4/6 inhibitor-treated solid cancers. Whilst this might in part relate to tumour cell secretion of chemoattractant cytokines, these agents can also directly stimulate T cell function. A consistent observation across studies has been a relative depletion of immunosuppressive T_{reg} cells (Figure 4a), resulting in an increase in the $CD8^+$:T_{reg} cell ratio that favours immune activation[130,164,206,210]. This is largely explained by an increased sensitivity of T_{reg} cells to the anti-proliferative effect of CDK4/6 inhibitors[130]. T lymphocytes from CDK4/6 inhibitor-treated mouse models of mammary and lung carcinoma models also show increased effector activity, reduced expression of numerous exhaustion markers, and increased expression of stimulatory receptors[130,158,206] – related in part to direct stimulation of T cell effector function by the CDK6 substrates nuclear factor of activated T-cells 2 (NFAT2) and NFAT4[158] (Figure 4b). Finally, recent preclinical and clinical data have described RB-dependent and independent mechanisms by which CDK4/6 inhibition promotes $CD8^+$ T cell memory differentiation (Figure 4b), supporting the prior preclinical observation that these agents protect against tumour re-challenge and might confer long-lasting protective immunity[130,211,212].

A key challenge now is to leverage the above observations to develop new clinically effective therapies. Several studies, even one suggesting that CDK4/6 inhibitors might foster immune evasion[161], have demonstrated synergy between CDK4/6 inhibition and

immunotherapies (both ICIs and agonists of stimulatory receptors)[130,158,204–206]. Clinical development will require identification of ideal immunotherapy partners and tumour types for this approach. Notably, metastatic luminal breast cancer is notoriously unresponsive to immune-directed strategies, and it will be important to direct efforts towards other, more immunogenic tumour types.

A noteworthy example supporting clinical development of CDK4/6 inhibitors as immunostimulatory agents is a randomised trial demonstrating that the addition of trilaciclib to chemotherapy improved overall survival of patients with advanced triple-negative breast cancer[213]. Biomarker studies from this trial suggested that this unexpected result might have been driven by trilaciclib-mediated immune-stimulation, and a larger confirmatory trial is underway (NCT04799249)[214].

Converting senescence to cell death

Like classically senescent cells, cancer cells that have entered CDK4/6 inhibitor-induced senescence show relative resistance to apoptotic insults *in vitro*[130,135,139,142]. This phenomenon is supported by the clinical observation that the addition of palbociclib to ET suppresses indices of apoptosis in luminal breast cancers[105]. Efforts are now underway to improve tumour eradication through the use of senolytics, agents that selectively kill senescent cells. This has been described as a ‘one-two punch’ approach, rendering a cancer cell senescent with a CDK4/6 inhibitor before killing it with a senolytic.

To date, anti-apoptotic BH3-family proteins have been implicated as the primary mediators of CDK4/6 inhibitor-induced apoptotic evasion. In luminal breast cancer cells, CDK4/6 inhibition induces the RB-dependent commissioning of a superenhancer spanning the *BCL2L1* locus, increasing *BCL2L1* expression and cellular levels of BCL-X_L[142]. This in turn primes tumour cells away from apoptosis at the level of the inner mitochondrial membrane. Consistent with this, CDK4/6 inhibitor-pretreated tumour cells can be re-primed towards their apoptotic threshold and, in some instances, killed by agents that selectively inhibit BCL-X_L[142]. Similarly, selective BCL-2 or dual BCL-2 and BCL-X_L inhibitors also show senolytic activity when given after CDK4/6 inhibitor therapy[182,210,215–217], and trials combining CDK4/6 and BCL-2 inhibitors in breast cancer are underway (e.g. NCT03900884)[214]. Although BCL-X_L inhibitors induce significant thrombocytopenia, which has limited their clinical development, novel formulations that selectively target senescent cells are currently under development[215].

Targeting tumour metabolism and autophagy

An additional function of the cyclin D1–CDK4 axis in mammary epithelial cells is the suppression of cellular autophagy[218]. Indeed, both genetic and pharmacological studies have shown that CDK4/6 pathway inhibition can induce autophagy in normal and malignant luminal mammary cells[218,219]. Similar observations have been made in RB-proficient tumour cells, where senescence and autophagy accompany one another in response to CDK4/6 inhibition[137]. These findings have prompted researchers to combine CDK4/6 inhibitors with inhibitors of autophagy (e.g., hydroxychloroquine and bafilomycin). Interestingly, such combinations demonstrate synergistic anti-tumour activity, manifest by

increases in both senescence and apoptosis. Notably, drugs such as hydroxychloroquine can also promote release of CDK4/6 inhibitors from lysosomes in which they are sequestered, which may in part explain these observations[220].

The impact of CDK4/6 inhibitors on tumour cell metabolism is an understudied topic, especially considering the profound metabolic derangements reported in senescent cells[139]. One study demonstrated RB-dependent metabolic reprogramming in pancreatic carcinoma cells in response to CDK4/6 inhibition, which was characterised by increased mitochondrial mass and consumption of glucose and glutamine[221]. Although this might explain the reported sensitivity of CDK4/6 inhibitor-treated cancer cells to glutaminase inhibition[222], the underlying biological mechanisms require further study before their clinical applicability is determined.

Combined inhibition of CDK4/6 and epigenetic proteins

As the impact of CDK4/6 inhibitors on the cancer cell epigenome is increasingly understood, so too is interest in determining how this might be leveraged for therapeutic benefit. At the time of writing, promising activity has been reported in preclinical studies combining CDK4/6 inhibitors with drugs targeting epigenetic readers and writers that drive transcriptional activity (e.g., bromodomain-containing protein 4 (BRD4) and p300 and CREB binding protein (CBP))[32,223,224], but information is limited.

Novel applications and future challenges

Combination with cytotoxic therapy

CDK4/6 inhibitors arrest RB-proficient cancer cells in G1, raising concerns that they might antagonise the effects of cytotoxic chemotherapy drugs, which typically exert their effects during S phase or G2/M. Several preclinical studies confirmed this notion, demonstrating that CDK4/6 inhibitor-induced proliferative arrest diminishes the efficacy of chemotherapy administered shortly thereafter[119,129,225,226]. Similar antagonism has been reported when administering CDK4/6 inhibitors prior to external beam radiation therapy[227].

More recent studies, however, have challenged the idea that CDK4/6 inhibitors should not be combined with cytotoxic therapies by employing alternative dosing schedules that enhance efficacy in RB-proficient tumours. For example, administering microtubule stabilisers or DNA damaging agents prior to CDK4/6 inhibition was shown highly effective in *in vitro* and *in vivo* models of pancreatic carcinoma[131]. These findings could be explained by impaired recovery from chemotherapy-induced DNA damage due to suppressed expression of E2F target genes whose protein products mediate DNA repair by homologous recombination[129,131] (Figure 5a). RB-independent effects of CDK4/6 inhibitors (e.g., inhibited phosphorylation of CDK4/6 substrates FOXM1 or FOXO3) might also be sufficient to impair DNA repair after chemotherapy. Similar findings have been reported when CDK4/6 inhibitors were given after radiotherapy in a variety of different tumour models[228–230].

CDK4/6 inhibitors for supportive care

The idea that CDK4/6 inhibitors could protect normal tissue from the harmful effects of cytotoxic therapy was first proposed in 2010. Preclinical modelling demonstrated that pre-treatment of solid tumour-bearing mice with palbociclib reduces the multi-lineage haematological toxicity of total body irradiation or carboplatin chemotherapy[226,227]. The primary mechanism underlying this myelopreservation is an RB-dependent G1 arrest in haematopoietic stem and progenitor cells (HSPCs), reducing their vulnerability to DNA damaging agents. These findings prompted the development of trilaciclib as a myelopreservative agent. Intravenous trilaciclib therapy induces a rapid but transient G1 arrest in HSPCs and is now FDA-approved to reduce haematological toxicity for patients receiving cytotoxic chemotherapy for SCLC[231] (Figure 5b). Of note, SCLCs are most commonly RB-deficient, thereby mitigating the risk of CDK4/6 inhibitor-chemotherapy antagonism in tumour cells.

The approval of trilaciclib will open new avenues for CDK4/6 inhibitors in the field of oncology supportive care, particularly for RB-deficient tumours. Indeed, preclinical studies have shown that palbociclib or ribociclib can protect against radiation-induced intestinal injury[232], minimise hair follicle damage after taxane chemotherapy[233], and mitigate acute kidney injury induced by cisplatin and other nephrotoxins[234,235].

Prevention of cancer relapse

Oral CDK4/6 inhibitors are only approved for the treatment of metastatic malignancy, and a critical question is whether they can be successfully used in the adjuvant setting to prevent cancer relapse. Three clinical trials of high-risk, early-stage luminal breast cancer have provided contrasting results. The MonarchE trial demonstrated a significant improvement in disease-free survival when abemaciclib is added to endocrine therapy in patients with high-risk HR-positive early breast cancer. Conversely, the PALLAS and PENELOPE trials failed to show similar improvements with palbociclib, albeit in slightly different patient populations. The reasons for these differences are not clear, and it is hoped that longer follow-up of these and other studies will provide clarity on the role of CDK4/6 inhibitors in this setting[236–238]. From a biological standpoint, the unanswered question is what impact CDK4/6 inhibitors have on micrometastatic dormant tumour cells. In one preclinical study, a short course of CDK4/6 inhibitor therapy delayed, but did not prevent, recurrence of HER2-driven mammary carcinomas[153], and it remains an open question in the clinical arena whether a cytostatic therapy can do any more than delay an inevitable relapse.

Selective CDK4 and CDK6 degradation

Novel technologies such as proteolysis targeting chimeras (PROTACs) have enabled synthesis of compounds that degrade CDK4/6 rather than inhibit their enzymatic activity. Although most currently available CDK4/6 degraders are based on the structure of approved CDK4/6 inhibitors, they show variable selectivity for degradation of CDK4, CDK6, or both[90,239–243]. Given this, these drugs are appealing for indications where selective inhibition of either CDK4 or CDK6 is desired. They also enable inhibition of kinase-independent effects of CDK4/6, which could offer further additional therapeutic potential[244], and the ability to ‘dial-in’ simultaneous degradation of other important cancer drivers[241].

Several CDK4/6 degraders inhibit RB phosphorylation and induce G1 arrest as well, if not more potently than, traditional inhibitors[90]. Of note, however, current evidence suggests that the kinase-inhibitor resistant, thermostable CDK6 is also resistant to CDK6 degradation[90].

De novo and acquired resistance

Despite the success of CDK4/6 inhibitors, therapeutic failures and acquired resistance represent major barriers to their more widespread implementation. In the case of advanced endocrine-sensitive breast cancer, most patients derive clinical benefit from CDK4/6 inhibitor therapy before eventually experiencing disease progression[107,109,245]. For other cancer types, a significant gap remains between the promise CDK4/6 inhibitors have shown in laboratory studies and their apparent clinical efficacy. The cellular and molecular mechanisms reported to drive CDK4/6 inhibitor resistance have been comprehensively reviewed elsewhere, and so we discuss them only at a high-level here[246].

Many of the tumour cell-intrinsic resistance mechanisms ultimately alter or ‘rewire’ cellular CDK dependence. First, genomic alterations conferring loss of RB function (mutations and/or deletions in *RB1*) are, not surprisingly, associated with *de novo* and acquired resistance to CDK4/6 inhibitors in breast cancer[72,247–250]. Second, various resistance mechanisms faciliate CDK2-mediated S phase entry despite sutained inhibition of CDK4/6. These include amplification or heightened expression of cyclin E genes[8,190,250,251], the formation of atypical cyclin D–CDK2 complexes that can phosphorylate RB[5,8], and increased translation of cyclin E proteins as a result of heightened mTOR activity[190]. The importance of CDK2 as a pharmacological target in *de novo* and acquired CDK4/6 inhibitor resistance is underscored by the recent development of selective inhibitors targeting either CDK2[252,253] or all interphase CDKs (i.e. CDK2/4/6 inhibitors)[254]. Third, a diverse series of mechanisms that increase CDK6 levels – including *CDK6* amplification, upstream mutations (e.g. in *FAT1*) that increase *CDK6* expression, and transforming growth factor β (TGFβ) pathway suppression – have all been linked to CDK4/6 inhibitor resistance[72,255–257]. How increased levels of wild-type CDK6 itself mediates CDK4/6 inhibitor resistance is a fascinating question, given that these agents are potent CDK6 inhibitors. A possible explanation is that CDK6 induces expression of INK4C, resulting in formation of enzymatically active INK4C–cyclin D–CDK4/6 complexes, which are not bound by CDK4/6 inhibitors[257]. Importantly, these complexes remain sensitive to CDK4/6 degraders[257].

Given the inherent plasticity of the cell cycle machinery, it is also possible that cancer cells might develop mechanisms to proliferate even in the face of combined inhibition of all interphase CDKs. For example, mammalian cells lacking CDK2, CDK3, CDK4, and CDK6 can proliferate using CDK1, and it remains to be seen whether cancer cells treated with combined CDK2/4/6 inhibitors, for example, might co-opt this mechanism[7]. A potential strategy to overcome this would be pharmacological inhibition of CDK7, which has dual roles as a CDK-activating kinase (phosphorylating CDK1, CDK2, CDK4 and CDK6) and a mediator of RNA polymerase II-mediated transcription[258,259]. Indeed, selective CDK7 inhibitors are in clinical development and have shown promise in initial trials of CDK4/6 inhibitor-resistant luminal breast cancer[260].

Laboratory studies in several cancer types have also uncovered numerous other mechanisms underlying acquired CDK4/6 inhibitor resistance. Some of these are tumour-cell intrinsic and include drug-induced activation of upstream mitogenic signalling pathways (described earlier), drug sequestration in the tumour cell lysosomal compartment[220,261], and changes in chromatin modifiers that faciliate re-expression of E2F target genes[32,262]. In addition, therapy-induced stromal changes, such as fibroblast senescence, have also been implicated to drive resumption of tumour cell proliferation despite CDK4/6 inhibition[140]. However, none of these mechanisms have been looked for or identified in clinical specimens of CDK4/6 inhibitor-resistant cancers.

A number of important yet unanswered questions face the CDK4/6 inhibitor resistance field. First, it is not clear how common genomic alterations found in clinical specimens of CDK4/6 inhibitor-resistant breast cancer (i.e. mutations and copy number alterations) actually drive resistance, as opposed to simply co-occur at the time of resistance. Estimates of the frequency of newly emergent mutations in CDK4/6 inhibitor-resistant breast cancers vary markedly, and resolving this issue is important given the plethora of non-genetic mechanisms reported and the tendency for clinical investigators to rely on DNA sequencing when studying drug resistant cancers[248,250]. Second, the extent to which preclinical mechanisms of breast cancer resistance to CDK4/6 inhibitor monotherapy can be extrapolated to the clinic, where patients are treated with combined CDK4/6 inhibition and endocrine therapy, is not known. For this reason, studies focusing on resistance to combination therapy are needed, as is a greater understanding of how these two treatments interact. Finally, it is likely that resistance mechanisms identified in breast cancer are not universally applicable to other cancer types, and lineage- and mutation-specific drivers of cell cycle progression further complicate progress in the field.

Conclusions and future perspectives

A significant amount of data regarding the use of CDK4/6 inhibitors in cancer has accumulated in recent years. Important studies in preclinical models have shed light into the cellular and molecular mechanisms underlying the basis for their success in the clinic. Validating these findings in clinical settings will be an important step in guiding the design of further trials based on preclinical results, especially as there is still a disconnect between preclinical results and clinical development strategies. Addressing this concern will also help identify and prioritize specific areas for further development. Indeed, the large number of clinical trials currently evaluating combinations of CDK4/6 inhibitors with other agents – both FDA-approved and those under development – underscores the importance of this challenge. Moreover, molecular pathways and synergies described *in vitro* may not be recapitulated in *in vivo* settings due to the impact of different microenvironments and other variables, such as genetic and epigenetic alterations. Another important consideration will be the potential toxicity of combination therapies, which could severely limit clinical success, as has already been observed in recent trials of combination CDK4/6 and PI3K or mTOR inhibitors. Critically, it will be important to consider the diverse determinants of CDK4/6 inhibitor response, as described by our conceptual framework, when designing future clinical trials with these agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing interests

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Glossary

Mitogenic signals
Signals arising from small extracellular proteins or peptides that induce a cell to begin cell division.

Interphase
The portion of the cell cycle including G1, S and G2 phases, but excluding M phase; interphase begins at the end of one mitotic division and ends at the beginning of the next mitotic division.

Endocrine therapy (ET)
A therapy that alters the effect of sex steroid hormones in cancer cells; in breast cancer, ET is used to block the effect of oestrogen in hormone receptor-positive breast tumours.

Lymphokines
A type of cytokine (i.e., a small secreted protein with autocrine, paracrine and/or endocrine functions) produced and secreted by lymphocytes.

Thrombocytopenia
Low blood count of platelets (thrombocytes), a type of blood cell important for clotting.

Homologous recombination
The exchange of genetic material between homologous chromosomes; an important mechanism used by cells to repair deleterious DNA damages such as double strand breaks and collapsed replication forks.

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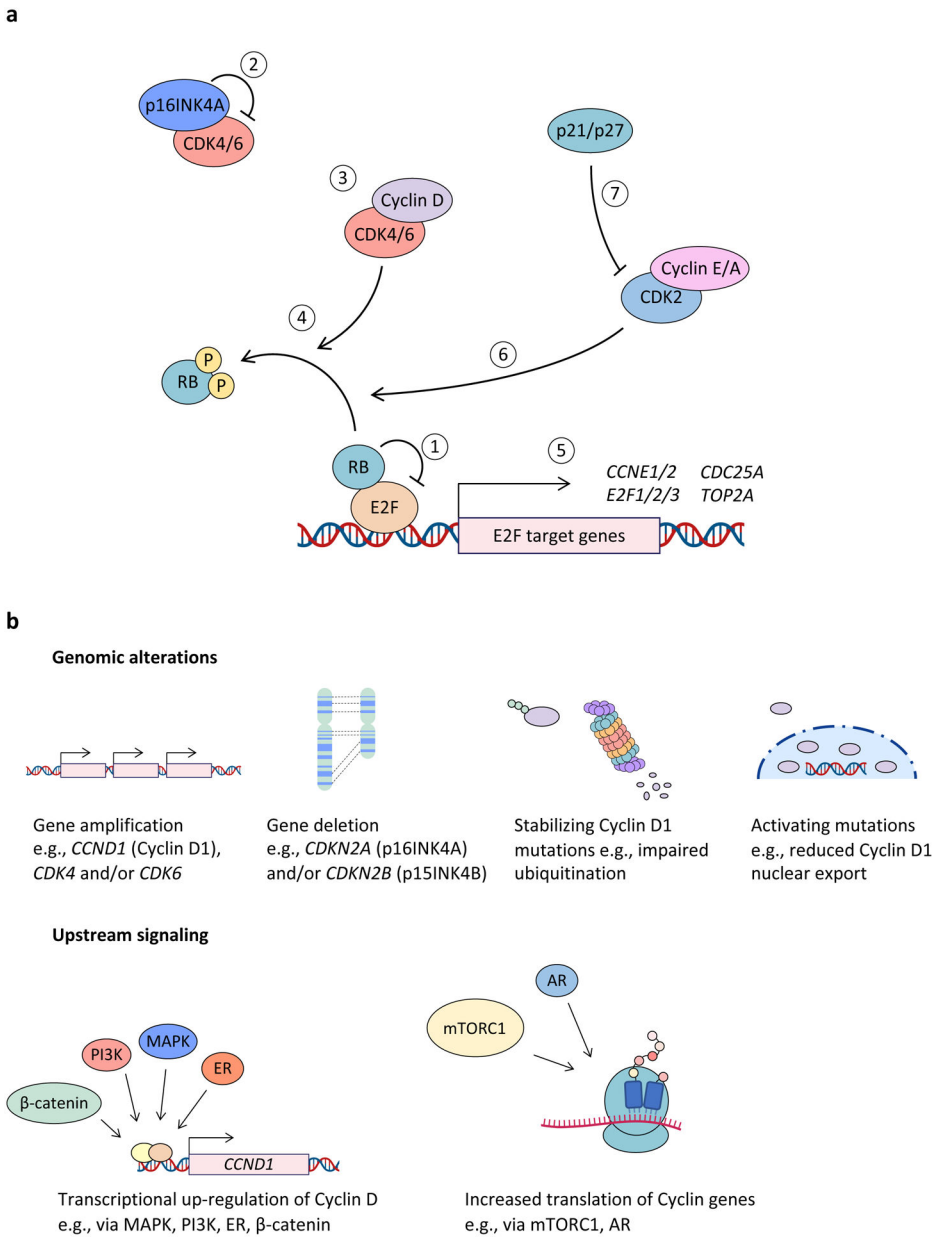


Figure 1. The CDK4 and CDK6 pathway in cancer.

a, Hypophosphorylated RB binds to and represses E2F family transcription factors (1). Negative regulation of cyclin-dependent kinase 4 (CDK4) and CDK6 (CDK4/6) activity is mediated primarily by the INK4 family of cell cycle inhibitors (INK4B (p15; encoded by cyclin-dependent kinase inhibitor 2B (*CDKN2B*)), INK4A (p16; encoded by *CDKN2A*), INK4C (p18), and INK4D (p19)), which bind to monomeric CDK4 and CDK6 to form inactive binary complexes (2). Mitogen or growth factor stimulation drives cyclin D up-regulation, leading to CDK4/6 activation (3). RB phosphorylation by cyclin D–CDK4/6 complexes promotes dissociation of RB–E2F binding (4). This in turn allows for E2F-mediated expression of genes required for cell cycle progression (5), which leads to progression through G1 phase and into S-phase. RB phosphorylation by cyclin E–CDK2

and cyclin A–CDK2 complexes (6) also promotes RB–E2F dissociation to drive progression into S phase. On the other hand, WAF1 and KIP family proteins, such as p21 (WAF1), p27 (KIP1) and p57 (KIP2), inhibit CDK2 and are important for inducing cell cycle arrest (7). Of note, p21 and p27 (p21/p27) have been shown to inhibit CDK4/6 activity in some instances and in other instances to stabilize cyclin D–CDK4/6 and thereby form an active trimeric holoenzyme. **b**, Major mechanisms responsible for dysregulated CDK4/6 activity in cancer include genomic alterations as well as activation of upstream signalling pathways that may up-regulate this pathway at the transcriptional, translational and post-translational levels. AR, androgen receptor; *CCN*, cyclin; ER, oestrogen receptor; mTORC1, mTOR complex 1.

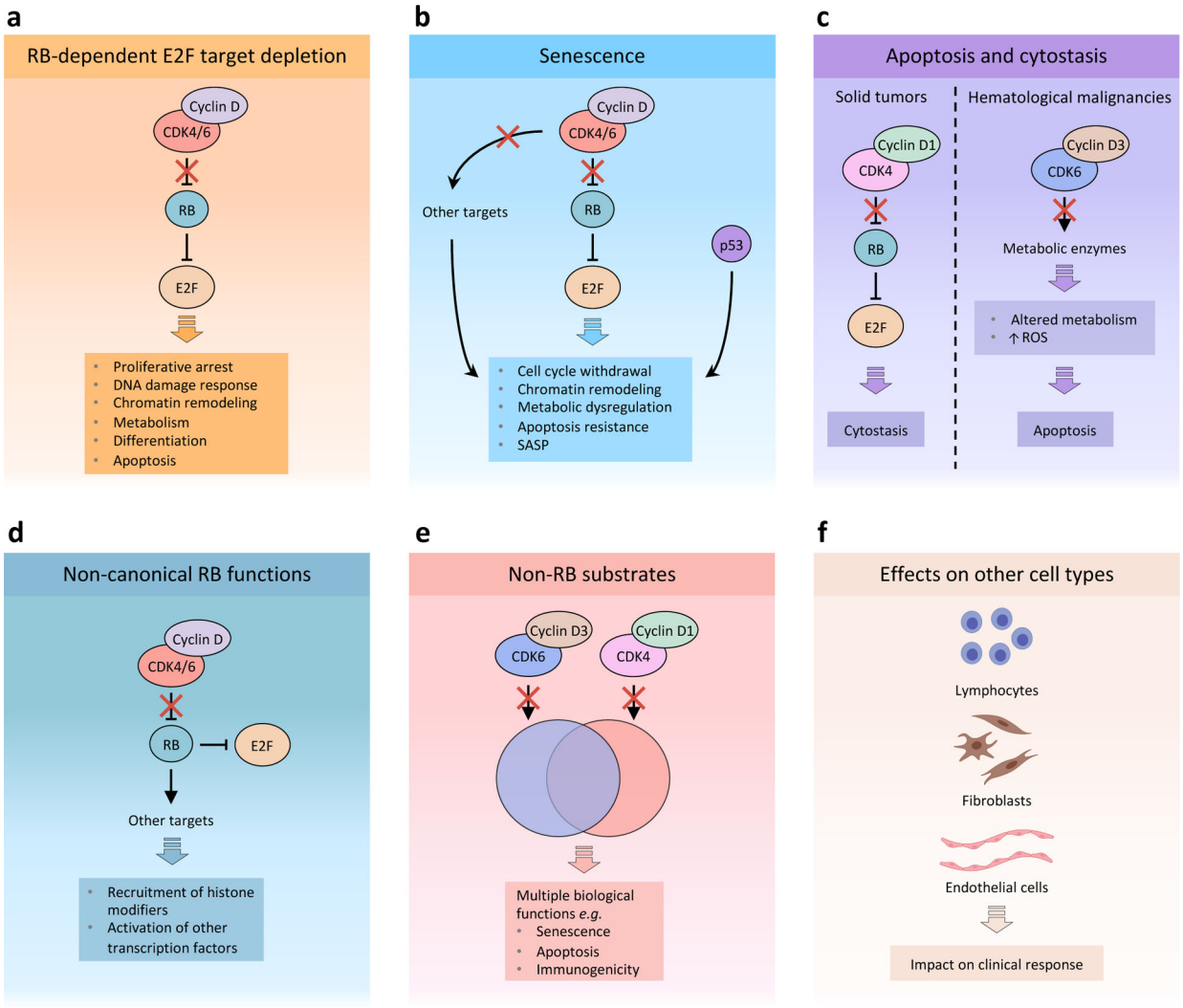
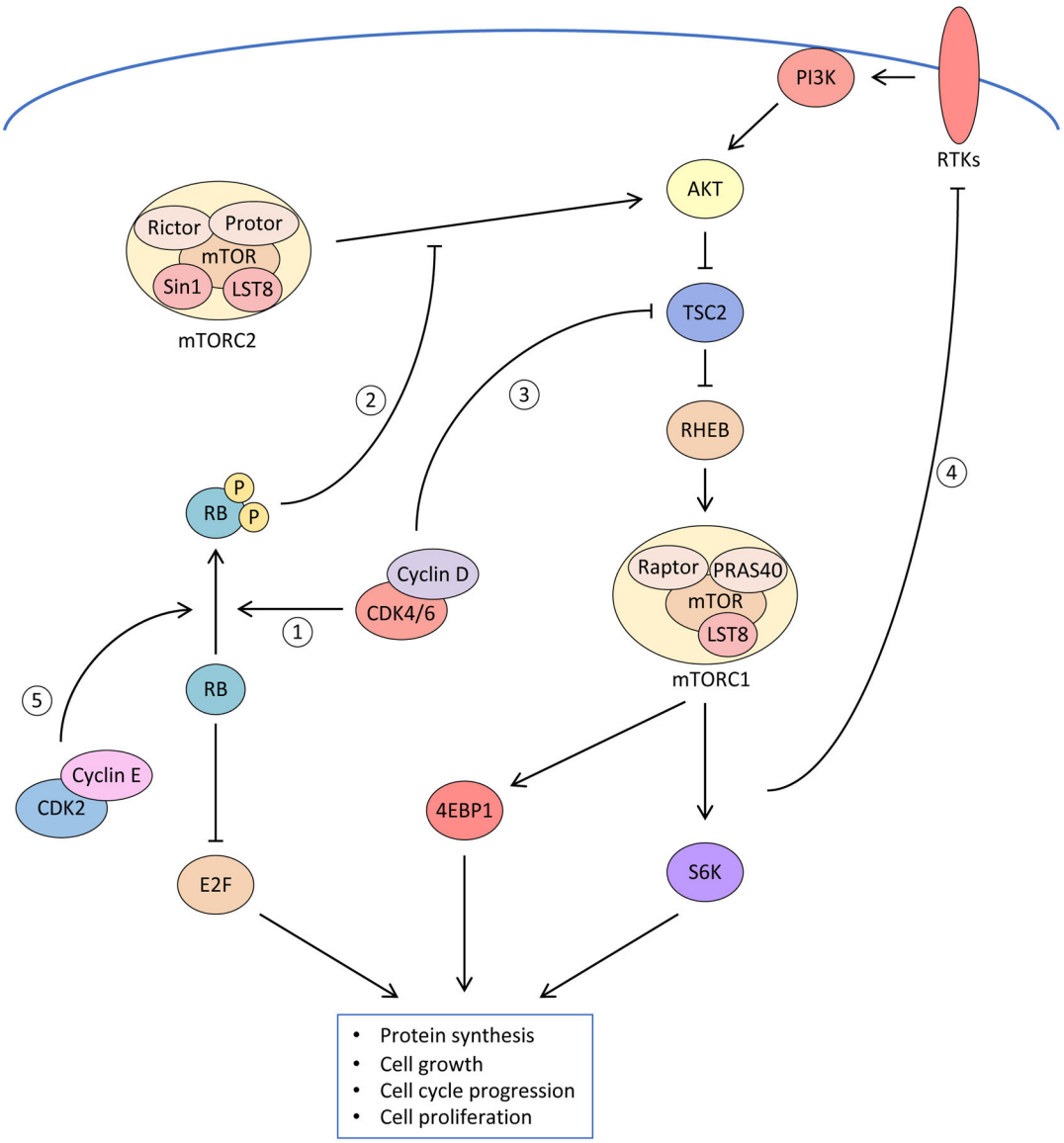


Figure 2. A conceptual framework to understand the effects of CDK4 and CDK6 inhibitors in cancer.

Response to cyclin-dependent kinase 4 (CDK4) and CDK6 (CDK4/6) inhibitors depends on multiple factors that affect the final outcome, including context-dependent tumour cell-intrinsic and -extrinsic features. **a**, RB-dependent E2F target depletion: Blockade of RB phosphorylation by cyclin D–CDK4/6 complexes leads to sustained binding to and inhibition of E2F by RB. A major consequence of E2F inactivation by RB is proliferative arrest. Moreover, E2F functions in various additional processes that are affected by CDK4/6 inhibition, including DNA damage response, chromatin remodelling, metabolism, differentiation and apoptosis. **b**, Senescence: CDK4/6 inhibitors may induce an RB-dependent senescent phenotype characterized by cell cycle withdrawal, chromatin remodelling, metabolic dysregulation, resistance to apoptosis and a senescence-associated secretory phenotype (SASP). Downregulation of other CDK4/6 targets, such as forkhead box protein M1 (FOXM1) and DNA methyltransferase 1 (DNMT1), is thought to enhance the senescent phenotype. In addition, p53 plays distinct and overlapping roles with RB in inducing senescence. Current evidence suggests that loss of p53 function may significantly

affect senescence induced by CDK4/6 inhibitors, although additional studies have shown that CDK4/6 inactivation can still induce senescent phenotypes in the absence of wild type p53. **c**, Apoptosis and cytostasis: Induction of apoptosis or cytostasis appears to be cell type-dependent and may depend on differences in signalling between CDK4 and CDK6. In haematological malignancies, inhibition of CDK6–cyclin D3 complexes may lead to an RB-independent state of metabolic dysregulation that results in apoptosis. On the other hand, solid tumours such as breast cancer often depend primarily on CDK4–cyclin D1 activity for proliferation. In this case, CDK4/6 monotherapy predominantly induces RB-dependent proliferative arrest as a result of sustained E2F inhibition. **d**, Non-canonical RB functions: In addition to inhibiting E2F, RB has been shown to exert other functions, such as mediating chromatin remodelling and activation of other transcription factors. **e**, Non-RB substrates: In addition to phosphorylating and inhibiting RB, CDK4 and CDK6 phosphorylate a set of unique and overlapping targets that play important functions in numerous biological processes, including senescence, apoptosis and immunogenicity. **f**, CDK4/6 inhibitors have been shown to exert direct effects on multiple cell types normally present within the tumour microenvironment, including lymphocytes, fibroblasts and endothelial cells. Several studies have shown CDK4/6 inhibitors can directly and indirectly promote anti-tumour T lymphocyte effector function and inhibit immunosuppressive regulatory T (T_{reg}) cells. CDK4/6 inhibitors also induce senescence phenotypes in fibroblasts, which could potentially impair response to therapy by promoting tumour growth, and cell cycle arrest in endothelial cells, which has the potential to impact on clinical response. ROS, reactive oxygen species.

a **Tumor cell-intrinsic signaling pathways affected by CDK4/6 inhibition**



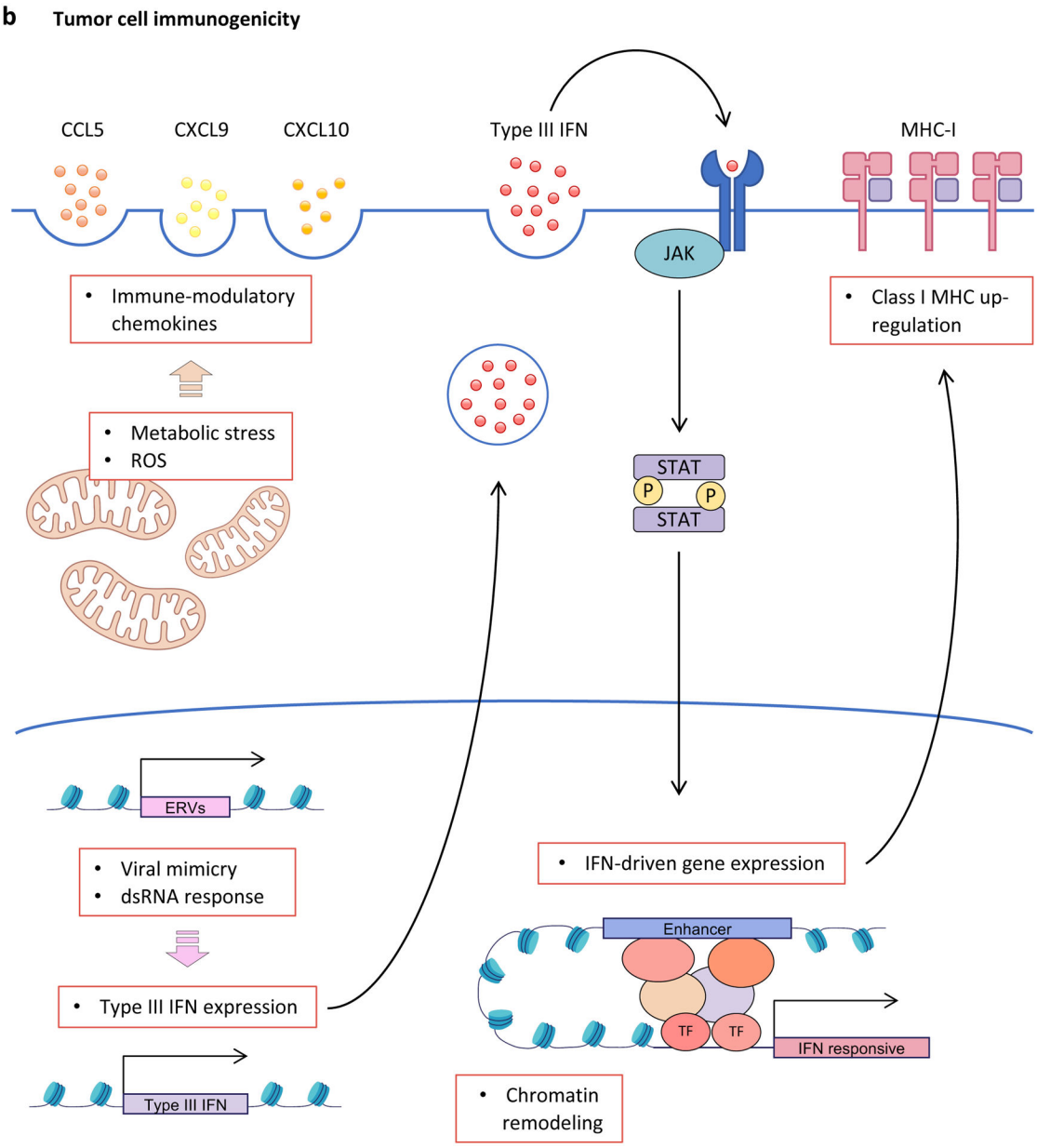


Figure 3. Effect of CDK4 and CDK6 inhibition upon tumour cell-intrinsic and -extrinsic signalling pathways.

a, Inhibition of cyclin-dependent kinase 4 (CDK4) and CDK6 (CDK4/6) activity induces rewiring of multiple interconnected kinase circuits in tumour cells. First, blocking CDK4/6 inhibits RB phosphorylation (1), thereby preventing E2F-mediated cell cycle progression. However, multiple mechanisms may counteract these effects. For example, decreased phosphorylated-RB levels also lead to enhanced AKT phosphorylation by mTORC complex 2 (mTORC2) (2), which can stimulate cell survival mechanisms. Similarly, decreased CDK4/6 activity can down-regulate mTORC1 signalling via enhanced tuberous sclerosis 2 (TSC2) activity (3), which may lead to loss of negative-feedback regulation of receptor tyrosine kinase (RTK) signalling (4), resulting in up-regulated upstream PI3K–AKT pathway activity. In addition, compensatory CDK2-mediated RB phosphorylation (5)

can stimulate cell cycle progression in the absence of CDK4/6 signalling. **b**, CDK4/6 inhibitors promote tumour cell immunogenicity via multiple mechanisms: (i) Metabolic stress and increased production of reactive oxygen species (ROS) up-regulate secretion of inflammatory chemokines CC-chemokine ligand 5 (CCL5), CXC-chemokine ligand 9 (CXCL9) and CXCL10; (ii) hypomethylation and therefore expression of endogenous retroviruses (ERVs) induces a double-stranded RNA (dsRNA) response, which leads to increased expression and secretion of type III interferon (IFN), activation of Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signalling, enhanced IFN-driven gene expression, and up-regulation of major histocompatibility complex (MHC) class I expression; and (iii) chromatin remodelling facilitates IFN-mediated expression of interferon-responsive genes. 4EBP1; eukaryotic translation initiation factor 4E binding protein 1; IFNLR1, interferon λ receptor 1; IL-10RB, interleukin-10 receptor subunit β; S6K, S6 kinase; TF, transcription factor.

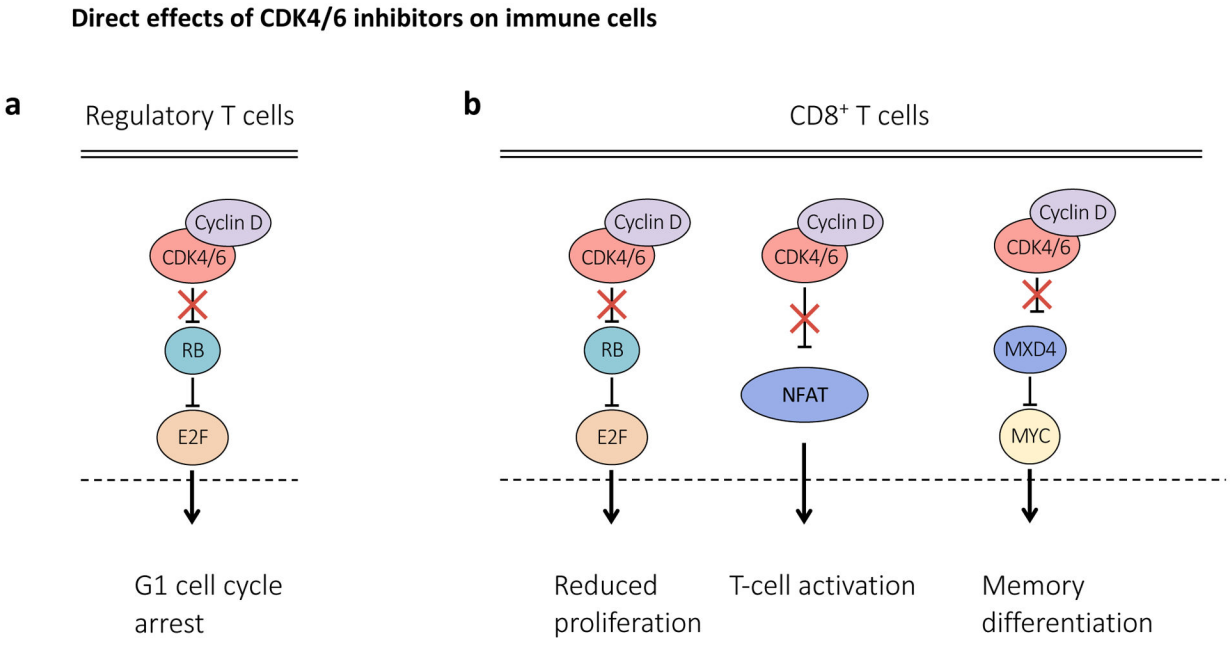


Figure 4. CDK4 and CDK6 inhibitors exert differential effects in distinct immune cell populations.

a, Upon treatment with cyclin-dependent kinase (CDK4) and CDK6 (CDK4/6) inhibitors, regulatory T (T_{reg}) cells preferentially undergo cell cycle arrest, likely due to increased reliance on CDK4/6 signalling for cell cycle progression. **b**, CDK4/6 inhibition enhances $CD8^+$ T-cell activation and induction of effector function via upregulation of nuclear factor of activated T-cells (NFAT) signalling. In addition, CDK4/6 inhibitors have been shown to promote memory T-cell differentiation through RB-dependent and RB-independent (increased MXD4 gene expression) mechanisms. MXD4, MAX dimerization protein 4.

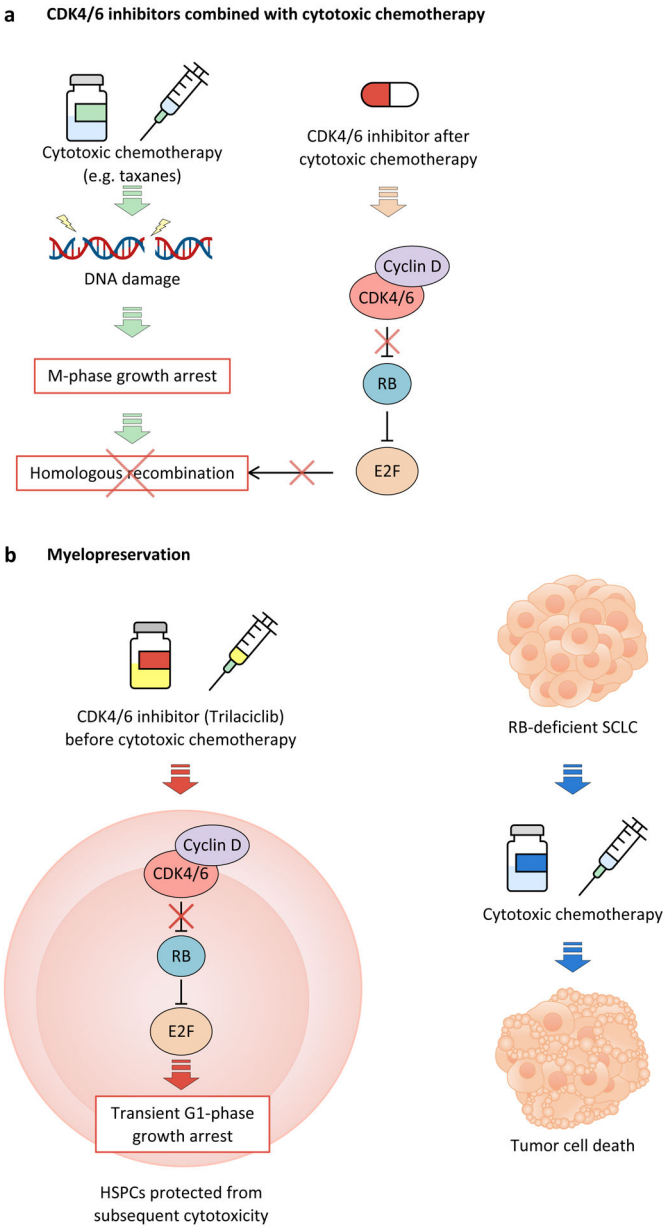


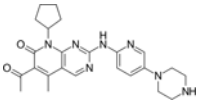
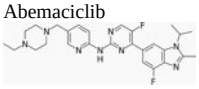
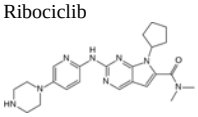
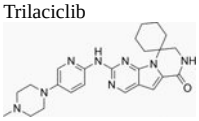
Figure 5. Novel approaches for the use of CDK4 and CDK6 inhibitors.

a, Administration of cyclin-dependent kinase 4 (CDK4) and CDK6 (CDK4/6) inhibitors after cytotoxic chemotherapy has been shown to enhance response by preventing E2F-mediated DNA damage repair after growth arrest during M-phase. Conversely, treatment with CDK4/6 inhibitors prior to chemotherapy would induce G1 cell cycle arrest, thus preventing chemotherapy-induced cytotoxicity that occurs during DNA replication or chromosome segregation. **b**, The CDK4/6 inhibitor trilaciclib can be used to protect hematopoietic stem and progenitor cells (HSPCs) from the cytotoxic effects of chemotherapy by inducing transient cell cycle arrest prior to treatment with chemotherapy. In this case, the target population would be patients with cancer types that are typically

RB-deficient, such as small-cell-lung cancer (SCLC), so as to not interfere with cytotoxicity against tumour cells.

Table 1.

CDK4 and CDK6 inhibitors approved or under clinical development.

Agent	Company	Selectivity (IC50)	Clinical development
Approved			
Palbociclib 	Pfizer	CDK4: 11 nM CDK6: 16 nM	Approved for HR+, HER2− advanced breast cancer in combination with hormonal therapy
Abemaciclib 	Eli Lilly	CDK4: 2 nM CDK6: 10 nM	Approved for HR+, HER2− advanced breast cancer in combination with hormonal therapy Approved as monotherapy for advanced HR+, HER2− breast cancer Approved as adjuvant therapy for high-risk, early-stage HR+, HER2− breast cancer in combination with hormonal therapy
Ribociclib 	Novartis	CDK4: 10 nM CDK6: 39 nM	Approved for HR+, HER2− advanced breast cancer in combination with hormonal therapy
Trilaciclib 	G1 Therapeutics	CDK4: 1 nM CDK6:4 nM	Approved to reduce chemotherapy-induced bone marrow suppression in patients with extensive-stage SCLC.
Phase III			
Dalpiciclib (SHR6390)	Jiangsu Hengrui Medicine	CDK4: 12 nM CDK6: 10 nM	Phase III for HR+, HER2− breast cancer in combination with hormonal therapy; phase I/II for multiple tumor types in combination with hormone, targeted or immune therapy
Phase II			
PF-06873600	Pfizer	CDK2: 0.09 nM (Ki) CDK4: 0.13 nM (Ki) CDK6: 0.16 nM (Ki)	Phase II for HR+, HER2− metastatic breast cancer, TNBC and gynecological cancers in combination with hormonal therapy
Phase I/II			
Lerociclib (G1T38)	G1 Therapeutics	CDK4: 1 nM CDK6: 2 nM CDK9: 28 nM	Phase I/II for HR+, HER2− metastatic breast cancer in combination with fulvestrant (SERD); phase I/II for EGFR-mutant metastatic NSCLC in combination with osimertinib (EGFR inhibitor)
Birociclib (XZP-3287)	Jilin Sihuan Pharmaceutical/ Xuanzhu Pharma	Not available	Phase I/II for HR+, HER2− advanced breast cancer
BPI-1178	Beta Pharma	Not available	Phase I/II for advanced solid tumors, and for HR+, HER2− advanced breast cancer in combination with hormone therapy
FCN-437C	Fochon Pharmaceuticals	Not available	Phase I/II for HR+, HER2− advanced breast cancer in combination with letrozole (aromatase inhibitor)
TQB3616	Chia Tai Pharmaceutical Group	Not available	Phase I/II for HR+, HER2− advanced breast cancer in combination with fulvestrant, and for advanced lung cancer in combination with anlotinib (VEGFR inhibitor) or standard chemotherapy
Phase **I**			
AMG-925 (FLX925)	Amgen	CDK4: 1.5 nM CDK6: 8 nM FLT3: 2.4 nM	Phase I/Ib for relapsed or refractory AML

Agent	Company	Selectivity (IC50)	Clinical development
R547	Hoffmann-La Roche	CDK1: 2 nM (Ki) CDK2: 3 nM (Ki) CDK4:1 nM (Ki)	Phase I for advanced solid cancers
BPI-16350	Betta Pharmaceuticals	Not available	Phase I for advanced solid cancers
CS3002	CStone Pharmaceuticals	Not available	Phase I for advanced solid cancers
HS-10342	Jiangsu Hansoh Pharmaceutical	Not available	Phase I for advanced solid cancers
PF-06842874	Pfizer	Not available	Phase I in healthy participants
TY-302	TYK Medicines	Not available	Phase I for advanced solid cancers, and for HR+, HER2− breast advanced cancer in combination with tamoxifen

AML, acute myeloid leukaemia; CDK, cyclin-dependent kinase; EGFR, epidermal growth factor receptor; FLT3, FMS-like tyrosine kinase 3; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; SCLC, small-cell lung cancer; SERD, selective estrogen receptor degrader; TNBC, triple-negative breast cancer; VEGFR, vascular endothelial growth factor receptor.