Targeting CDK4 and CDK6: From Discovery to Therapy

Charles J. Sherr, David Beach, and Geoffrey I. Shapiro

ABSTRACT

Biochemical and genetic characterization of D-type cyclins, their cyclin D–dependent kinases (CDK4 and CDK6), and the polypeptide CDK4/6 inhibitor p16INK4 over two decades ago revealed how mammalian cells regulate entry into the DNA synthetic (S) phase of the cell-division cycle in a retinoblastoma protein–dependent manner. These investigations provided proof-of-principle that CDK4/6 inhibitors, particularly when combined with coinhibition of allied mitogen-dependent signal transduction pathways, might prove valuable in cancer therapy. FDA approval of the CDK4/6 inhibitor palbociclib used with the aromatase inhibitor letrozole for breast cancer treatment highlights long-sought success. The newest findings herald clinical trials targeting other cancers.

Significance: Rapidly emerging data with selective inhibitors of CDK4/6 have validated these cell-cycle kinases as anticancer drug targets, corroborating longstanding preclinical predictions. This review addresses the discovery of these CDKs and their regulators, as well as translation of CDK4/6 biology to positive clinical outcomes and development of rational combinatorial therapies. Cancer Discov; 6(4):353–67. © 2015 AACR.

INTRODUCTION

Cyclin-dependent kinase 4 (CDK4) and closely related CDK6 play key roles in mammalian cell proliferation, where they help to drive the progression of cells into the DNA synthetic (S) phase of the cell-division cycle. Unlike CDKs 1 and 2, which act later in the cell cycle in response to periodic oscillations of cyclins E, A, and B to coordinate DNA replication with mitosis (Fig. 1), the enzymatic activities of CDK4 and CDK6 in the first gap phase (G1) of the cycle are governed by D-type cyclins expressed in response to various extracellular signals, including stimulatory mitogens, inhibitory cytokines, differentiation inducers, cell–cell contacts, and other spatial cues. The three D-type cyclins (D1, D2, and D3) are differentially expressed, alone or in combination, in distinct cell lineages, where they assemble with CDK4 and CDK6 to form enzymatically active holoenzyme complexes. An understanding of how the three different D-type cyclins act as environmental sensors in responding dynamically to extracellular cues in various cell types helps to explain how CDK4/6 activities are differentially regulated and predicts the basis of functional interactions between mitogen signaling pathways and CDK4/6 activity in both normal and cancer cells. More than two decades after discovery of CDK4 and CDK6, drugs inhibiting their activities are now demonstrating significant efficacy in cancer treatment (for other recent reviews, see refs. 1–3). The elucidation of how signal transduction pathways activate CDK4/6 in different tumor types should pave the way for combinatorial therapies that target both cyclin D and CDK4/6 simultaneously to improve therapeutic responses.

DISCOVERY OF D-TYPE CYCLINS

The D-type cyclins were identified in 1991 by three groups of investigators under widely different experimental circumstances. At the time, no “G1 cyclins” had yet been found in mammalian cells, whereas budding yeast (Saccharomyces cerevisiae) was recognized to synthesize three such cyclins (CLNs 1, 2, and 3). In yeast, the induced CLN proteins, like S phase and mitotic cyclins, associate with a single CDK (CDC28/CDK1), whose rise in activity in late G1 is associated with the commitment of cells (START in yeast) to enter S phase (4). Using a conditionally CLN-deficient yeast strain, a human cDNA that complemented the CLN genetic deficiency was designated cyclin D1 (CCND1; ref. 5). In independent studies, others recovered a differentially expressed cyclin-like cDNA (originally designated Cyl1) that was induced during G1 phase in murine macrophages synchronously entering the cell cycle in response to mitogen stimulation by colony-stimulating factor-1 (6). Two closely related genes, Cyl2 and Cyl3, were
to be equivalent to the subsequently identified human cyclin D2 (CCND2) and D3 (CCND3) genes, respectively (9, 10).

Taken together, these earliest reports defined a distinct family of three D-type cyclins (i) that acted as mitogen sensors during the G1 phase of the cell cycle; (ii) that were expressed in various combinations within a cell lineage–specific manner; (iii) whose functions were evolutionarily conserved but with no one of them being essential for cell-cycle progression; (iv) that were predicted to activate then-novel CDKs, and (v) that had latent proto-oncogenic capabilities.

**DISCOVERY OF CDK4 AND CDK6**

The presumption that D-type cyclins might allosterically regulate a novel CDK was validated by the discovery of CDK4, which was revealed to physically bind to, and be enzymatically activated by, any of the three D-type cyclins (11). A related cyclin D–dependent kinase, CDK6, with similar properties was identified 2 years later (12). Although CDKs 1 and 2, in complexes with cyclins E, A, and B, drive cell-cycle progression through S phase and M phase (mitosis), the cyclin D–dependent CDKs act during G1 phase to propel quiescent cells that have entered the cell cycle, or proliferating cells that have completed mitosis, toward S phase (Fig. 1).

Unlike CDKs 1 and 2 that phosphorylate many hundreds of cellular protein substrates (13), CDK4 is a surprisingly fastidious enzyme that has a restricted propensity to phosphorylate the retinoblastoma protein (RB1, hereafter RB) and two other RB-family proteins [RB2 (p130), RBL1 (p107); refs. 11, 14–16], but very few other substrates (17).

RB is a canonical tumor-suppressor gene in retinoblastoma and in many other cancers as well (18, 19). The RB protein undergoes periodic phosphorylation as cells traverse the division cycle. RB is dephosphorylated as cells exit mitosis, and the hypophosphorylated form detected in G1 phase becomes hyperphosphorylated (inactivated) in late G1 and remains so throughout progression through S phase to mitosis (refs. 20–23; Fig. 1). The role of hypophosphorylated (active) RB to restrict proliferation and act as a potent tumor-suppressor gene was highlighted by studies indicating that RB’s growth-suppressive function could be inactivated by its binding to DNA tumor virus oncoproteins (human papillomavirus E7, adenovirus E1A, and SV40 T antigen; refs. 24–27). In mammalian cells stimulated by mitogens to enter the division cycle from a quiescent state (G0), CDK4/6-mediated RB phosphorylation was first detected in mid-G1 phase after induction of cyclin D but prior to activation of cyclin E– and A–dependent CDK2 (12, 28). Together, these results implied that the role of CDK4/6 was to phosphorylate RB, priming it for inactivation by other CDKs later in G1, and releasing E2F transcription factors from RB constraint to allow their coordinate induction of a suite of genes whose activities are jointly required for initiation of S phase (reviewed in detail in refs. 16, 29–31).

**ENTER p16INK4a**

Early controversies quickly arose around the issue of how, and even whether, the D-type cyclins regulated the cell cycle, the respective roles that CDK4 and other CDKs might play as RB kinases, and what the putative G1 signaling pathways

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**Figure 1.** The cell cycle. The four phases of the mitotic cell division cycle are indicated in the inner circle, including mitosis (M phase), the cellular DNA synthesis (S) phase, and their separation by two gap (G) phases, the first (G1) between M and S phases and the second (G2) between S and M phases. The levels of total CDK activity are lowest in early G1 phase and progressively increase under the agency of different cyclin–CDK complexes, reaching maximal net CDK activity as cells enter mitosis. States of RB phosphorylation (P) throughout the cell cycle are schematized. RB is dephosphorylated in M phase (green arrow) and progressively rephosphorylated in G1, first by cyclin D–dependent CDK4/6 and later by cyclin E–dependent CDK2. RB becomes fully phosphorylated in late G1 (red arrow), resulting in inactivation of its proliferation-suppressive function and triggering the cell’s subsequent entry into S phase. The point in the cell cycle (sometimes called “the restriction point”; red arrow) at which RB becomes fully phosphorylated temporally corresponds to a late G1 phase transition when cells lose their marked dependency on extracellular mitogens and commit to enter S phase and complete the cycle. During the S and G2 phases, RB phosphorylation is maintained by the progressive activation of other CDKs, including cyclin A–CDK2 and cyclins A/B–CDK1. Degradation of cyclins A and B in mitosis results in the collapse of CDK activity and restores the G0 state. INK4 proteins (the prototype p16INK4A is shown) specifically inhibit the cyclin D–dependent kinases to inhibit RB phosphorylation and arrest cells in G0 phase. Arrested cells can return to a noncycling but reversible quiescent state (G0) after mitogen withdrawal in which D-type cyclins are usually degraded or, in response to particular stress conditions, can undergo durable cell-cycle arrest (senescence). Quiescent cells reconstituted with mitogens restore cyclin D synthesis and re-enter the cell cycle in early G1, whereas senescent cells are refractory to mitogen reactivation and resist oncogenic challenge. Asynchronously dividing cells maintain mitogen-dependent cyclin D synthesis and have a contracted G0 phase when compared with quiescent cells re-entering the division cycle (for pertinent detailed reviews, see refs. 4, 16, 41, 65).

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**CANCER DISCOVERY**

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CDK4 and CDK6 as Anticancer Drug Targets

might be. The discovery of a highly specific 16-kDa polypeptide inhibitor of CDK4 encoded by the INK4a (formally CDKN2A) gene (32) provided compelling data that CDK4 acts upstream of RB. Key features were that p16^{INK4a} bound to and potently inhibited cyclin D–CDK4 kinase activity but spared holoenzyme complexes containing other CDKs, and that expression of p16^{INK4a} inhibited RB phosphorylation and arrested cells in the G1 phase of the cell cycle. Importantly, cells lacking functional RB were resistant to p16^{INK4a}-mediated cell-cycle arrest, implying that the ability of CDK4 (and later, CDK6) to drive G1 phase progression required RB (33–35) and predicting that chemical CDK4/6 inhibitors would show efficacy only if RB were functionally intact.

Given the role of RB as a tumor suppressor, it was intuitively that p16^{INK4a} would play a similar role in inhibiting tumor formation. Within months of its discovery, the INK4a (CDKN2A) and the genetically linked INK4b gene (CDKN2B; ref. 36) were implicated in a reverse genetic screen to play tumor-suppressive roles in familial melanoma (37). A flurry of subsequent papers soon identified p16^{INK4a} as a frequent target of inactivating mutations and deletions in many human cancers and revealed that loss of function of p16^{INK4a} and RB generally occur as mutually exclusive events in tumor cells. In turn, the realization that CCND1 in particular was a target of translocation in certain tumors (for example, in mantle cell lymphoma [MCL]) or was amplified (for example, in breast cancer) reinforced the view that cyclin D1 (and, by presumption, CDK4) were oncoproteins. After compiling data from numerous independent reports, mutations in the “RB pathway” were soon proposed to be a hallmark of cancer (19, 38).

REGULATION OF CDK4 AND CDK6 BY D-TYPE CYCLINS: IMPLICATIONS FOR CANCER TREATMENT

In many cell types, transcription of CCND1 and cyclin D1 assembly with CDK4 each depend on activation of a RAS-dependent kinase cascade that relies on the sequential activation of Raf1, MEK1, MEK2, and ERKs (39–42). In serum-deprived fibroblasts lacking endogenous cyclin D expression, ectopically expressed cyclin D1 does not associate with CDK4 (28), but assembly of cyclin D–CDK complexes occurs in response to enforced expression of constitutively active MEK (43). HSC70 associates with newly synthesized cyclin D1 and is a component of the mature catalytically active cyclin D1–CDK4 complex (44). CDK4, like several other kinases, similarly requires molecular chaperones to be properly folded and to assemble into productive complexes. In the cytoplasm, newly synthesized CDK4 is detected within high–molecular weight complexes that also contain HSP90 and CDC37 (45, 46). Release from the chaperone complex enables CDK4 to interact with D-type cyclins or, alternatively, to dimerize with p16^{INK4a}, yielding inactive CDK4. Under normal physiologic circumstances in young animals, p16^{INK4a} is not expressed; however, it is induced by a variety of hyperproliferative stress signals whose oncogenic effects are counteracted by p16^{INK4a}-induced cell-cycle arrest (47). Competition between mitogen-activated D-type cyclins and stress-activated p16^{INK4a} for CDK4 binding determines whether cells undergo G1 arrest or enter S phase. Presumably, HSP90 inhibition might also complement CDK4 inhibitors in preventing RB phosphorylation and enforcing cell-cycle arrest.

Naturally occurring pan-CDK inhibitors, including p21\cc

and p27\cc

, facilitate cyclin D–CDK assembly and the nuclear import of the resulting complexes without inhibiting CDK4/6 kinase activity (41, 48–51). Posttranslational modification of these CIP/KIP proteins by mitogen-triggered tyrosine phosphorylation may explain their loss of CDK-inhibitory activity when acting as “assembly factors” in binding cooperatively to D cyclins and CDK4/6 (52, 53). The subsequent importation of assembled cyclin D–CDK complexes into the nucleus allows them to access and phosphorylate RB. Under normal circumstances, asynchronously dividing cells periodically express peak nuclear cyclin D1 levels at G1–S, after which cyclin D1 export into the cytoplasm and its increased turnover is triggered during S phase (54).

A separate RAS signaling pathway commanded by PI3K and AKT (protein kinase B) negatively regulates glycogen synthase kinase 3β (GSK3β) to prevent its phosphorylation of cyclin D1 on a single threonine reside (Thr286; refs. 55, 56). PI3K/AKT-mediated inhibition of cyclin D1 Thr286 phosphorylation prevents the nuclear export of cyclin D1–CDK complexes and blocks cyclin D1 recognition by the FBX4-containing ubiquitin ligase that triggers cyclin D degradation (55–58). Moreover, certain cyclin D mutants or C-terminally truncated variants encoded by an alternatively spliced mRNA (D1b) that lacks the Thr286 codon are retained in the nucleus during S phase and are proposed to confer a neo-oncogenic function that triggers DNA re-replication, aneuploidy, and tumorigenesis (59–61). Thus, altered cyclin D turnover in tumor cells may be reflected by persistent and intense nuclear cyclin D1 expression.

RAS signaling, highlighted in the above discussion, is by no means the only arbor of the life history of D-type cyclins in cells, as many receptor-mediated signals—for example, the T-cell receptor, cytokine and hormone receptors (HR), and the machinery that monitors cell adhesion—all converge to positively or negatively regulate the levels of individual D-type cyclins in different cell types. In short, cyclin D transcription, assembly with CDK4/6, nuclear transport, and stability are each mitogen-dependent steps governed by distinct signaling pathways. The central conclusion is that D-type cyclins act as mitogen sensors to govern G1 phase progression. Cancer-specific mutations, such as those affecting receptor tyrosine kinases (RTK), RAS, RAF, PI3K, or PTEN, or genetic alterations leading to aberrant hormone and cytokine receptor signaling can enhance cyclin D–dependent CDK4/6 activity. Conversely, cell type–specific RTK, RAF/MEK/ERK, and PI3K/AKT inhibitors, particular hormone or interleukin antagonists, or antiproliferative cytokines, such as TGFβ, can increase the threshold for CDK4/6 activation and synergize with CDK4/6 inhibitors to induce G1 phase cell-cycle arrest (19).

Despite the high frequency of mutations epistatically targeting the RB signaling pathway in cancer cells, inactivation of the individual Ccnd1, Cdk4/6, and Cdkn2a genes in mice, while leading to specific developmental defects when disrupted alone or in combination, established that their functions were nonessential for the cell cycle per se (62–64). In contrast, the demonstration that inactivation of these genes can prevent oncogene-induced tumor development in mouse models reinforced the view that these enzymes might be suitable cancer-specific drug targets (65).
EARLY ATTEMPTS TO DEVELOP A CHEMICAL CDK4/6 INHIBITOR

Based on founding discoveries in the early 1990s that provided proof-of-principle that CDK4 inhibition might retard cancer cell development, David Beach and Giulio Draetta founded Mitotix, Inc. Although inhibitors, such as flavopiridol and roscovitine, that broadly targeted CDKs were then available (1), the Mitotix scientific advisory board (Beach D, Draetta G, Sherr CJ, Bishop JM, Kirschner M, Rothstein R, Nicolau KC, and Folkman J) decided at its first meeting in late 1993 to try to develop a drug that would specifically inhibit CDK4. At this early stage, the significance of RB phosphorylation by CDK4 still remained a subject of intense debate, particularly given that cyclin E/A–dependent CDK2 phosphorylation by CDK4 still remained a subject of intense debate. Moreover, CDK6 had not yet been discovered (12), and the demonstration that the ability of p16INK4a to inhibit CDK4. At this early stage, the significance of RB phosphorylation by CDK4 still remained a subject of intense debate. Moreover, CDK6 had not yet been discovered (12), and the demonstration that the ability of p16INK4a to inhibit CDK4, but not other CDKs, and whose pharmacologic and medicinal properties would facilitate further drug development. However, no suitable drugs were identified, and Mitotix was sold during the dot-com bust in 2000–2001. Soon thereafter, chemists at Parke-Davis developed a specific CDK inhibitor (PD0332991) that would eventually become Pfizer’s palbociclib (Ibrance; refs. 66, 67). Palbociclib is an orally bioavailable, low nanomolar reversible inhibitor of CDK4/6 (Table 1), which exhibited no significant activity against a wide panel of other kinases; these included cyclin E– and A–driven CDK2 and cyclin B–CDK1 that are more than 1,000-fold less sensitive to the drug (Table 1). Palbociclib arrested the proliferation of tumor cell lines that retain functional RB, blocking its phosphorylation on CDK4/6–specific sites. Treated breast, colon, and lung cancer cell lines, which are primarily driven by cyclin D1–CDK4, as well as myeloid and lymphoid leukemia cells that primarily depend on cyclins D2/D3 and CDK6, accumulated in G1 phase, exhibited loss of the proliferation marker Ki67, and downregulated canonical E2F target genes (IC50 values for cultured cells: 40 to 170 nmol/L). In several xenograft models, the drug was efficacious in inducing tumor stasis or regression and was tolerated without significant toxicities at daily doses up to 150 mg/kg for up to 50 days of treatment (66, 67). Although withdrawal of palbociclib after several weeks was accompanied by tumor regrowth, the re-emerging cancers remained drug-sensitive, suggesting that recurring tumors did not acquire therapeutic resistance. Tumor xenografts lacking CDKN2A were sensitive to the drug, whereas those lacking functional RB were refractory to palbociclib treatment. Although several lines of evidence argue for CDK4/6–independent roles of D-type cyclins as transcriptional cofactors for HRs (68) and

Table 1. Key characteristics of CDK inhibitors

<table>
<thead>
<tr>
<th>Drug</th>
<th>Palbociclib (Pfizer) (PD0332991, Ibrance)</th>
<th>Ribociclib (Novartis) (LEE011)</th>
<th>Abemaciclib (Eli Lilly) (LY2835219)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50</td>
<td>CDK4: 11 nmol/L</td>
<td>CDK4: 10 nmol/L</td>
<td>CDK4: 0.6–2 nmol/L</td>
</tr>
<tr>
<td></td>
<td>CDK4: 9 nmol/L</td>
<td>CDK6: 39 nmol/L</td>
<td>CDK6: 2.4–5 nmol/L</td>
</tr>
<tr>
<td></td>
<td>CDK6: 15 nmol/L</td>
<td>CDK1: 100 μmol/L</td>
<td>CDK9: 57 nmol/L</td>
</tr>
<tr>
<td></td>
<td>CDK: 10 μmol/L</td>
<td>CDK2: 50 μmol/L</td>
<td>CDK1: 1 μmol/L</td>
</tr>
<tr>
<td></td>
<td>CDK2: 10 μmol/L</td>
<td>1 (89)</td>
<td>CDK2: 500 nmol/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1, 88)</td>
</tr>
<tr>
<td>PK</td>
<td>Tmax: 4–6 h</td>
<td>Tmax: 6 h</td>
<td>Tmax: 4–6 h</td>
</tr>
<tr>
<td></td>
<td>t1/2: 17–38 h</td>
<td>t1/2: 17–38 h</td>
<td>t1/2: 17–38 h</td>
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<td></td>
<td>(crosses blood–brain barrier; refs.</td>
<td></td>
<td>(crosses blood–brain barrier; refs.</td>
</tr>
<tr>
<td></td>
<td>92, 93)</td>
<td></td>
<td>92, 93)</td>
</tr>
<tr>
<td>PD</td>
<td>Reduced RB phosphorylation in paired</td>
<td>Reduced RB phosphorylation</td>
<td>Reduced RB phosphorylation and</td>
</tr>
<tr>
<td></td>
<td>tumor biopsies, along with reduced</td>
<td>and Ki67 expression in</td>
<td>topoisomerase II expression in</td>
</tr>
<tr>
<td></td>
<td>fluorothymidine-PET uptake (75)</td>
<td>paired tumor biopsies (90)</td>
<td>paired tumor and skin biopsies (92)</td>
</tr>
<tr>
<td>Dosing</td>
<td>125 mg daily (3 weeks, 1-week drug</td>
<td>600 mg daily (3 weeks, 1-week</td>
<td>200 mg twice daily (continuous</td>
</tr>
<tr>
<td></td>
<td>holiday) or 200 mg daily (2 weeks, 1-week drug holiday; refs. 69, 70)</td>
<td>drug holiday; ref. 90)</td>
<td>dosing; ref. 92)</td>
</tr>
<tr>
<td>Major dose-limiting toxicities</td>
<td>Neutropenia, thrombocytopenia</td>
<td>Neutropenia, thrombocytopenia</td>
<td>Fatigue</td>
</tr>
<tr>
<td>Other reported adverse events</td>
<td>Anemia, nausea, anorexia, fatigue, diarrhea (69, 70)</td>
<td>Mucositis, Prolonged EKG QTc interval, Elevated creatinine, Nausea (90)</td>
<td>Diarrhea, Neutropenia (92)</td>
</tr>
</tbody>
</table>
for phosphoproteins, such as FOXM1, as alternative CDK4/6 substrates (17), the observed preclinical effects of palbociclib were consistent with the notion that inhibition of CDK4/6 is a key mechanism underlying antitumor activity (66). Clinical development of CDK4/6 inhibitors, briefly summarized in Table 2, is discussed below.

**PALBOCICLIB CLINICAL DEVELOPMENT**

Phase I studies of palbociclib were conducted in patients with advanced cancers that express RB, establishing recommended phase II doses of 125 mg daily for 3 weeks on/1 week off (3/1 schedule) or 200 mg daily for 2 weeks on/1 week off (2/1 schedule; refs. 69, 70). Dose-limiting toxicities were neutropenia and thrombocytopenia that precluded continuous dosing. Changes in absolute neutrophil count and platelets as related to plasma palbociclib exposure were established using an $E_{\text{max}}$ model. Given the reliance of myeloid development in the mouse on cyclin D2- and D3-driven CDK6 (62, 71, 72), these results might have been anticipated. Other side effects were mild, including fatigue, diarrhea, anemia, and nausea, with overall good tolerability on both dosing schedules. Palbociclib exhibited linear pharmacokinetics, with a median time to maximal concentration ($T_{\text{max}}$) of ~5 hours, and was eliminated with a mean plasma half-life of ~26 hours (Table 1). In the phase I experience, a partial response

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**Table 2. Highlights of representative completed and ongoing clinical trials with CDK4/6 inhibitors**

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Drug(s) (trial phase)</th>
<th>Description and outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monotherapy trials</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Advanced solid tumors (various)</td>
<td>Palbociclib (phase I, completed)</td>
<td>Drug dosage, PK/PD, and dose-limiting toxicities were established. Stable disease realized in 19 of 74 patients.</td>
<td>(69, 70)</td>
</tr>
<tr>
<td>Advanced solid tumors (various)</td>
<td>Ribociclib (phase I)</td>
<td>Drug dosage, PK/PD, and dose-limiting toxicities established in 85 patients. Reductions in Ki67 and phosphorylated RB documented in paired pre- and posttreatment tumor biopsies. Stable disease for $&gt;6$ treatment cycles in 14% of patients.</td>
<td>(90, 91)</td>
</tr>
<tr>
<td>Advanced solid tumors (various)</td>
<td>Abemaciclib (phase I)</td>
<td>Drug dosage, PK/PD, and dose-limiting toxicities determined. Drug efficiently crosses the blood:brain barrier to equal plasma concentrations.</td>
<td>(92)</td>
</tr>
<tr>
<td>Advanced solid tumors or hematologic malignancies (various)</td>
<td>Ribociclib (phase II)</td>
<td>SIGNATURE: to determine efficacy of treatment in previously treated patients preidentified as having CDK4/6 pathway–activated tumors (including p16 loss or CDK4/6 or cyclin D1/D3 amplification).</td>
<td>(133) Outcome not reported</td>
</tr>
<tr>
<td>MCL</td>
<td>Palbociclib (PD study, completed)</td>
<td>Of 17 patients receiving drug on the 3/1 schedule, reductions in RB phosphorylation and tumor proliferation (Ki67 and fluorothymidine PET) occurred during the first cycle in most patients. Five heavily pretreated patients achieved PFS of $&gt;1$ year.</td>
<td>(75)</td>
</tr>
<tr>
<td>MCL</td>
<td>Abemaciclib (phase II)</td>
<td>Of 22 patients with relapsed or refractory disease who received $&gt;6$ treatment cycles, there were five partial responses, and 9 patients with stable disease.</td>
<td>(105)</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>Palbociclib (phase II)</td>
<td>Of 30 patients who had progressed on prior therapy, 66% were progression-free after 12 weeks on a 2/1 schedule. Eight patients remained on study for $&gt;40$ weeks with tumor regressions in 4 patients and one complete response.</td>
<td>(69, 77, 80)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Palbociclib (phase II)</td>
<td>Of 37 patients enrolled with 2 partial responses and 5 with stable disease for a clinical benefit rate of 19% overall and 29% in HR+/HER2− disease.</td>
<td>(82)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Abemaciclib (phase I)</td>
<td>Expansion cohort of phase I trial. Evaluation in 25 heavily pretreated patients with ER+/HER2− disease in which 72% exhibited overall clinical benefit. Drug was also evaluated in 11 patients with HR+/HER2− disease (with 100% control rate); 5 patients with HR+/HER2− disease exhibited stable disease of only brief duration. Median duration of response for all treated HR+ patients was 13.4 months with 8.8-month PFS.</td>
<td>(92, 95, 96)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Abemaciclib (phase II)</td>
<td>MONARCH-1. Based on monotherapy responses seen in expansion cohort of the phase I trial. Evaluating monotherapy for patients whose disease has progressed despite prior chemotherapy.</td>
<td>Not reported</td>
</tr>
</tbody>
</table>

(continued)
### Table 2. Highlights of representative completed and ongoing clinical trials with CDK4/6 inhibitors (Continued)

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Drug(s) (trial phase)</th>
<th>Description and outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>Abemaciclib (phase I)</td>
<td>Designed to exploit traversal of the blood-brain barrier by abemaciclib. Assessment in breast cancer patients with brain metastases in 3 cohorts: (1) HR+/HER2--; (2) HR+/HER2+; (3) patients eligible for resection, 5-14 days prior to surgery.</td>
<td>Not reported</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Palbociclib (phase II)</td>
<td>16 patients enrolled with advanced disease and evidence of CDKN2A loss on the 3/1 schedule. 8 patients were progression-free &gt;4 months.</td>
<td>(101)</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Abemaciclib (phase I)</td>
<td>Expansion cohort in phase I trial. In 15 of 31 patients who remained on trial for &gt;6 months, overall disease control rate was 49% with 6-month PFS in 26%. Patients with tumors harboring KRAS mutation showed greater disease control.</td>
<td>(92, 102)</td>
</tr>
<tr>
<td>GBM</td>
<td>Abemaciclib (phase I)</td>
<td>Expansion cohort in phase I trial. Two of 17 patients showed decreases in tumor size and prolonged time to progression.</td>
<td>(92)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Abemaciclib (phase I)</td>
<td>Expansion cohort in phase I trial. 26 patients enrolled. Partial response observed in a patient with tumor harboring NRAS mutation and CDKN2A loss.</td>
<td>(92)</td>
</tr>
<tr>
<td>Germ cell tumor</td>
<td>Palbociclib (phase II)</td>
<td>30 patients enrolled to 3/1 schedule, based on preliminary efficacy seen in patients with growing teratoma syndrome in phase I trial. 24-week PFS rate 28%, with efficacy predominantly in patients with teratoma or teratoma with malignant transformation.</td>
<td>(73, 74)</td>
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</table>

#### Hormonal combinations in ER+ breast cancer

| Breast cancer             | Palbociclib Letrozole (phase II/III) | PALOMA-1: 165 postmenopausal women with advanced ER+/HER2- disease who had not received systemic treatment for advanced disease were randomized to receive letrozole vs. letrozole/palbociclib. Mean PFS was 10.2 months with letrozole alone and 20.2 months for the combination. CCND1 amplification and CDKN2A loss did not predict benefit. Provisional FDA approval for this indication was obtained in early 2015, and data are awaited from phase III evaluation (PALOMA-2). | (83)       |
| Breast cancer             | Palbociclib Fulvestrant (phase III)  | PALOMA-3: Interim analysis of ongoing phase III study of pre- and postmenopausal women with advanced ER+/HER2- disease reported PFS of 9.2 months with combination vs. 3.8 months with fulvestrant alone.                                                      | (84)       |
| Breast cancer             | Abemaciclib Fulvestrant (phase III)  | MONARCH-2: Fulvestrant with or without abemaciclib.                                                                                                                                                                     | Not reported |
| Breast cancer             | Abemaciclib Aromatase inhibitors (phase III) | MONARCH-3: Anastrozole or letrozole with placebo or abemaciclib.                                                                                                                                                       | Not reported |
| Breast cancer             | Ribociclib Letrozole (phase II, completed) | MONALEESA 1: Presurgical study of letrozole vs. letrozole/ribociclib in early breast cancer patients.                                                                                                                    | Results pending |
| Breast cancer             | Ribociclib Letrozole (phase III)      | MONALEESA 2: First-line metastatic trial in postmenopausal patients randomizing letrozole to letrozole/ribociclib.                                                                                                       | Not reported |
| Breast cancer             | Ribociclib Fulvestrant (phase III)    | MONALEESA 3: Randomized double-blind, placebo-controlled study in postmenopausal women with HR+/HER2- advanced disease who have received no or 1 line of endocrine treatment.                                                   | Not reported |
| Breast cancer             | Ribociclib Tamoxifen Goserelin (phase III) | MONALEESA 7: Randomized double-blind, placebo-controlled study of tamoxifen or an aromatase inhibitor with goserelin along with ribociclib or placebo in pre- or perimenopausal women with HR+/HER2- breast cancer. | Not reported |
| Breast cancer             | Ribociclib Letrozole BYL719 (phase II) | Example of triplet therapy combining CDK4/6 inhibition, hormonal therapy, and an α isoform-selective PI3K inhibitor.                                                                                                   | (116)      |
was achieved in a patient with growing teratoma syndrome (73), with clinical benefit later confirmed in a larger group of patients with teratoma (74): Stable disease was noted in 19 of 74 patients with advanced solid tumors, with 9 patients receiving 10 or more cycles on either the 3/1 or 2/1 schedule.

### Mantle Cell Lymphoma

In order to document pharmacodynamic effects of palbociclib-mediated CDK4/6 inhibition, a pilot study was conducted in patients with MCL (75), a subtype associated with a [t(11:14(q13;q32)] translocation that drives ectopic cyclin D1 expression in B cells that normally express cyclins D2 and D3 (76). Seventeen previously treated patients received palbociclib on the 3/1 schedule. All patients underwent 2-deoxy-2-[(18)F]fluoro-D-glucose (FDG) and 3-deoxy-3[(18)F]fluorothymidine (FLT) PET to study tumor metabolism and proliferation, respectively, in concert with pretreatment and on-treatment lymph node biopsies to assess RB phosphorylation and markers of proliferation and apoptosis. Substantial reductions in the summed FLT-PET maximal standard uptake value (SUV_{max}), as well as in RB phosphorylation and Ki67 expression, occurred after 3 weeks in most patients, with significant correlations among these end points. These results definitively demonstrated that palbociclib mediated CDK4/6 inhibition with consequent G_{1} arrest in patients’ MCL cells. Five of 17 heavily pretreated patients [including those who had received prior chemotherapy (bortezomib) and stem cell transplant] achieved progression-free survival (PFS) of >1 year (range, 15–30 months), with one complete and two partial responses. Of note, responses were not immediate but occurred after 4 to 8 cycles. Although those patients with prolonged clinical benefit demonstrated marked first-cycle reductions in summed FLT SUV_{max} and in expression of phosphorylated RB and Ki67, such decreases also occurred in patients who did not go on to achieve prolonged benefit, suggesting population heterogeneity in responses after initial CDK4/6 inhibition.

### Liposarcoma

Palbociclib has also been evaluated in patients with RB-positive, CDK4-amplified well-differentiated or dedifferentiated liposarcoma, who had progressed on prior therapy (77). Among 30 patients treated on the 2/1 schedule, 66% were progression-free at 12 weeks, exceeding the protocol predefined endpoint of 12-week PFS of 40%. Of note, 8 patients remained on study for more than 40 weeks, including three with well-differentiated and five with dedifferentiated disease. In addition, regressions were noted in 4 patients; in two instances, these were documented late in the treatment course, reminiscent of delayed responses observed in the MCL study. One patient with a dedifferentiated tumor achieved partial response at 74 weeks and went on to achieve a complete response; another patient demonstrated reduction of a dedifferentiated component within a well-differentiated lesion that resulted in a 30% tumor decrease over a 1-year period. These results suggest that gradual tumor regression can occur after initial CDK4/6 inhibitor-mediated tumor growth inhibition. A fundamental question concerns how continuous administration of a seemingly cytostatic drug can sometimes lead to

### Table 2. Highlights of representative completed and ongoing clinical trials with CDK4/6 inhibitors (Continued)

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Drug(s) (trial phase)</th>
<th>Description and outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAP kinase pathway combinations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF-mutant melanoma</td>
<td>Ribociclib</td>
<td>Phase I study followed by the randomized phase II of LGX818 vs. LGX818/ribociclib in BRAF inhibitor-naive population and assessment of LGX818/ribociclib in BRAF inhibitor-resistant population.</td>
<td>Not reported</td>
</tr>
<tr>
<td>NCT01777776 (phase I/II)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAS-mutant melanoma</td>
<td>Ribociclib</td>
<td>Preliminary phase I results among 14 patients demonstrated 6 with partial response and 6 with stable disease (4 of whom had &gt;20% regression).</td>
<td>(114)</td>
</tr>
<tr>
<td>NCT01781572 (phase Ib/II)</td>
<td>Binimetinib</td>
<td>Phase I trial with expansion in KRAS-mutant NSCLC.</td>
<td>Not reported</td>
</tr>
<tr>
<td>RAS-mutant cancers</td>
<td>Palbociclib</td>
<td>Phase I trial with expansion in NRAS-mutant melanoma.</td>
<td>(137)</td>
</tr>
<tr>
<td>NCT02022982 (phase I/II)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RAS-mutant cancers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT02085063 (phase I)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Other combinations</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MCL</td>
<td>Palbociclib/ibrutinib</td>
<td>Trial of palbociclib and ibrutinib in previously treated MCL.</td>
<td>Not reported</td>
</tr>
<tr>
<td>NCT02159755 (phase I)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Small cell lung cancer</td>
<td>G1T28 (ref.130)</td>
<td>Phase I followed by randomization of etoposide/carboplatin ± G1T28; first trial utilizing CDK4/6 inhibitor to protect bone marrow function from effects of chemotherapy in an RB-negative tumor type.</td>
<td>Not reported</td>
</tr>
<tr>
<td>NCT02499770</td>
<td>Etoposide</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Carboplatin (phase I)</td>
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</tbody>
</table>
| **Abbreviation:** GBM, glioblastoma multiforme.
tumor regression, whether in xenograft models or in patients. An issue is whether reversible G1 arrest (quiescence), which occurs in all RB-positive cell lines exposed to CDK4/6 inhibitors, can lead to a durable state of cell-cycle exit (senescence) marked by resistance to mitogenic stimulation or oncogenic challenge. Although it is relatively easy to define senescence in culture, it is considerably more problematic in a clinical setting to evaluate the significance of suboptimal senescence-associated biomarkers, whether expressed alone or in combination (78). Despite these caveats, there is considerable evidence that senescent cells secrete cytokines that attract immune cells and lead to tumor clearance, providing a potential explanation for tumor regression (79).

In an intriguing study (80), palbociclib treatment of seven RB-positive liposarcoma cell lines, each with chromosome 12q14 amplification of linked CDK4 and MDM2, arrested in a G1 state within 48 hours of drug exposure. Three of the cell lines previously expressed several senescence biomarkers and failed to resume proliferation when palbociclib was withdrawn. Similar results were obtained with other CDK4/6 inhibitors (Table 1, and see below). Proteolytic turnover of MDM2 was required for the induction of senescence, whereas cell lines that only underwent transient G1 arrest did not reduce MDM2 in response to palbociclib. MDM2 turnover was found to depend on its E3 ligase activity and the expression of ATRX, and is postulated to facilitate stabilization of a senescence-activating protein(s) in a p53-independent fashion. Importantly, in an analysis of seven paired tumor biopsies among patients in the liposarcoma trial, among 3 patients without clinical benefit (time to progression < 84 days), there was no evidence of reduced MDM2 in the on-treatment biopsy. In contrast, among 4 patients who achieved demonstrable clinical benefit, remaining progression free for 168, 376, 500+, and 800+ days (including the patient with complete response), the on-treatment biopsies demonstrated marked reduction in MDM2 expression. Of note, all of the on-treatment biopsies showed reduced expression of phosphorylated RB. Therefore, similar to the experience in MCL, reduced RB phosphorylation appears necessary but not sufficient to define patients ultimately destined to achieve clinical benefit with sustained disease control. Elucidating the factors that distinguish transient CDK4/6-inhibitory drug-induced cytostasis from durable cell-cycle withdrawal in various cancer types remains a formidable challenge.

Breast Cancer

The use of palbociclib in breast cancer is relatively advanced. Preclinical data utilizing palbociclib demonstrated the substantial sensitivity of HR-positive cell lines, compared with HR-negative cell lines, in part related to a higher incidence of RB negativity in the latter breast cancer subgroup (81). In a phase II study of palbociclib on the 3/1 schedule in RB-positive advanced breast cancer, 31 patients with HR+/HER2- disease and 4 patients with HR+/HER2- disease were enrolled (82). Of the HR+/HER2- group, 1 patient achieved partial response and 5 had stable disease for 26 months, with median PFS of 3.8 and 1.5 months for the HR+/HER2- and HR+/HER2- groups, respectively. Stratification related to degree of prior treatment demonstrated that patients with HR tumors who had received more than 2 lines of anti-estrogen therapy had significantly longer PFS than those who had received fewer therapeutic cycles (5 vs. 2 months); the degree of prior cytotoxic regimen exposure did not affect outcome. Notably, in this trial, 24% of patients had treatment interruption and 51% had dose reduction, all for cytopenias. In the initial survey of breast cancer cell lines with palbociclib, synergistic growth-inhibitory activity was noted with the estrogen antagonist tamoxifen, including activity in a model of acquired tamoxifen resistance. This work prompted extensive clinical investigation of CDK4/6 inhibitors with anti-estrogen agents in estrogen receptor (ER)-positive breast cancer. After phase 1 work demonstrated that full-dose palbociclib on the 3/1 schedule could be combined with the standard dose of the aromatase inhibitor letrozole (2.5 mg once daily), a randomized phase II study was conducted (PALOMA-1/TRIO-18; NCT00721409) comparing letrozole with the combination of palbociclib and letrozole (1:1) in postmenopausal women with ER+/HER2- breast cancer who had not received systemic treatment for advanced disease (83). Two cohorts were sequentially enrolled. In the first group, 66 patients were selected based on ER+/HER2- status alone; in the second, the 99 patients enrolled were also required to have breast cancer harboring CCND1 amplification, loss of CDKN2A, or both. PFS was the primary endpoint. In cohort 1, median PFS was 5.7 months for letrozole alone, compared with 26.1 months for the combination group (hazard ratio, 0.299), whereas in cohort 2, median PFS was 11.1 months for the letrozole group compared with 18.1 months for the combination group (hazard ratio, 0.508). When the entire population of 165 patients was considered, median PFS was 10.2 months for letrozole alone, compared with 26.1 months for the combination. PFS was significantly improved for patients who received letrozole in first-line systemic treatment for advanced ER+/HER2- breast cancer. Based on these data, a phase III, double-blind, placebo-controlled study in a similar population of 650 patients is ongoing and awaiting further analysis (PALOMA-2; NCT0170427). Nonetheless, given the significant benefit of the combined regimen in extending PFS in the phase II trial, palbociclib was provisionally approved by the FDA in early 2015 for use in patients with ER+/HER2- breast cancer.

It is noteworthy that CCND1 amplification and CDKN2A loss do not appear to contribute to the ability to select patients who are most likely to benefit from combined hormonal treatment and CDK4/6 inhibition. Instead, the study confirmed monotherapy data suggesting that ER positivity may be the most effective predictive marker for identification of patients with breast cancer for CDK4/6 inhibitor–based treatment, reflective of the importance of CDK4 activity in the proliferation of ER+ breast cancer cells, irrespective of genomic alterations in the pathway. Possibly, CCND1 amplification and CDKN2A loss may contribute to determining response to letrozole alone, again reflecting substantial biology linking cyclin D1 and the ER (68); however, larger studies will be required for definitive conclusions to be drawn. Moreover, these findings highlight an emerging theme that genetic alterations in the “RB signaling pathway,” except for RB loss itself, may not serve as informative biomarkers in distinguishing patient responses to CDK4/6 inhibition.

Palbociclib combined with the ER antagonist fulvestrant has also been studied in a 2:1 randomized, placebo-controlled
phase III study enrolling 521 patients with advanced ER+/HER2− breast cancer who had progressed during prior endocrine therapy (PALOMA-3; NCT01942135; ref. 84). Consistent with the results of the study with letrozole, an interim analysis reported PFS of 9.2 months with palbociclib–fulvestrant and 3.8 months with placebo–fulvestrant (hazard ratio, 0.42). This study enrolled premenopausal and perimenopausal patients, with benefit of the combination observed across groups.

Many additional questions about these trials require further clinical assessment. For example, although combination treatment was well tolerated overall, even without increased incidence of febrile neutropenia, grade 3–4 neutropenia was still significantly more common in the palbociclib-containing combinations compared with hormonal therapy alone, necessitating dose interruptions and reductions and suggesting that confirmation of pharmacodynamic effects utilizing lower doses of palbociclib may prove worthwhile. Although diabetogenic effects in long-term treated patients have not been reported, predicted side effects of targeted therapy may involve glucose intolerance, based on requirements of pancreatic β cells for CDK4 (85, 86) and effects of p16INK4a on age-dependent islet cell regeneration (87). In addition, to date, the effect of palbociclib on overall survival is unknown, with follow-up ongoing in the PALOMA-2 and PALOMA-3 trials. It will also be of interest to determine whether palbociclib, used alone or in combination with other agents, has utility in women whose tumors are resistant to anti-estrogen treatment based on ESR1 mutation. Finally, the success of the addition of palbociclib to hormonal therapy in metastatic disease has also generated interest in the use of such combinations in the adjuvant setting; a pilot study assessing the feasibility of 2 years of combined palbociclib and hormonal treatment is currently under way (NCT01864746).

**DEVELOPMENT OF ADDITIONAL CDK4/6 INHIBITORS: RIBOCICLIB AND ABEMACICLIB**

Two additional CDK4/6 inhibitors, ribociclib (LEE011) and abemaciclib (LY2835219; Table 1), are in active clinical development (Table 2). Like palbociclib, these orally bioavailable and highly selective CDK4/6-targeting agents exhibit IC50 values in the low nanomolar range, whereas other CDK family members are far less sensitive (1, 88, 89). All three inhibitors compete for binding of ATP to CDK4/6, so variations in potency and specificity reflect their somewhat different chemical structures (illustrated in ref. 1).

**Ribociclib**

Phase I clinical development of ribociclib in patients with tumors with documented RB positivity utilized a Bayesian logistic regression model incorporating an overdose control principle to guide dose escalation (NCT01237236; refs. 90, 91). Among 85 patients treated, dose-limiting toxicities occurred in 10, including neutropenia, thrombocytopenia, pulmonary embolism, hyponatremia, QTcF prolongation, and elevated creatinine, resulting in a recommended phase II dose of 600 mg daily on the 3/1 schedule. Plasma exposure increases were slightly higher than dose-proportional; the mean half-life at the 600 mg dose level was 36.2 hours. Among paired tumor biopsies from 40 patients, reductions of ≥50% from baseline in Ki67 and phospho-pRB were documented in 55% and 42% of samples, respectively; correlations with clinical outcome have not been reported. Partial responses were seen in a patient with PIK3CA-mutated, CCND1-amplified, ER+ breast cancer and in a second patient with wild-type BRAF/NRAS, CCND1-amplified melanoma. Stable disease for 26 cycles was observed in 14% of the treated population. Despite the expanded use of ribociclib in the clinical arena alone or in concert with other drugs (Table 2), published outcome data are not yet available.

**Abemaciclib**

In contrast with palbociclib and ribociclib, the dose-limiting toxicity of abemaciclib is fatigue (NCT01394016; ref. 92). Diarrhea and hematologic toxicity also occur with this agent, although the former is manageable with supportive medications and/or dose reduction, and the latter is milder than with the other compounds, allowing continuous daily dosing without interruption. The reasons for reduced hematologic toxicity and other observed nonhematologic toxicities are under active investigation, but may be related to a greater selectivity for CDK4 over CDK6 with abemaciclib (88), its greater relative potency and potential to target CDK9 (Table 1), and/or its distribution into the central nervous system (93). Both once-daily and twice-daily schedules have been evaluated with a recommended phase II dose of 200 mg twice daily (92). The agent demonstrates dose-proportional pharmacokinetics, with a median time to maximal plasma concentration of 4 to 6 hours and terminal elimination half-life ranging from 17 to 38 hours. Abemaciclib is widely distributed (93) such that concentrations can be detected in cerebrospinal fluid that approximate those in plasma (92), forecasting its potential use in treating brain tumors. Pharmacodynamic assessment of target engagement in keratinocytes from skin biopsies demonstrated both reduced RB phosphorylation and topoisoerase IIα expression by 4 hours after treatment; however, despite the half-life, in patients treated once daily, there was partial reversibility in samples obtained directly prior to the next dose (92). Therefore, 200 mg twice daily was selected as the dose and schedule for further development to ensure persistent target coverage over the dosing interval, as was shown to be important in preclinical pharmacokinetic/pharmacodynamics modeling in mice bearing human tumor xenografts (94).

In an expansion cohort in the phase I trial, abemaciclib has also been evaluated as monotherapy in advanced breast cancer among heavily pretreated patients with a median of 7 prior systemic therapies (92, 95, 96). In the initial experience, among 25 patients with HR+/HER2− disease, the clinical benefit rate was 72%, including 7 partial responses (28%). Only one of the partial responders received concomitant hormonal therapy, suggesting a higher response rate for abemaciclib as a single agent than palbociclib. Although dose reductions for fatigue and diarrhea from 200 to 150 mg twice daily occurred, 150 mg still produced pharmacodynamic CDK4/6 inhibition and was administered continuously without interruption, raising the possibility of the importance of continuous target inhibition. Importantly, activity was observed irrespective of concomitant PIK3CA mutation. Like the palbociclib experience, among 4 patients with HR+/HER2− cancers, all still had progressive disease. Abemaciclib has also been combined...
successfully at full dose with fulvestrant in a small group of patients, with acceptable safety and promising efficacy (96, 97), underscored the potential value of combinatorial regimens as seen with palbociclib (see below).

HER2-amplified breast cancer cell lines have also demonstrated sensitivity to CDK4/6 inhibition. In addition, genetically engineered mouse models clearly revealed the importance of cyclin D1–CDK4 activity for the initiation and maintenance of HER2-driven breast cancers (98–100). The initial abemaciclib trial enrolled 11 patients with HR+/HER2+ and 5 patients with HR-/HER2- disease. The disease control rate in the HR+/HER2+ group was 100%, with 4 partial responses, whereas 3 patients with HR-/HER2- disease had stable disease of brief duration (92, 95, 96). Although patients with HR+ /HER2+ disease are likely to derive benefit from CDK4/6 inhibition, further work will be required to determine if activity is driven primarily by HER2 status and whether there is benefit for patients with HR-/HER2- disease. Of note, for the entire HR+ population treated with abemaciclib, the median duration of response was 13.4 months and median PFS was 8.8 months.

As with breast cancer, patients with non–small cell lung cancer (NSCLC) have been evaluated in a phase II trial of palbociclib (NCT01291017) and in a phase I expansion cohort of abemaciclib. In the palbociclib study, among previously treated patients, 8 achieved stable disease ≥ 4 months (101). With abemaciclib (92, 102), 68 patients have been treated with a range of one to ten prior systemic therapies. Two partial responses were observed, including 1 patient with KRAS-mutant NSCLC and 1 patient with squamous NSCLC and copy-number loss of CDKN2A. Thirty-one (46%) patients had stable disease, including 15 patients who remained on trial ≥ 6 months (4 of whom for >12 months), for an overall disease control rate of 49% and 6-month PFS rate of 26%. In addition, patients with KRAS-mutant NSCLC fared best; the disease control rate for the KRAS mutation–positive population was 55% (16/29 patients), whereas that for the population with wild-type KRAS was 39% (13/33 patients). These data are consistent with the importance of CDK4 activity in preclinical mouse models of KRAS-driven NSCLC, where CDK4 ablation or inhibition has induced synthetic lethality (103).

The activity of abemaciclib has also been explored in glioblastoma multiforme (GBM) and melanoma (92). Among 17 GBM patients, 2 patients had a decrease in tumor size and received treatment without progression for >16 months. In preclinical models, codelletion of CDKN2A and CDKN2C, encoding p16INK4A and p18INK4C, respectively, dictated increased sensitivity to CDK4/6 inhibition, and it will be of interest to determine whether responding cases met this prediction (104). Among 26 melanoma patients, 1 patient with a tumor harboring NRAS mutation and CDKN2A/B alteration achieved a confirmed partial response. In melanoma models, CDK4/6 inhibition has been shown to cause destabilization of FOXM1, an event linked to the induction of senescence (17); whether FOXM1 expression was altered by abemaciclib in this tumor is unknown.

Abemaciclib has also been evaluated in a population of patients with relapsed or refractory MCL similar to those treated with palbociclib (NCT01739309; ref. 105). Among 22 patients, grade 3–4 neutropenia and thrombocytopenia were more common than in the solid tumor treatment setting, occurring in approximately one third of patients. Overall, there were 5 partial responses and 9 patients who achieved stable disease as the best response; among these 14 patients, 8 received ≥ 6 cycles. Taken together, the palbociclib and abemaciclib trials demonstrate that CDK4/6 inhibition can achieve durable disease control in patients with relapsed or refractory MCL.

### ADDITIONAL APPLICATIONS OF COMBINATORIAL APPROACHES WITH CDK4/6 INHIBITORS

#### MEK Inhibitor Combinations in RAS-Driven Cancers

Consistent with early findings indicating that the cellular life history of D-type cyclins is highly dependent on RAS signaling, preclinical synergistic effects of CDK4/6 inhibition and MAP kinase inhibition in melanoma and pancreatic cancer models have stimulated substantial interest in the development of these combinations (106–108). Possibly, the predominantly cytostatic effects of CDK4/6 inhibitors might be reprogrammed to induce senescence or apoptosis in response to drugs targeting RTKs and/or downstream RAS signaling pathways that are essential for cell viability. For example, in an inducible mouse model of NRAS-mutant melanoma, pharmacologic inhibition of MEK activates apoptosis, but not cell-cycle arrest (109). Therefore, cell death is balanced by continued proliferation, leading to tumor stasis in vivo. In contrast, genetic extinction of NRAS induces both of these effects. CDK4 was identified as the critical driver of this differential phenotype, so that combined inhibition of CDK4 and MEK led to apoptosis with blockade of continued proliferation, resulting in net tumor regression and substantial synergy in therapeutic efficacy. Consistent with these results, combined CDK4 and MEK inhibition has led to increased apoptosis and/or reduced viability in colony formation assays in human melanoma and pancreatic cancer cell lines. However, although potent inhibitors of RAS–RAF–MEK–ERK signaling efficiently silence ERK phosphorylation, interruption of feedback circuits can result in rebound of ERK activity (110). Determinants of primary resistance are not the same in different tumor types that upregulate various RTKs or their ligands in response to pathway inhibition (111–113). Drug dosing schedules or the addition of specific RTK inhibitors may hold the key to circumventing these potential problems.

This work was translated to a phase I trial combining ribociclib (3/1 schedule) with the MEK inhibitor binimetinib (twice daily continuously) in NRAS-mutant melanoma (NCT01781572; ref. 114). In a preliminary report of 14 patients receiving the first two dose levels, including ribociclib at 200 or 300 mg with binimetinib at 45 mg, 6 patients had achieved partial response and 6 had stable disease, including 4 with ≥20% tumor shrinkage. Tumor regression was often early and accompanied by major symptomatic improvement. The drug combination was not without severe adverse effects, with dose-limiting toxicities, including acute renal injury, elevated creatine phosphokinase, peripheral edema, and arrhythmia most likely due to binimetinib or to consequences of combinatorial treatment. Other common treatment-related toxicities included rash, anemia, nausea, diarrhea, and fatigue. Possibly, antitumor drug synergy between various CDK4/6 and MEK inhibitors may allow reduced dosing schedules...
CDK4 and CDK6 as Anticancer Drug Targets

REVIEW

Combined Inhibition of CDK4/6 and PI3K Pathway Signaling

Combined CDK4/6 and PI3K or PI3K/mTOR signaling has been investigated preclinically primarily in models of breast cancer, where activation of the PI3K pathway occurs frequently, with the most promising results emerging with PI3Kα isoform-selective drugs. Recently, it has been recognized that in sensitive cells, as well as in tumors from patients who responded to PI3K inhibition, there is repression of RB phosphorylation, whereas in cell lines with reduced sensitivity or in tumors from patients who did not respond to PI3K inhibition, RB phosphorylation is sustained. A combinatorial drug screen utilizing multiple PIK3CA-mutant cell lines with only modest sensitivity to PI3K inhibitors revealed that combined ribociclib-mediated CDK4/6 and PI3K inhibition synergistically reduces cell viability in vitro and leads to tumor regressions in vivo (115). In addition to overcoming intrinsic resistance in these models, the combination also reverses adaptive resistance to PI3K inhibition. The addition of a PI3K inhibitor to combined CDK4/6 inhibition and hormonal therapy is particularly attractive for PIK3CA-mutant ER+ breast cancers (116).

Combined PI3K and CDK4/6 inhibition may also be of substantial interest in squamous cell NSCLC and head and neck cancers, where amplification of both CCND1 and PIK3CA are common events (117, 118), as well as in pancreatic cancer, where genetically engineered mouse models have suggested the importance of the PI3K pathway downstream from activated KRAS (119). p16INK4A-deficient pancreatic cancer cell lines may be inherently resistant to CDK4/6 inhibitors; synergistic compromise of cell proliferation and viability have been demonstrated with PI3K inhibitors (108), as well as with inhibitors of IGF1R both in vitro and in vivo (120). Of note, sensitivity to combined CDK4/6 and IGF1R inhibition correlated with reduced activity of mTORC1, and combined inhibition of CDK4/6 with temsirolimus recapitulated the effects of the IGF1R combination (120). Although these data are provocative, recent results in pancreatic patient-derived xenografts suggest that CDK4/6 inhibition alone may be highly effective in suppressing proliferation, raising the question of whether established cell lines are adequate for assessing therapeutic sensitivities (121). A combinatorial drug screen in dedifferentiated liposarcoma cell lines also identified CDK4 and IGF1R as synergistic drug targets. In this work, the phosphorylation of multiple proteins and cell viability in response to systematic drug combinations were measured in order to derive computational models of the signaling network in dedifferentiated liposarcoma. The models predicted that the observed synergy of CDK4 and IGF1R inhibitors depends on activated AKT; consistent with this prediction, combined inhibition of CDK4 and IGF1R cooperatively suppressed activation of proteins within the AKT pathway (122).

Combined inhibition of CDK4/6 and PI3Kδ has been examined in models of MCL, where idelalisib (Zydelig) monotherapy achieves transient inhibition of AKT phosphorylation but only modest effects on cell proliferation. MCLs express low levels of the negative PI3K regulator PIK3AP1 compared with normal peripheral B cells; however, prolonged CDK4/6 inhibition has been shown to induce expression of PIK3AP1 in these cells and thereby cooperates with PI3Kδ inhibition to lead to robust apoptosis (123). Similar mechanistic considerations may underlie synergism of CDK4/6 inhibition with ibrutinib-mediated BTK inhibition; in addition, combined CDK4/6-PI3Kδ inhibition may also be an effective strategy following the development of acquired ibrutinib resistance (124).

ADDITIONAL THERAPEUTIC OPTIONS

Enthusiasm for the use CDK4/6 inhibitors in cancer treatment raises obvious questions about how they might be leveraged in combination with other therapeutic modalities, including cytotoxic chemotherapy, irradiation, immune checkpoint blockade, and angiogenesis inhibition. Because CDK4/6 inhibitors induce G1 phase arrest in tumors expressing functional RB, they may well blunt the effects of cytotoxic drugs or ionizing irradiation (IR), which kill cancer cells in S- or M-phase (2). Preclinical studies revealed that CDK4/6 inhibitors protect both cancer cells and normal hematopoietic cells from cytotoxicity induced by IR and DNA-damaging agents (125); in turn, immune checkpoint inhibitors rely on the ability of antibodies to PD-1/PD-L1, CTLA-4, and other analogous negative regulatory molecules to restore proliferative T-cell expansion and differentiation, both of which depend on cyclin D2/D3 and CDK4/6 (126, 127). Perhaps clever combinatorial drug sequencing schedules might circumvent these potential problems (128), but relevant data are largely unavailable.

Conversely, CDK4/6 inhibition might be used to transiently arrest normal hematopoietic stem and progenitor cells during chemotherapy or radiation exposure. For example, in a mouse RB-competent MMTV–HER2-driven breast cancer model, the antitumor activity of carboplatin was compromised by palbociclib administration, whereas in an RB-deficient model, palbociclib coadministration protected against carboplatin-induced hematologic toxicity (129). Given that multilineage myelosuppression is a major dose-limiting toxicity of chemotherapy, transient administration of a selective CDK4/6 inhibitor may render CDK4/6-dependent stem and progenitor cells resistant to chemotherapy to preserve hematopoietic function (130). Such trials have recently been initiated in small cell lung cancer, a routinely RB-negative tumor type (NCT02499770).

CONCLUSIONS

It has taken well over two decades to exploit the scientific insights that provided early proof-of-principle that CDK4/6 inhibitors might prove useful for cancer therapy. The founding discoveries, although firmly supported by biochemical and genetic data, predated the available chemistry technologies required to exploit them. Not for failure of trying, it took a decade before chemists at Parke-Davis developed palbociclib, and the merger of the company with Warner-Lambert and
then Pfizer, coupled with a lack of enthusiasm for palbociclib monotherapy based on early phase I trials, challenged the company to champion this drug candidate over many others in their burgeoning pipeline (131). In retrospect, one might have predicted that combinatorial therapies with drugs targeting mitogen-dependent signaling pathways regulating D-type cyclins would synergize with CDK4/6 inhibitors to prevent tumor cell proliferation. Whether chosen purposely or empirically, the combined use of palbociclib and letrozole, a “cyclin D1 inhibitor,” in patients with ER+ breast cancer revealed potent antitumor activity and ultimately led to FDA approval of palbociclib in early 2015.

Many tumors lacking p16INK4a or overexpressing D-type cyclins have shown exquisite sensitivity to CDK4/6 inhibitors, whereas many normal cells are relatively resistant, implying that cancer cells become “addicted” to RB pathway mutations (132). As indicated by trials with breast cancer (83) and under study in a SIGNATURE trial (NCT02187783), amplification of D-type cyclins or CDK4/6 and p16 inactivation may not predict objective responses to CDK4/6-inhibitory therapy (133). Although profound resistance to CDK4/6 inhibitors is conferred by RB inactivation per se, it may well prove that mutations affecting other cell-cycle regulators, such as amplification of cyclin E, loss of p27KIP1 or p21CIP1, and activation of CDK2, would bypass the requirement of tumor cells for CDK4/6 activity, thereby also conferring de novo resistance (62–64). In addition, alterations in expression of such cell-cycle regulators, including cyclin E and p27KIP1, as well as D-type cyclins themselves, have been demonstrated in adaptation to CDK4/6 inhibition, potentially contributing to acquired resistance (108, 134–136). Of note, some of these adaptations are reversible after drug removal, suggesting, perhaps counterintuitively, the potential value of intermittent dosing schedules for maintenance of cell-cycle arrest.

At present, it remains unclear whether the cytostatic effects alone of CDK4/6 inhibition can efficaciously control tumor progression or whether definitive tumor cell elimination under the duress of continued target engagement is a prerequisite for durable clinical responses. Additional drugs targeting mitogenic signaling pathways in RB-positive tumors may be able to convert cytostatic responses to durable cell-cycle arrest (senescence) or cell death (apoptosis). In the next few years, we are likely to see combinations of targeted therapies—some obvious combinations being RAF/MEK/ERK and PI3K inhibitors used in conjunction with those targeting CDK4/6—for which trials are now under way. Despite the decades required for drug discovery and clinical applications, much remains to be learned. Future work will indicate whether the promise of CDK4/6 inhibitors, most advanced for breast cancer, can be validated and extended to other cancers.

Disclosure of Potential Conflicts of Interest
C.J. Sherr has consulted at a 2-day workshop for Eli Lilly. G.I. Shapiro reports receiving a commercial research grant from Pfizer and is a consultant/advisory board member for Eli Lilly, EMD Serono, G1 Therapeutics, and Vertex Pharmaceuticals. No potential conflicts of interest were disclosed by the other author.

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