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Perspective



# CDK4/6 Inhibitors: The Mechanism of Action May Not Be as Simple as Once Thought

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CDK4/6 inhibitors are among a new generation of therapeutics. Building upon the striking success of the combination of CDK4/6 inhibitors and the hormone receptor antagonist letrozole in breast cancer, many other combinations have recently entered clinical trials in multiple diseases. To achieve maximal benefit with CDK4/6 inhibitors it will be critical to understand the cellular mechanisms by which they act. Here we highlight the mechanisms by which CDK4/6 inhibitors can exert their anti-tumor activities beyond simply enforcing cytostatic growth arrest, and discuss how this knowledge may inform new combinations, improve outcomes, and modify dosing schedules in the future.

## The Importance of Cyclin D-CDK4/6 in the Cell Cycle

With the war on cancer declared almost 50 years ago, a mechanistic understanding of the processes underlying the duplication and segregation of DNA was long considered a useful area for the identification of druggable targets for therapy. Nearly 20 years later the cyclin/cyclin-dependent kinase (CDK) holoenzyme was identified as a key driver of a number of cell-cycle transitions and the first non-specific CDK inhibitors made it to the clinic over the next 10 years. Such pan-CDK inhibitors had limited success, due in part to the dose-limiting toxicities that hampered their use (Asghar et al., 2015). Today, far more specific inhibitors that target CDK4 and CDK6 (palbociclib, abemaciclib, and ribociclib) exist. These have more limited toxicities, which allows for their broad use to treat a variety of neoplasms. Currently CDK4/6 inhibitors are being used both as single agents, and in a vast number of clinical trials evaluating their efficacy when combined with signaling pathway inhibitors. Thus, it is crucial to understand how they exert their anti-tumorigenic effects.

CDK4/6 activity bridges numerous extracellular signaling pathways to the cell cycle (Sherr and Roberts, 1999). Both non-immortal non-transformed cells (hereafter referred to as "normal" cells) and many transformed tumor cells commit irreversibly to the mitotic cell cycle in the $G_1$ phase. Commitment depends on the phosphorylation and inactivation of the retinoblastoma tumor-suppressor protein, Rb. The growth suppressive properties of Rb are largely, but not completely, associated with its binding to the transcription factor E2F and repressing transcription at target promoters (Classon and Harlow, 2002; Harbour and Dean, 2000; Stevaux and Dyson, 2002). Phosphorylation of Rb destabilizes its interaction with E2F and other transcriptional regulators. In normal cells, the phosphorylation of Rb is typically carried out by the sequential actions of the CDK4 or CDK6 kinases in complex with a positive regulatory D-type cyclin subunit, followed by cyclin E/CDK2 complexes (Harbour et al., 1999; Lundberg and Weinberg, 1998). Additionally, extracellular signals regulate the expression of cyclins and CDK inhibitors, such as $p16^{Ink4a}$, $p21^{Cip1}$, and $p27^{Kip1}$, the first of which inhibits the CDK4/6 kinases whereas the latter two inhibit the CDK2 kinases (Sherr and Roberts, 1999).

In virtually all human cancer cells, this circuit is dysregulated by either overexpression of cyclin D1, loss of $p16^{Ink4a}$, the mutation of CDK4 to an Ink4-refractory state, or the loss of Rb itself (Classon and Harlow, 2002). This not only affects how the cancer cell responds to extracellular signals; it can also affect the requirement for sequential ordered phosphorylation by the CDKs during the inactivation of Rb. Thus, in some cells CDK2 may be dispensable, or compensated for by other CDKs.

Although Rb is the primary cell-cycle target of CDK4/6, Rb and other proteins that control the commitment decision have non-cell-cycle-related roles (Besson et al., 2008; Denicourt and Dowdy, 2004). CDK4/6 kinases can phosphorylate factors involved in cell differentiation affecting their transcriptional activity, apoptotic factors affecting their activity, and other factors that can directly affect mitochondrial activity (Hydbring et al., 2016; Lim and Kaldis, 2013). Where applicable we include potential non-Rb targets that could be participating in the immunological, senescence-promoting, and metabolic outcomes associated with these drugs. However, our focus in this Perspective is on the outcome of inhibiting the CDK4/6 kinases in Rb-proficient tumors, and we encourage readers with an interest in alternative substrates and interactions to seek out additional reviews.

## CDK4/6 Inhibitors: A Trio of Compounds with Distinct Advantages

Although palbociclib was the first CDK4/6 inhibitor to demonstrate clinical efficacy (Finn et al., 2016), two others soon followed. Ribociclib is structurally very similar to palbociclib, and abemaciclib is significantly less similar to either one (Table 1).

**Table 1. Drug Characteristics of CDK4/6 Inhibitors**

| | Structure | $IC_{50}$ in Cell-Free Assay | $C_{max}$ (nM) | $t_{max}$ (hr) | $t_{1/2}$ (hr) | $K_{p,uu}$ in Mouse Models | Toxicities in Phase 3 Trials | Dosing Schedule |
|---|---|---|---|---|---|---|---|---|
| Palbociclib (PD0332991) |  | CDK4 (11 nM) CDK6 (15 nM) | 200–260 | 4–8 | 28 | 0.01 | neutropenia | 125 mg p.o. daily for 21 out of every 28 days (in combination with hormone therapy) |
| Ribociclib (LEE011) |  | CDK4 (10 nM) CDK6 (39 nM) | 4,000–7,000 | 2–5 | 30–50 | 0.12 | neutropenia | 600 mg p.o. daily for 21 out of every 28 days (in combination with hormone therapy) |
| Abemaciclib (LY2835219) |  | CDK4 (2 nM) CDK6 (9.9 nM) | 500–600 | 4 | NR (21 hr for a single dose) | 0.03 | GI distress, neutropenia (not dose-limiting) | 200 mg p.o. daily continuously (as a monotherapy) |

p.o., by mouth; NR, not reported; GI, gastrointestinal.

*In vitro* studies using cyclin D1/CDK4 and various cyclin D/CDK6 kinases determined that both abemaciclib and ribociclib are more potent against CDK4 than CDK6 (Gelbert et al., 2014; Tripathy et al., 2017) (Table 1). Palbociclib, on the other hand, has similar potency when comparing its activity on cyclin D1/CDK4 and cyclin D2/CDK6 (Fry et al., 2004). In such assays, abemaciclib also has modest activity, relative to its CDK4 inhibitory activity, against cyclin T1/CDK9, cyclin E2/CDK2, p25/CDK5, and p35/CDK5 (Gelbert et al., 2014) (Table 2). However, the remarkable specificity of all of these drugs to inhibit the proliferation of Rb-positive tumor cells but not Rb-negative tumor cells suggests that differences in the *in vitro* profiles might not contribute that much to their *in vivo* activity.

All three CDK4/6 inhibitors are orally available, but each has differing pharmacokinetics and clinical toxicities (Table 1), necessitating different dosing strategies. Both palbociclib and ribociclib are dosed once daily whereas abemaciclib is dosed twice daily. Ribociclib is notable for achieving high maximum plasma concentrations (exceeding 2 μg/mL) with a long half-life (greater than 30 hr). This may translate to higher cerebrospinal fluid concentrations for ribociclib compared with palbociclib and abemaciclib, as noted in mouse models (DiPippo et al., 2016; Raub et al., 2015; Yin et al., 2017). Other rodent models indicate a strong efflux of palbociclib out of the CNS, and less so for abemaciclib (Raub et al., 2015). In the few human patients receiving abemaciclib in whom drug levels in the CNS were measured, accumulations in the range of 2.2–14.7 nmol/L were achieved (Patnaik et al., 2016).

There are marked differences in the toxicity profiles of the inhibitors for reasons that are not completely clear. Grade 3–4 neutropenia is observed in approximately 60% of patients taking palbociclib and ribociclib (Asghar et al., 2015; Hortobagyi et al., 2016). Abemaciclib appears to be better tolerated overall, with only 55% of patients experiencing significant adverse events (compared with 70%–80% with ribociclib and palbociclib) and only 21% with grade 3–4 neutropenia. However, 10% of the patients treated with abemaciclib develop grade 3 diarrhea, which is very rare with the other two inhibitors (Asghar et al., 2015; Hortobagyi et al., 2016). Unraveling the toxicities and overcoming them may lead to a better understanding of these drugs and their biological availability.

Due to the significant myelotoxicity of palbociclib and ribociclib, both drugs require dose interruption and are administered on a 3-weeks-on/1-week-off schedule to allow marrow recovery. In contrast, abemaciclib is dosed continuously. When considering the cytostatic effect of CDK4/6 inhibitors, interrupted dosing can provide an opportunity for potent synergy with combination therapies dedicated to interfering with other cell-cycle effects. For example, DNA-damaging agents typically require active cell-cycle progression. While co-administration of CDK4/6 inhibitors has been used *in vivo* to "protect" healthy cells from the toxic side effects of chemotherapy (He et al., 2017), interrupted dosing may provide an additional opportunity to use cytotoxic DNA-damaging and other cell-cycle targeting therapies as the cells become somewhat synchronized upon release, resulting in increased susceptibility to those compounds (Francis et al., 2017; Huang et al., 2012; Yang et al., 2015). Such alternative dosing strategies are being investigated in early-phase multi-agent clinical trials in a variety of different tumor

**Table 2. $IC_{50}$ of CDK4/6 Inhibitors in Cell-Free Assays**

| | $IC_{50}$ by Cell-Free Assay | | |
|---|---|---|---|
| CDK Family Kinase Complex | Palbociclib | Ribociclib | Abemaciclib |
| CDK4/cyclin D1 | 11 nM | 8 nM | 2 nM |
| CDK4/cyclin D3 | 9 nM | NR | NR |
| CDK6/cyclin D1 | NR | NR | 9.9 nM |
| CDK6/cyclin D2 | 15 nM | NR | NR |
| CDK6/cyclin D3 | NR | 39 nM | NR |
| CDK1/cyclin B | >10 μM | >1.5 μM | 1,627 nM |
| CDK2/cyclin A | >10 μM | >1.5 μM | NR |
| CDK2/cyclin E2 | >10 μM | >1.5 μM | 504 nM |
| CDK5/p25 | >10 μM | >1.5 μM | 355 nM |
| CDK5/p35 | NR | >1.5 μM | 287 nM |
| CDK7/cyclin H1 | NR | >1.5 μM | 3,910 nM |
| CDK9/cyclin T1 | NR | 1,510 nM | 57 nM |
| References | Fry et al., 2004 | Tripathy et al., 2017 | Gelbert et al., 2014 |

NR, not reported.

types including breast cancer, leukemia, and soft tissue sarcomas (Table 3).

In summary, all three inhibitors target the proliferative function of the cyclin D-associated kinases in Rb-positive tumor cells to induce cell-cycle exit and are largely inactive in Rb-negative cells. While this suggests that Rb is the sole important substrate, it is conceivable that other substrates of these kinases contribute to phenotypes after the cells have exited the cell cycle. The key to improving combination therapies may lie in recognizing the consequences of growth arrest induced by CDK4/6 inhibitors and understanding how signaling pathways contribute to maintaining cells in a quiescent state.

## CDK4/6 Inhibitors Reinforce Cytostasis Induced by Signaling Pathway Inhibitors

Many early-phase clinical trials are combining CDK4/6 inhibitors with signaling pathway-targeted inhibitors (Table 3). In cell lines and xenografts, intrinsic and acquired resistance to signaling pathway inhibitors against estrogen, RAF, epidermal growth factor receptor (EGFR), phosphoinositide 3-kinase (PI3K), and others is sometimes associated with mutations in $p16^{Ink4a}$, upregulated expression of cyclin D1 or other D-type cyclins, or upregulation of CDK4 or CDK6 (Jiang et al., 2016; Long et al., 2014; Yadav et al., 2014). In some of these models, resistance can be overcome by including CDK4/6 inhibitors in the treatment (Finn et al., 2009; Goel et al., 2016; Kwong et al., 2012; Vora et al., 2014; Zhou et al., 2017). Thus, increased therapeutic efficiency can occur by enforcing a more durable cell-cycle exit (Figure 1A) as has been extensively reviewed (e.g., Sherr et al., 2016).

However, a number of recent investigations provide an alternative mechanistic explanation for the clinical activity of CDK4/6 inhibitors in combination with other drugs. Outcomes can depend on the nature of the cytostatic effect in tumor cells, vis-à-vis whether it undergoes a reversible quiescence or a more stable senescence. CDK4/6 inhibition can also alter cellular metabolism, depleting antioxidants, increasing reactive oxygen species (ROS), and triggering apoptosis. CDK4/6 inhibition can also affect both the maturation of sentinel cells of the immune system and the expansion of regulatory T cells. These mechanisms are summarized in Figure 1, and how they might affect combinatorial cancer therapies in the future is discussed individually below.

## Senescence after CDK4/6 Inhibitor-Induced Growth Arrest

Recently, a number of groups have become interested in the decisions that cells make when they exit from the $G_1$ phase of the cell cycle into quiescence. Depending on the cell type and the transforming event, some Rb-positive cells undergo quiescence and others undergo senescence when treated with CDK4/6 inhibitors (Baughn et al., 2006; Choi et al., 2012; Kovatcheva et al., 2015; Michaud et al., 2010; Puyol et al., 2010). Unlike quiescent cells, senescent cells will not return to the cell cycle following removal of the inducing signal and are generally refractory to other proliferation-inducing signals (Rodier and Campisi, 2011). This outcome may be an important consideration when deciding whether to treat with abemaciclib or palbociclib/ribociclib, given their differing dosing schedules.

While exploring the effects of palbociclib and other CDK4/6 inhibitors, a decision point was identified at which quiescent cells decide whether or not to progress to senescence (Figure 1B). The transition from quiescence into senescence has been termed geroconversion and can also be described as senescence after growth arrest (SAGA). Which outcome is achieved depends on a cell-type intrinsic program that is activated following the withdrawal of the cell from the cell cycle. Specifically, downregulation of MDM2, redistribution of the chromatin-remodeling enzyme ATRX, and repression of *HRAS* transcription are necessary for the transition of CDK4 inhibitor-induced quiescence into senescence in a number of mesenchymal and epithelial cell lines derived from different tumor types, including breast, non-small cell lung cancer, soft tissue sarcoma, and glioma (Kovatcheva et al., 2015, 2017).

**Table 3. List of Ongoing Clinical Trials with CDK4/6 Inhibitors in Combination with One or More Other Therapies**

| Combination | Dosing Schedule | Disease | Phase | Identifier |
|---|---|---|---|---|
| Palbociclib | | | | |
| Trastuzumab-DM1 (HER2 antibody) | palbociclib days 5–18 (21-day cycle)<br>trastuzumab day 1 | $HER2^+$ breast cancer | Ib | NCT1976169 |
| Tucatinib (HER2 inhibitor)<br>+ letrozole (aromatase inhibitor) | palbociclib days 1–21 (28-day cycle)<br>letrozole and tucatinib days 1–28 | $HR^+$, $HER2^+$ breast cancer | Ib/II | NCT03054363 |
| Anastrozole (aromatase inhibitor)<br>+ trastuzumab<br>+ pertuzumab (HER2 inhibitor) | palbociclib days 1–21 (28-day cycle)<br>anastrozole days 1–28<br>trastuzumab and pertuzumab<br>once every 21 days | $HR^+$, $HER2^+$ breast cancer | I/II | NCT03304080 |
| Baxedoxifene (ER modulator) | not stated | $HR^+$ breast cancer | Ib/II | NCT02448771 |
| SAR439859 (ER degrader) | palbociclib days 1–21 (28-day cycle)<br>SAR439859 days 1–28 | $ER^+$ breast cancer | I/II | NCT03284957 |
| GDC-0810 (ER downregulator) | palbociclib days 1–21 (28-day cycle)<br>GDC-0810 days 1–28 | $ER^+/HER2^-$ breast cancer | I/II | NCT01823835 |
| Gedatolisib (PI3K/mTOR inhibitor)<br>+ fulvestrant (ER antagonist) | palbociclib days 1–21 (28-day cycle)<br>gedatolisib days 1, 7, 14, 21;<br>Fulvestrant day 1 | $ER^+/HER2^-$ breast cancer | I | NCT02626507 |
| Gedatolisib (PI3K/mTOR inhibitor) | palbociclib days 1–21 (28-day cycle)<br>gedatolisib days 1, 7, 14, and 21 | Solid tumors | I | NCT03065062 |
| Copanlisib (PI3K inhibitor) + letrozole | palbociclib days 1–21 (28-day cycle)<br>copanlisib days 1, 8, and 15;<br>letrozole days 1–28 | $HR^+$, $HER2^-$ breast cancer | Ib/II | NCT03128619 |
| GDC-0077 (PI3K inhibitor) + letrozole | palbociclib days 1–21 (28-day cycle)<br>GDC-0077 and Letrozole days 1–28 | PIK3CA mutant, $HR^+$, $HER2^-$<br>breast cancer | I/II | NCT03006172 |
| AZD2014 (mTORC1/2 inhibitor)<br>+ fulvestrant | not stated | $ER^+$ breast cancer | I/II | NCT02599714 |
| Everolimus (mTOR inhibitor)<br>+ exemestane (aromatase inhibitor) | palbociclib days 1–21 (28-day cycle)<br>everolimus and exemestane days 1–28 | $ER^+$, $HER2^-$ breast cancer | Ib/IIa | NCT02871791 |
| PD-0325901 (MEK inhibitor) | palbociclib and PD-0325901 days 1–21<br>(28-day cycle) | KRAS mutant non-small cell<br>lung cancer, solid tumors | I/II | NCT02022982 |
| Binimetinib (MEK inhibitor) | palbociclib days 1–21 (28-day cycle)<br>binimetinib days 1–28 | KRAS mutant non-small<br>cell lung cancer | I/II | NCT03170206 |
| Neratinib (pan-ERBB inhibitor) | palbociclib and neratinib days 1–21<br>(28-day cycle) | EGFR, HER2/3/4 amplified/mutated<br>advanced cancers | I | NCT03065387 |
| Ibrutinib (BTK inhibitor) | palbociclib days 1–21 (28-day cycle)<br>ibrutinib days 1–28 | mantle cell lymphoma | I | NCT02159775 |
| Erdafitinib (FGFR inhibitor)<br>+ fulvestrant | palbociclib days 1–21 (28-day cycle)<br>erdafitinib days 1–28; fulvestrant day 1 | $ER^+/HER2^-$/FGFR amplified<br>breast cancer | I | NCT03238196 |
| Cetuximab (EGFR inhibitor) | palbociclib days 1–21 (28-day cycle)<br>cetuximab once weekly | squamous cell carcinoma<br>of the head and neck | II | NCT02499120 |

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**Table 3.** ***Continued***

| Combination | Dosing Schedule | Disease | Phase | Identifier |
|---|---|---|---|---|
| Sorafenib (RTK inhibitor) $_{OR}$ decitabine $_{OR}$ dexamethasone | palbociclib days 1–21 (28-day cycle); sorafenib days 1–28<br>palbociclib days 1–7 (28-day cycle); decitabine days 8–12<br>palbociclib days 1–21 (28-day cycle); dexamethasone days 1–4 and days 15–18 | relapsed and refractory leukemias | I | NCT03132454 |
| Bicalutamide (anti-androgen) | palbociclib days 1–21 (28-day cycle)<br>bicalutamide days 1–28 | $AR^+$ breast cancer | I/II | NCT02605486 |
| Anastrozole (aromatase inhibitor) | palbociclib days 1–21 (28-day cycle)<br>anastrozole days 1–28 | $HER2^-$ breast cancer | II | NCT02942355 |
| Tamoxifen (anti-mitotic) | palbociclib days 1–21 (28-day cycle)<br>tamoxifen days 1–28 | $HR^+$, $HER2^-$ breast cancer | II | NCT02668666 |
| Cisplatin $_{OR}$ carboplatin | palbociclib days 2–22 (28-day cycle)<br>cisplatin or carboplatin day 1 | advanced solid tumors | I | NCT02897375 |
| Carboplatin | palbociclib days 1–14 (21-day cycle)<br>carboplatin day 1 | squamous cell carcinoma of the head and neck | II | NCT03194373 |
| 5-FU (nucleotide analog) + Oxaliplatin (platinum-based) | palbociclib days 1–7 (14-day cycle)<br>5-FU/oxaliplatin day 8 | advanced solid tumors | I | NCT01522989 |
| Bortezomib (proteasome inhibitor) | palbociclib days 1–12 (21-day cycle)<br>bortezomib days 8,11,15,18 | mantle cell lymphoma | I | NCT01111188 |
| Paclitaxel (anti-mitotic) | palbociclib days 1–21 (28-day cycle)<br>paclitaxel days 1, 8, 15 | pancreatic ductal adenocarcinoma | I | NCT02501902 |
| Paclitaxel | not stated | advanced breast cancer | I | NCT01320592 |
| Avelumab (anti-PD-L1) + fulvestrant | palbociclib days 2–22 (28-day cycle)<br>avelumab once every two weeks; fulvestrant day 1 | $ER^+/HER2^-$ metastatic breast cancer | II | NCT03147287 |
| Pembrolizumab (PD-1 inhibitor) + letrozole | palbociclib days 1–21 (28-day cycle)<br>pembrolizumab every 21 days; letrozole days 1–28 | $ER^+$, $HER2^-$ breast cancer | II | NCT02778685 |
| Ribociclib | | | | |
| Trastuzumab (HER2 antibody) | ribociclib days 5–18 (21-day cycle); trastuzumab day 1 | $HER2^+$ breast cancer | I/II | NCT02657343 |
| LSZ102 (ER degrader) | not stated | $ER^+$ breast cancer | I | NCT02734615 |
| Everolimus (mTOR inhibitor) | ribociclib days 1–21 (28-day cycle)<br>everolimus days 1–28 | pancreatic adenocarcinoma | I/II | NCT02985125 |
| Everolimus + letrozole | all drugs days 1–28 (28-day cycle) | endometrial cancer | II | NCT03008408 |
| Everolimus | ribociclib days 1–21 (28-day cycle)<br>everolimus days 1–28 | dedifferentiated liposarcoma and leiomyosarcoma | II | NCT03114527 |
| Everolimus | ribociclib days 1–21 (28-day cycle)<br>everolimus days 1–28 | neuroendocrine tumors | II | NCT03070301 |

(*Continued on next page*)

**Table 3. *Continued***

| Combination | Dosing Schedule | Disease | Phase | Identifier |
|---|---|---|---|---|
| Everolimus<br>+ exemestane (aromatase inhibitor) | ribociclib days 1–21 (28-day cycle)<br>everolimus + exemestane days 1–28 | $HR^+$, $HER2^-$ breast cancer | I | NCT01857193 |
| BLY719 (PI3K inhibitor) + letrozole | ribociclib days 1–21 (28-day cycle)<br>BLY719 + letrozole days 1–28 | $ER^+$ breast cancer | I | NCT01872260 |
| BLY719 $_{OR}$<br>BKM120 (pan-PI3K inhibitor)<br>+ fulvestrant | ribociclib days 1–21 (28-day cycle)<br>BLY719 or BKM120 days 1–28;<br>fulvestrant day 1 | $ER^+/HER2^-$ breast cancer | I/II | NCT02088684 |
| Trametinib (MEK inhibitor) | not stated | advanced solid tumors | I/II | NCT02703571 |
| MEK162 (MEK inhibitor) | ribociclib days 1–21 (28-day cycle)<br>MEK162 days 1–28 | NRAS mutant melanoma | Ib/II | NCT01781572 |
| LGX818 (RAF inhibitor) + MEK162 | ribociclib days 1–21 (28-day cycle)<br>LGX818 + MEK162 days 1–28 | BRAF-dependent advanced solid tumors | I/II | NCT01543698 |
| EGF816 (EGFR inhibitor) | not stated | EGFR mutant non-small cell lung cancer | I | NCT03333343 |
| Ceritinib (ALK inhibitor) | not stated | ALK-positive non-small cell lung cancer | I | NCT02292550 |
| Enzalutamide (anti-androgen) | ribociclib days 1–21 (28-day cycle);<br>enzalutamide days 1–28 | prostate cancer | I/II | NCT02555189 |
| Bicalutamide (anti-androgen) | ribociclib days 1–21 (28-day cycle);<br>bicalutamide days 1–28 | $AR^+$ triple-negative breast cancer | I/II | NCT03090165 |
| Carboplatin<br>+ paclitaxel (anti-mitotic) | ribociclib days 1–4, 8–11, 15–18<br>(28-day cycle)<br>paclitaxel + carboplatin days 1, 8, 15 | ovarian cancer | I | NCT03056833 |
| Paclitaxel | not stated | advanced breast cancer | I | NCT02599363 |
| Doxorubicin | ribociclib days 1–7 (21-day cycle)<br>doxorubicin day 10 | advanced soft tissue sarcoma | I | NCT03009201 |
| Tamoxifen (anti-mitotic) | ribociclib days 1–21 (28-day cycle)<br>tamoxifen days 1–28 | $ER^+$, $HER2^-$ breast cancer | I | NCT02586675 |
| Gemcitabine (nucleotide analog) | ribociclib days 8–14 (21-day cycle)<br>gemcitabine days 1, 8 | advanced solid tumors | I | NCT03237390 |
| Docetaxel (anti-mitotic)<br>+ prednisone | ribociclib days 2–14 (21-day cycle)<br>docetaxel and prednisone days 1–21 | prostate cancer | I/II | NCT02494921 |
| PDR001 (anti-PD1 antibody)<br>± fulvestrant | ribociclib days 1–21 (28-day cycle)<br>PDR001 days 1–28 | $HR^+$, $HER2^-$ breast and ovarian cancer | I | NCT03294694 |
| Abemaciclib | | | | |
| LY3023414 (PI3K/mTOR inhibitor) | not stated | pancreatic ductal adenocarcinoma | II | NCT02981342 |
| LY3214996 (ERK1/2 inhibitor) | not stated | advanced solid tumors | I | NCT02857270 |
| Ramucirumab (anti-VEGFR2) | abemaciclib days 1–28 (28-day cycle)<br>ramucirumab days 1, 15 | advanced solid tumors and lymphoma | I | NCT02745769 |
| Xentuzumab (IGF1/2 inhibitor) | abemaciclib daily, xentuzumab<br>once a week | advanced solid tumors, $HR^+$ breast cancer | I | NCT03099174 |
| LY3039478 (Notchi) | both drugs daily | advanced solid tumors | Ib | NCT02787495 |

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**Table 3. *Continued***

| Combination | Dosing Schedule | Disease | Phase | Identifier |
|---|---|---|---|---|
| Exemestane (aromatase inhibitor) OR exemestane + everolimus OR LY3032414 + fulvestrant OR Letrozole (aromatase inhibitor) OR anastrozole (aromatase inhibitor) OR tamoxifen (anti-mitotic) OR trastuzumab (HER2 antibody) | all drugs daily | metastatic breast cancer | Ib | NCT02057133 |
| Anastrozole OR letrozole | all drugs daily | $HR^+$, $HER2^-$ breast cancer | III | NCT02246621 |
| Tamoxifen | both drugs daily | $HR^+$, $HER2^-$ breast cancer | II | NCT02747004 |
| Premetrexed (anti-folate) OR gemcitabine OR Ramucirumab OR LY3023414 OR pembrolizumab (PD-1 inhibitor) | abemaciclib daily (21-day cycle) premetrexed day 1 gemcitabine days 1, 8 ramucirumab days 1, 8 pembrolizumab day 1 | non-small cell lung cancer | I | NCT02079636 |
| LY3300054 (anti-PD-L1) | abemaciclib daily (28-day cycle); LY3300054 days 1, 15 | advanced solid tumors | I | NCT02791334 |

In addition to creating a permissive senescence environment by fulfilling the requisite of cell-cycle exit, CDK4/6 inhibition may elicit senescence via alternative CDK4/6 substrates. CDK4/6 inhibition leads to the loss of multi-site phosphorylation and the destabilization of the transcription factor Forkhead Box M1 (FOXM1) (Anders et al., 2011). FOXM1 suppression following CDK4/6 inhibition leads to the accumulation of ROS and senescence in transformed melanomas, but not in normal melanocytes. Nevertheless, knockdown of FOXM1 does not completely recapitulate the senescence effect of CDK4/6 inhibition, suggesting that CDK4/6 inhibitors have functions in addition to suppression of FOXM1. Recent work by others has shown that the induction of senescence after CDK4/6 inhibition is Rb dependent in melanoma (Yoshida et al., 2016), thus raising the question of whether FOXM1 suppression requires a cell that has first been growth arrested, or whether there are further context-specific effects of this pathway that need to be understood before it is targeted therapeutically.

It has also been suggested that the mammalian target of rapamycin (mTOR) can toggle the decision between quiescence and senescence (Korotchkina et al., 2010; Leontieva et al., 2012). This may be cell-type specific with the contextual clues not yet understood. For example, growth inhibition by overexpression of the CDK inhibitor p21 in the context of active mTOR signaling has been described to lead to a "futile" period of cell growth, ultimately leading to senescence (Korotchkina et al., 2010). Inhibition of mTOR indirectly following treatment with nutlin-3 or directly by treatment with rapamycin drives the cells into a quiescent state. In contrast to this, however, mTOR inhibition is crucial to reach oncogene-induced senescence in mouse models of melanoma, and mTOR inhibition can cooperate with CDK4/6 inhibition to drive senescence in melanoma cell lines (Damsky et al., 2015; Yoshida et al., 2016). As several mitogenic pathways—all frequently hyperactivated in cancer—impinge on mTOR activity, it may be valuable to further investigate how combining inhibitors of these pathways with CDK4/6 inhibitors affects mTOR activity and senescence, and which other pathways can affect this outcome.

Collectively, these studies suggest that the cooperation of CDK4/6 inhibitors and signaling pathway inhibitors may be affecting sequential decisions in the tumor cell. Inhibitors that have minimal effect in cycling tumor cells may have more of an impact in non-cycling CDK4/6 inhibitor-treated cells, perhaps pushing them into senescence. Indeed, quiescent CDK4/6 inhibitor arrested liposarcoma tumor cells are pushed into senescence upon knocking down HRAS, and enforced expression of near physiological levels of HRAS mRNA is able to prevent cells from undergoing senescence following treatment with CDK4/6 inhibitors, albeit they still exit the cell cycle (Kovatcheva et al., 2017). Identifying the proteins that control the transition from quiescence into senescence may nominate new targets for combinatorial drug therapy, and changing dosing schedules whereby cells are first induced to exit the cell cycle and then treated with a second drug targeting such signaling events may even provide a therapeutic gain by minimizing the necessary dose and the subsequent toxicity associated with such inhibitors.

The consequences of driving a cell into SAGA are particularly interesting. Senescent cells express a cell-type- and

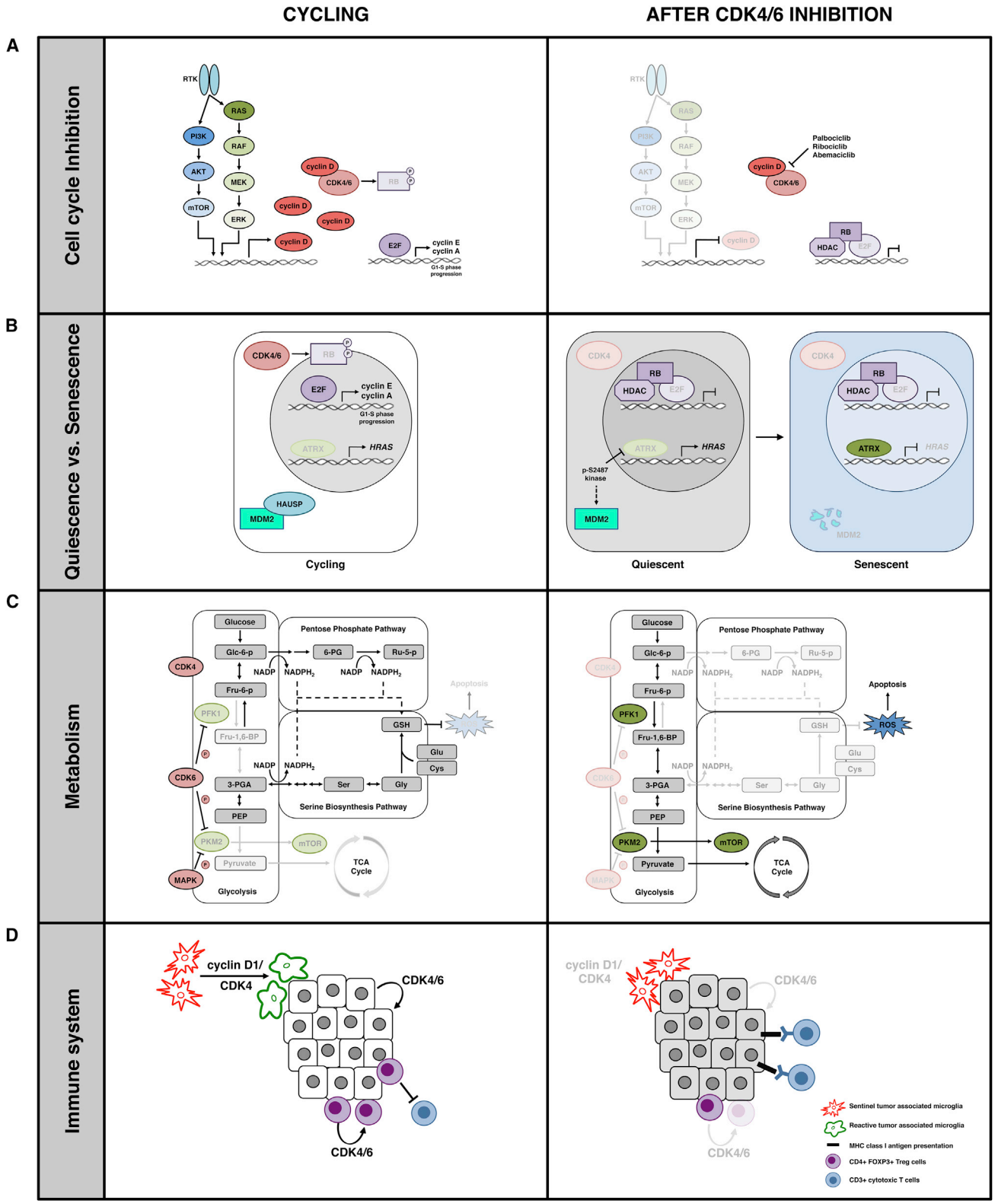


**Figure 1. The Consequences of CDK4/6 Inhibition**
The four cellular mechanisms, labeled A–D, that contribute to the efficacy of CDK4/6 inhibitors are shown. Details are provided in the accompanying text.

signal-specific program of gene expression and cytokine secretion (the senescence-associated secretory phenotype [SASP]), which can sculpt the immune response (Ohtani et al., 2012). On one hand, the SASP can induce the recruitment of immune cells that will mediate tumor clearance (Xue et al., 2007), or promote paracrine senescence (Acosta et al., 2008, 2013). On the other hand, the SASP can create a pro-tumorigenic environment (Coppe et al., 2010; Krtolica et al., 2001; Ruhland et al., 2016). Consequently, the SASP is often referred to as a "double-edged sword," and a better understanding of this biology will be paramount for its clinical manipulation.

Furthermore, the SASP can induce cellular plasticity (Ritschka et al., 2017), and cancer cells that manage to escape senescence have a more aggressive, cancer stem cell-like identity (Milanovic et al., 2018). Indeed, persistent senescent cells may contribute to detrimental short and long-term side effects of treatment, as well as to metastases and relapse (Demaria et al., 2017). Collectively, these observations suggest that the most effective approach to harness the senescence-promoting effects of CDK4/6 inhibitors may be to first induce senescence and then eliminate the persistent senescent cells. Evidence that BCL2 inhibitors can directly eliminate senescent cells (Chang et al., 2016; Yosef et al., 2016) suggests that combining this with other cancer therapies in a sequential manner might be useful. Future proteomic, transcriptomic, and metabolomic data from senescent cells holds promise to identify additional vulnerabilities.

## The Impact of CDK4/6 Inhibitors on Cellular Metabolism

It has long been recognized that cell division is coordinated with metabolic state. A number of non-Rb targets for CDK4/6 have been identified in the metabolic machinery. For example, phosphorylation of AMPK$\alpha$2 by CDK4 is associated with increased glycolysis and decreased fatty acid oxidation in mouse embryonic fibroblasts (Lopez-Mejia et al., 2017). In contrast, CDK4 phosphorylation of GCN5 can lead to acetylation of PGC-1$\alpha$ and is associated with decreased glucose metabolism in hepatic cells (Lee et al., 2014).

There is some evidence that manipulating metabolic pathways may be a useful addition of CDK4/6 inhibition (Figure 1C). Inhibition of CDK4/6 with any of the three drugs in pancreatic ductal adenocarcinoma cell lines alters glycolytic and oxidative metabolism, leading to an increase in ROS in an Rb-dependent manner (Franco et al., 2016). Combining CDK4/6 inhibition with an mTOR inhibitor, a BCL2 inhibitor, or reduction of the ROS scavengers drives these cells into apoptosis, whereas treatment with single agents alone is not sufficient. Interestingly, combined treatment with an MEK inhibitor uniquely drove these cells into senescence.

In T cell acute lymphoblastic leukemia (T-ALL), inhibition of CDK6 or genetic repression of cyclin D3 induces apoptosis (Choi et al., 2012; Sawai et al., 2012). These cells express very low levels of cyclin D1, cyclin D2, and CDK4, and the proliferative decision is dependent upon cyclin D3 and CDK6 (Wang et al., 2017; Wolowiec et al., 1996). The cyclin D3/CDK6 kinase complex can phosphorylate 6-phosphofructokinase and pyruvate kinase M2 (Wang et al., 2017). This has the effect of pushing glycolytic intermediates into the pentose phosphate and serine pathways, and inhibition of CDK6 (through treatment with palbociclib, ribociclib, or knockdown of CDK6) depletes the antioxidants NADPH and glutathione, increasing ROS and apoptosis. It is likely that the changes in metabolism after CDK4/6 inhibition will be context specific and may be dependent on the oncogenic drivers that set up unique metabolic pathways and vulnerabilities.

## CDK4/6 in the Tumor Microenvironment

Our understanding of the relationship between cancer cells and the supporting cells that create a tumor microenvironment has significantly advanced during the last 10 years. The extraordinarily complex microenvironment is not only supportive for growth but can also drive the transition of slow-growing, indolent tumors into a more aggressive state.

The importance of CDK4 activity within the microenvironment was first demonstrated by crossing an RCAS-PDGF/nestin-TvA mouse model of oligodendroglioma into a CDK4-deficient background (Ciznadija et al., 2011). CDK4 is required for the proliferation of the tumor cells; however, reconstituting incipient CDK4-deficient tumor cells with CDK4 expression vectors is not sufficient for tumors to progress to a more aggressive state when the rest of the animal is CDK4 deficient. This suggests there are tumor-extrinsic roles for CDK4. The lack of progression in this model is associated with a defect in the maturation of tumor-associated microglia, which remain in sentinel mode in the absence of CDK4. This is consistent with findings that the maturation of microglia from sentinel to reactive supports the progression of oligodendroglioma into its more aggressive form (Ghosh and Chaudhuri, 2010). Further reinforcing the notion that this is a cyclin D/CDK4-dependent phenomenon, similar observations were made in cyclin D1-deficient mice. Thus, CDK4 is required for both the proliferation of tumor cells and for the maturation of the tumor microenvironment, and both are necessary for the progression of disease in this model.

Recently, in a variety of breast cancer models, including patient-derived xenografts and an MMTV-HER2 mouse, it was demonstrated that abemaciclib or palbociclib induces growth arrest and upregulation of antigen processing and presentation in the tumor cells (Goel et al., 2017). This enhances the immunogenicity of the tumor cells. Consistent with this, the number of $CD3^+$ cells recruited into the tumor mass increases after treatment, allowing for the stimulation of cytotoxic T cells (CTLs). Additionally, CDK4 is necessary for the development of $CD4^+FOXP3^+$ regulatory T (Treg) cells that can suppress CTL responses. In both tumor-bearing and non-tumor-bearing animals, CDK4 deficiency is associated with a reduced number of infiltrating and circulating $CD4^+FOXP3^+$ Treg cells with minimal impact on other T cell subsets (Chow et al., 2010; Goel et al., 2017). Thus, by enhancing the antigenicity of the tumor cell and suppressing the negative regulatory cells, one can achieve a substantial effect on tumor growth using CDK4/6 inhibitors (Figure 1D).

Interestingly, the action of CDK4/6 inhibitors on the microenvironment might not be only through its ability to block Rb phosphorylation and promote cell-cycle exit. CDK4 was shown to have kinase activity toward SPOP, a cullin E3 ubiquitin ligase adaptor protein that can interact with PD-L1 (Zhang et al., 2018). Treatment with palbociclib inhibits SPOP phosphorylation, promoting its degradation and blocking PD-L1

proteasome-mediated degradation. CDK4/6 inhibition is also able to stimulate PD-1-expressing T cells *in vitro* and enhance T cell infiltration (Deng et al., 2017). This enhancement is driven at least in part by CDK6-induced phosphorylation of NFAT4. Combining palbociclib with a PD-1 blockade enhanced tumor regression and improved overall survival in mouse xenograft models of colon adenocarcinoma (Deng et al., 2017; Zhang et al., 2018).

These examples illustrate the profound effect that CDK4/6 inhibition can have on tumor growth by acting on the cells of the tumor microenvironment, either by affecting proliferation and maturation or by affecting antigen processing and other immunological features of the cells, both tumor and normal. Such observations empower new opportunities to combine CDK4/6 inhibitors with immune checkpoint blockades or other types of immunotherapy. Indeed, it also raises the mechanistic possibility that the effect of the signaling pathway inhibitor combinations may also strike at two (or more) different cellular targets to achieve response.

For example, although individually CDK4/6 inhibitors and PI3K inhibitors have little effect in a mouse model of triple-negative breast cancer, the combination of CDK4/6 and PI3K inhibitors increases the expression of HLA antigens, leading to an increase in tumor-infiltrating cytotoxic $CD4^+$ and $CD8^+$ T cells and natural killer cells, and a decrease in the $CD4^+FOXP3^+$ Treg suppressor cells (Teo et al., 2017). While these investigators did not experimentally address why the combination was better, they further showed that adding an immune checkpoint blockade induces complete and durable regression of established tumors.

Given the importance of CDK4 in the tumor microenvironment, it may be somewhat surprising that CDK4/6 inhibitors have no significant effect on Rb-negative tumors. It is possible that growth arrest in the tumor cell is necessary for the changes in histocompatibility to manifest. Alternatively, and by no means exclusively, the ability of CDK4/6 to sculpt the microenvironment may not be sufficient to reduce tumor burden. In the oligodendroglioma model described above (Ciznadija et al., 2011), ectopic PDGF expression is sufficient to drive the initiation of disease in CDK4-positive tumor cells, but if the microenvironment is CDK4 deficient these low-grade lesions would not progress to high-grade lethal malignancies. It is possible that the experiments examining the effect of CDK4/6 inhibitors in Rb-negative tumors in both animals and humans are simply not powered to identify more subtle changes in tumor grade. Regardless, multiple cellular targets of the CDK4/6 inhibitors, in the tumor cell and in the tumor microenvironment, can also cooperate to yield optimal clinical success.

## Conclusion

It is clear that the mechanisms by which CDK4/6 inhibitors can retard cancer progression are far more diverse than originally thought. The *in vivo* functions of CDK4/6 inhibition are likely to extend beyond simply enforcing reversible cytostasis. Nevertheless, it is tempting to speculate that the alternative mechanisms discussed here may not be completely separate. Senescent cells are characterized by metabolic changes and elaboration of cytokines that modulate the immune response. Thus, the ability of CDK4/6 inhibitors to drive tumor cells into senescence may lead to changes in the immune response and cellular metabolism, yielding a unified mechanistic cellular response. Consequently, it will be exciting to examine each mechanism as we learn how drug cooperativity benefits each patient population.

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## DECLARATION OF INTERESTS

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