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Selective CDK4/6 Inhibitors: Biologic Outcomes, Determinants of Sensitivity, Mechanisms of Resistance, Combinatorial Approaches, and Pharmacodynamic Biomarkers

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Abstract

CDK4/6 inhibitors are now part of the standard armamentarium for hormone receptor-positive breast cancer. In this article, we review the biologic outcomes imposed by these drugs on cancer cells, determinants of response, mechanisms of intrinsic and acquired resistance, as well as combinatorial approaches emanating from mechanistic studies that may allow use of these agents to extend beyond breast cancer. In addition, we will address tumor-, imaging-, and blood-based pharmacodynamic biomarkers that can inform rationally designed trials as clinical development continues.

CELL CYCLE CONTROL, THE RETINOBLASTOMA PATHWAY, AND THE CANONICAL VIEW OF CDK4/6 INHIBITION

The mitotic cell cycle underlies the division of all somatic cells in the body. Because uncontrolled cell division is a hallmark of cancer, it has been posited that targeting the cell cycle could represent a universal mechanism for cancer therapy.^{1,2} The signaling pathways that control the cell cycle are complex, but most mitogenic and oncogenic signals act by driving cells through the G0/G1 to S-phase transition (Fig. 1). The key machinery of the cell cycle includes the cyclin-dependent kinases (CDKs) and the associated cyclins required for their catalytic activity.³ Cell cycle traversal during mid-G1 is governed by CDK4 or CDK6 kinase complexes that are highly responsive to a host of cancer-relevant perturbations.^{4,5} For example, multiple oncogenic factors hijack normal regulation of cyclin D1, which promotes

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST AND DATA AVAILABILITY STATEMENT
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CDK4/6 activity.⁶ In addition, genetic permutations directly target CDK4/6 activity, including amplification of genes that encode CDK4, CDK6, or cyclin D1, which have been observed in more than 15% of all tumors, as well as deletion, mutation, or methylation of *CDKN2A*, which encodes the endogenous CDK4/6 inhibitor p16^{INK4A}.⁴ Thus, much of the oncogenic activity observed in the cell cycle is targeted at net CDK4/6 activity. This is also borne out because the retinoblastoma (RB) tumor suppressor is one of the key substrates of CDK4/6⁷ and is also a commonly mutated tumor suppressor in a fashion that is mutually exclusive with CDK4/6 deregulation.⁸

The canonical view of the RB pathway is presented in Figure 1, where the activation of CDKs 4 and 6 by multiple mechanisms is required for the initiation of RB phosphorylation. In the absence of phosphorylation, RB, in an activated state, elicits potent transcriptional repression over a gene expression program required for subsequent cell cycle progression.⁹ This list, which is ever evolving, includes genes required for DNA synthesis, mitosis, and cytokinesis.⁵ In essence, the RB activation state is a bottleneck for the cell cycle; when RB is activated and unphosphorylated, cells cannot progress through the cell cycle because of the absence of required proteins.

If such a model is accurate, it follows that pharmacologically inhibiting CDK4/6 could have potent effects for blocking tumor cell division.^{3,4} Although the class of CDK4/6 inhibitors is expanding, palbociclib, ribociclib, and abemaciclib are the only currently clinically approved agents, all of which are highly selective for CDK4 and CDK6 relative to other CDK complexes.^{3,4} Classically, each of these agents arrests tumor cells in a G1-like state. Concordantly, they generally elicit a cytostatic, as opposed to cytotoxic, mechanism of action in solid tumor cell lines, animal models, and in patient samples.^{10–17} Ample preclinical data indicate that this activity in restraining cell division is mediated by the activation of RB.^{13,18,19} This model suggests that CDK4/6 inhibitors should have potent clinical activity against a wide variety of cancers; however, the evaluation of this class of agents in a multitude of tumor types has demonstrated a varying degree of clinical efficacy.^{3,20} This may be related to diverse biologic outcomes that may occur after CDK4/6 inhibitor-mediated quiescence. Some cell types may more easily adapt than others and readily demonstrate resistance, whereas other cells may convert to a senescent state, resulting in more long-term disease control.^{21–23}

CLINICAL USE OF CDK4/6 INHIBITORS AND BIOMARKERS OF SENSITIVITY

CDK4/6 inhibitors as single agents are particularly effective in suppressing the proliferation of luminal-like breast cancer models.¹³ In addition, CDK4/6 inhibition can be combined effectively with endocrine therapies used in the management of breast cancer and can clearly exert activity in tumor models resistant to endocrine therapy.^{13,24,25} This preclinical efficacy has been validated in the clinic, which led to the U.S. Food and Drug Administration approvals for palbociclib, ribociclib, and abemaciclib in combination with endocrine therapy in the context of HR+/HER2– breast cancer,^{4,20} where they are highly effective in delaying progression of disease. In several cases, these treatments have improved overall survival^{26–29}

compared with endocrine therapy alone. Abemaciclib has also demonstrated monotherapy activity.^{16,30}

Despite these striking clinical results, biomarkers predicting activity of CDK4/6 inhibitors have been difficult to identify, other than preserved expression of the RB protein, a primary CDK4/6 target. In the PALOMA-1 study, the improvement afforded by combined palbociclib and letrozole compared with letrozole alone was similar in unselected patients and in those whose tumors harbored *CCND1* amplification or *CDKN2A* loss.³¹ However, more recent analyses among a large number of cell lines and preclinical models of varying tumor types suggested that tumors with D-cyclin activating features (DCAF) might be particularly sensitive to CDK4/6 inhibition, including those with 3'UTR alterations that stabilize D-cyclin mRNAs and encoded proteins, as can occur in mantle cell lymphoma (MCL) or endometrial cancer. Other DCAF features include *CCND2* or *CCND3* amplification, as in multiple hematopoietic malignancies, or less commonly among solid tumors.^{32,33}

CELL CYCLE PLASTICITY AND MECHANISMS OF INTRINSIC AND ACQUIRED RESISTANCE

Loss of RB has emerged as a mechanism of both intrinsic and acquired resistance. At the genetic level, *RB1* loss is rare in advanced ER+ breast cancer, occurring in less than 10% of patients. Although a rare event, in one report, in 9 of 338 patients with *RB1* loss who were subsequently treated with CDK4/6 inhibitors, progression-free survival (PFS) was only 3.6 months compared with 10.1 months for patients with intact *RB1*.³⁴ Similarly, *RB1* mutations have been reported in samples obtained from patients after development of acquired resistance to CDK4/6 inhibitors.³⁵

However, *RB1* mutation or loss does not account for most of the acquired resistance observed to date in breast cancer; other mechanisms must govern the evolution to a presumed CDK4-/6-independent state. In addition, in many other tumor types, it appears that a CDK4-/6-independent state is either preexisting or evolves rapidly,^{5,36} thereby limiting clinical efficacy of monotherapy. Work in mouse models has provided insight into mechanisms by which CDK4/6 inhibition is bypassed, demonstrating that cells in the mouse can adapt to the loss or reduction of CDK4/6 activity so that other cyclin and/or CDK complexes take their place and mediate the phosphorylation of RB, referred to as “cell cycle plasticity.”^{37,38}

The ability to bypass pharmacologic CDK4/6 inhibition, whether intrinsic or acquired by cell cycle plasticity, occurs through two broad mechanisms. First, high levels of CDK4/6 and D-cyclin complexes can either titrate the pharmacologic inhibitor or escape inhibition and have been described in multiple preclinical and clinical settings. For example, FAT1, a putative tumor suppressor and a member of the cadherin superfamily that interacts with the Hippo signaling pathway, has recently been shown to regulate the expression of CDK6, and its loss may mediate resistance to CDK4/6 inhibitors. Knockout of *FAT1* leads to the downregulation of the Hippo pathway and overexpression of CDK6.³⁴ Genetic sequencing

of 348 biopsies from patients who were subsequently treated with CDK4/6 inhibitor-based therapy revealed that *FAT1* was mutated in approximately 6% of these patients.

Patients with *FAT1* mutation had a PFS of only 2.4 months compared with 10.1 months for patients without *FAT1* mutations.³⁴ In preclinical models of acquired CDK4/6 inhibitor resistance, both *CDK6* amplification³⁹ and CDK6 overexpression via microRNA-mediated modulation of the transforming growth factor beta (TGF- β) pathway have been described; the latter has also been validated in samples from patients whose tumors demonstrated CDK4/6 inhibitor resistance.⁴ In addition, in KRAS-dependent models of pancreatic and lung cancer, adaptation to CDK4/6 inhibition has resulted in increased functional cyclin-CDK complexes that mediate resistance.^{41,42}

Similarly, upstream alterations that likely modulate cyclin D1-CDK4/6 activity have also been observed among resistant tumors, including those activating the fibroblast growth factor receptor (FGFR) signaling pathway. Next-generation sequencing of circulating tumor DNA from patients enrolled in the MONALEESA-2 trial demonstrated that patients with *FGFR1* amplification exhibited a shorter PFS compared with patients with wild-type *FGFR1*.⁴³ Similarly, following progression on CDK4/6 inhibitors, *FGFR1/2* amplification or activating mutations were identified in 14 of 34 (41%) postprogression specimens.⁴³ FGFR-mediated signaling also played a role in resistance in KRAS-dependent models.⁴² In contrast, the role of mutations in other upstream proteins that could affect cyclin D1-CDK4/6 activity is less clear, including *ESR1* and *PIK3CA* mutations. For example, in the PALOMA-3 study, the phase III clinical trial testing the combination of palbociclib and fulvestrant, there was no correlation between response and PFS and the presence of *ESR1* or *PIK3CA* mutations, which indicated that palbociclib was equally active regardless of mutational status.^{44,45} Similar data were reported with ribociclib used as first-line therapy in hormone receptor-positive breast cancer (MONALEESA-2).⁴⁶

Second, in addition to alterations in the expression of the target cyclin-CDK complexes, CDK2 can assume the role of CDK4/6 and therefore render tumor cells resistant.⁴⁷ Cyclin E, a regulatory subunit of CDK2, is central to the initiation of DNA replication at the G₁/S checkpoint. Cyclin E-CDK2 can also phosphorylate RB and can result in the activation of E2F target genes. Because cyclin E-CDK2 phosphorylation events are downstream of those mediated by cyclin D-CDK4/6, overexpression of cyclin E renders inhibition of CDK4/6 ineffective in inducing G₁ arrest or subsequent growth inhibition. Hence, tumors with high levels of cyclin E are likely to be intrinsically resistant to CDK4/6 inhibition. Gene expression profiling on 302 archival formalin-fixed, paraffin-embedded samples from the PALOMA 3 trial revealed that in patients treated with combined palbociclib and fulvestrant, those with high cyclin E1 mRNA expression had a median PFS of 7.6 months compared with those with low cyclin E mRNA, who had a PFS of 14.1 months.⁴⁸

Cyclin E is also post-translationally modified by neutrophil elastase-mediated proteolytic cleavage to generate the low molecular-weight isoforms of cyclin E (LMW-E) that are detected in many cancer types.⁴⁹ LMW-E lacks the *N*-terminal cyclin E nuclear localization signal and promotes its accumulation in the cytoplasm. Compared with full-length cyclin E, the aberrant localization and unique stereochemistry of LMW-E dramatically alters the

substrate specificity and selectivity of CDK2, increasing tumorigenicity in experimental models. Cytoplasmic LMW-E, which can be assessed by immunohistochemistry, is prognostic of poor survival independent of breast cancer subtype^{50,51} and also predicts lack of response to neoadjuvant chemotherapy in breast cancer.⁵² Immunohistochemical analysis of cytoplasmic cyclin E on archival tumor tissue from 109 hormone receptor-positive patients with advanced breast cancer who were treated with palbociclib as first line (+ letrozole) or second line (+ fulvestrant) endocrine therapy revealed that 49.5% of these patients were positive for cyclin E, which was associated with a worse prognosis.⁵³ Specifically, patients with cytoplasmic cyclin E-positive tumors had a median PFS of 13.4 months compared with a PFS of 36.5 months in those with cytoplasmic negative tumors.

GUIDELINES FOR BIOMARKER IDENTIFICATION

As evident by these examples, biomarkers of resistance to CDK4/6 inhibitors are heterogeneous in nature and currently have been primarily examined in pretreatment biopsies, which are largely prognostic. To better develop biomarkers of resistance to CDK4/6 inhibitors, it is critical to first identify if patients are early or late progressors. To this end, it is reasonable to define time to progression in groups of patients based on the historic median PFS of 24.8 months for first-line therapy and 9.5 months for second-line therapy. Progressors can be subdivided into three groups that likely have distinct molecular characteristics (Fig. 2): (1) early progressors (6 months post-therapy for all patients); (2) intermediate progressors (6–24 months post-therapy for first-line therapy patients [Fig. 2A] and 6–9 months post-therapy for second-line therapy patients [Fig. 2B]); and (3) late progressors (. 24 months post-therapy for first-line therapy patients [Fig. 2A] and . 9 months post-therapy for second-line therapy patients [Fig. 2B]). Early progressors are considered to be intrinsically resistant to CDK4/6 inhibitors or endocrine therapy (first or second line) as single agents, whereas intermediate and late progressors are considered to have acquired resistance to the combination of CDK4/6 inhibitors and endocrine therapy. Having distinguished the early from the late progressors, the selected biomarkers must be assessed in matched pretreatment and post-progression biopsy material from these patients. By examining only the pretreatment samples, the biomarkers selected will likely identify the intrinsically resistant patient cohort. However, if postprogression matched samples are also assessed for the same biomarkers, novel insights into the utility of that specific biomarker for potentially predicting acquired resistance to CDK4/6 inhibitor-based therapy can be attained. When matched tumor samples (n = 25) from patients with breast cancer who progressed on palbociclib were examined for deregulation of cyclin E, estrogen receptor, DNA repair, and interleukin-6/STAT3 signaling, results revealed that these pathways were all altered compared with pretreatment tumor samples, which suggested that the alterations of these pathways were specific to palbociclib resistance.⁵⁴

Therefore, there is a continued need for development of reliable biomarkers to identify patients with resistance to endocrine therapy and CDK4/6 inhibitors as single agents and in combination that will be clinically meaningful to guide patient care. In addition, exploring mechanisms of disease resistance could considerably improve development of novel treatments effective in delaying or reversing resistance and ultimately improving survival.

COMBINATION THERAPIES TO ENHANCE THE ACTIVITY AND INDICATIONS FOR CDK4/6 INHIBITORS

How cell cycle plasticity is fundamentally controlled or can be predicted is unclear. However, the framework of resistance outlined provides a basis for combination therapy, with the hope of enhancing clinical efficacy of CDK4/6 inhibitors beyond HR+/HER2– breast cancer.

To expand the therapeutic efficacy of CDK4/6 inhibitors, multiple combinatorial approaches have been undertaken and are summarized in Table 1. These efforts largely fall among three different classes of agents: endocrine therapy, kinase inhibitors, and chemotherapy.

Similar to combined CDK4/6 inhibition and endocrine therapies in breast cancer, it has been shown that CDK4/6 inhibitors will cooperate with androgen-deprivation strategies in preclinical models of prostate cancer⁵⁵ and with estrogen depletion in specific gynecologic malignancies. Whether there is clinical benefit of the combination of CDK4/6 inhibition and these approaches is being tested in several clinical trials (Table 1).

In addition to endocrine therapy, a host of kinase inhibitors have been shown to positively interact with CDK4/6 inhibitors. In many such contexts, the kinase inhibitor is targeting an oncogenic pathway present in the associated tumor type.

Examples include the combination of EGFR and CDK4/6 inhibition in *EGFR*-mutant lung cancer or MEK inhibition with CDK4/6 inhibition in a RAS-driven tumor.^{42,56–58}

Considerable preclinical data support a wide spectrum of such combination therapies that either build off U.S. Food and Drug Administration-approved targeted therapies or represent new combination approaches to target a specific cancer (Table 1). Although many of these strategies are strongly supported by preclinical data, emerging clinical results remain uneven and will require randomized studies to clarify the contribution of CDK4/6 inhibition. In addition, toxicity of certain combinations represents a substantial challenge.

Chemotherapy and CDK4/6 inhibition at face value would appear to be antagonistic, because most chemotherapy kills tumor cells based on ongoing cell cycle progression and mitotic division. CDK4/6 inhibition has been shown to antagonize responses to chemotherapy in RB-expressing cells.^{59,60} However, most of these studies investigated short-term endpoints and did not necessarily model how to best combine the agents for efficacy. A number of recent studies have suggested that CDK4/6 inhibitors could be used metronomically or as maintenance strategies following different chemotherapy regimens.^{12,61–64} The nature of the positive interaction of chemotherapy and CDK4/6 inhibition is complex. RB regulates the expression of many factors associated with DNA repair or recovery from mitotic stress,⁹ and therefore, if RB is active, it could prevent recovery from chemotherapy-mediated damage. Conversely, it is well known that chemotherapy will elicit mechanisms to downregulate CDK activity and could therefore cooperate with CDK4/6 inhibition. Irrespective of these mechanisms, clinical studies with combination strategies have produced some degree of efficacy, and multiple trials are ongoing (Table 1).⁶⁵

NONCANONICAL EFFECTS OF ACTIVATING THE RB-PATHWAY AND NEW VULNERABILITIES WITH CDK4/6 INHIBITION

With the extensive research in the activity of CDK4/6 inhibitors, it is not surprising that new discoveries are being made that could be clinically actionable beyond effects on the cell cycle.^{5,21,66} Analysis of gene expression data from cells and tumors in which the RB pathway is activated by CDK4/6 inhibition revealed the induction of an immunologic gene expression program, including genes involved in antigen presentation and interferon response, which suggests that the tumor cell may become more susceptible to immunologic surveillance.^{67–71} Parallel studies demonstrated that systemic CDK4/6 inhibition led to changes in T-cell activity through modulation of NFAT (nuclear factor of activated T cells), which can drive a more potent antitumor response.⁶⁸ These results have been extended by illustrating cooperation of CDK4/6 inhibition with the immune-checkpoint blockade in a variety of tumor cell types. In addition, correlative analysis from tumors treated with CDK4/6 inhibitors have indicated enhanced immunologic infiltrates.⁶⁷ These findings, in concert with the overall excitement surrounding immunotherapy, have led to a proliferation of clinical studies combining CDK4/6 inhibitors, alone or in combination with other targeted agents and with immunotherapy (Table 2). Because many of these studies have only recently been initiated, the data on clinical efficacy and tolerability are not yet mature; however, current preliminary findings appear promising.⁷²

The RB pathway, in addition to controlling cell cycle progression, also plays a role in coordinating different features of metabolism. Evolutionarily, this makes sense because cellular division is tightly linked to nutrient availability to support the biologic mass of two daughter cells. In the case of CDK4/6 inhibition, there is a shift toward oxidative^{73–75} or autophagic metabolism.^{53,76} These findings could be leveraged using selective inhibitors targeting these processes to essentially shift the cytostatic state induced by CDK4/6 inhibit into a selective vulnerability.⁵³ Similarly, because RB activation can limit the expression of multiple DNA repair-associated genes, treatment with CDK4/6 inhibitors could yield a functional state similar to multiple forms of DNA repair deficiency, which ostensibly can be exploited by chemotherapy or radiation therapy. Recent studies have also suggested that CDK4/6 inhibitors could yield sufficient deficiency in homologous recombination-mediated repair to drive cooperative interactions with PARP inhibitors.⁷⁷ Evaluation of these vulnerabilities are being explored clinically (Table 2).

PHARMACODYNAMIC MONITORING OF CDK4/6 INHIBITION IN CLINICAL TRIALS

As clinical trials of CDK4/6 inhibitors study multiple tumor types in combination with other agents, the assessment of pharmacodynamic markers capable of monitoring target engagement and/or pathway modulation will be critical. Several candidates have been identified for this purpose, including expression of phosphorylated RB (phospho-RB) protein, 3'-deoxy-3' [¹⁸F]-fluorothymidine (FLT)-positron emission tomography (PET) and serum thymidine kinase-1 (TK1) activity.

Clinical studies support the monitoring of phospho-RB for evidence of CDK4/6 inhibition. This was initially performed in a pharmacodynamic study of palbociclib in MCL, which demonstrated an 89% reduction in phospho-RB in on-treatment biopsies taken after 3 weeks of palbociclib treatment (125 mg daily for 3 of 4 weeks), compared with pretreatment, with no corresponding changes in total RB.¹⁵ There was a concurrent reduction in the proliferative marker Ki-67 that correlated with the degree of phospho-RB reduction and that confirmed phospho-RB as a potential marker for monitoring CDK4/6 inhibitor-mediated antiproliferative responses. However, further correlative analyses indicated that reductions in Ki-67 and phospho-RB were necessary but not sufficient to predict long-term clinical benefit in heavily pretreated patients with MCL, likely because of subsequent development of resistance in a subset of patients that limited the use of phospho-RB early after treatment initiation as a predictive biomarker. However, the absence of reduced RB phosphorylation early after the start of treatment may correlate with ultimate lack of response. For example, in a short-term preoperative breast cancer trial with palbociclib as a single agent, nonresponders (measured by change in Ki67) were characterized by no change in levels of phospho-RB.⁷⁸ Similar results were seen in the initial trial of abemaciclib monotherapy, in which there was a significant correlation between clinical efficacy and modulation of phospho-RB in proliferating keratinocytes and tumor biopsies. Here, a 60% phospho-RB reduction threshold was able to separate most patients with stable disease or response from those who progressed.¹⁶

The role of phospho-RB as a marker for CDK4/6 inhibitor activity was also assessed in a randomized phase II study of palbociclib (125 or 100 mg) in combination with either fulvestrant or tamoxifen in metastatic hormone receptor-positive breast cancer (TBCRC 035).⁷⁹ Findings were in line with those observed in patients with MCL; there was a considerable decrease in phospho-RB and Ki-67 staining observed in both skin and tumor biopsies. The degree of change was comparable between the 125- and 100-mg palbociclib dose levels, as was clinical outcome, which indicated that dose reduction of palbociclib, if required for hematologic toxicity, was unlikely to compromise pharmacodynamic effects and efficacy signals. In another phase I clinical trial of palbociclib and the MEK inhibitor PD-0325901 in *RAS*-mutant solid tumors that compared continuous and intermittent (3 weeks of every 4 weeks [3/1 dosing]) palbociclib dosing, serial analysis of skin keratinocytes during the first treatment cycle demonstrated that phospho-RB and Ki-67 staining mirrored palbociclib exposure. There was a sustained decrease in patients who underwent continuous dosing, whereas intermittent dosing resulted in a recovery of phospho-RB staining during the off week of palbociclib.⁸⁰

The ability to use noninvasive or minimally invasive biomarkers is preferential to biopsy-based biomarkers, and [¹⁸F] FLT identifies cells undergoing active DNA synthesis, which allows for its potential use as a surrogate marker for *S*-phase and the quantification of cellular proliferation.⁸¹ The MCL study also examined the role of pre- and on-treatment [¹⁸F] FLT-PET as an imaging biomarker to demonstrate the biologic effect of G1 arrest mediated by palbociclib.¹⁵ Of 16 evaluable patients, there were significant reductions in the summed [¹⁸F] FLT-PET maximal standard uptake value in most patients, and in patients with PFS longer than 1 year (n = 5, including 1 patient with complete response and 2 patients with partial responses), there was a more than 70% reduction in summed [¹⁸F] FLT

maximal standard uptake value. However, correlative analysis with clinical response indicated that as with phospho-RB and Ki-67 reductions, the reduction in [^{18}F] FLT uptake was necessary but not sufficient for predicting long-term survival benefit.

TK1 is an enzyme critical for DNA synthesis and is expressed in all human cells during normal cell division through E2F-dependent transcription, with small amounts found in serum. Tumors, because of their higher replication rates compared with normal tissues, are capable of secreting pathologic levels of TK1 detectable in serum. Serum TK1 activity can be measured in patients with cancer using the DiviTum Assay (Biovica, Uppsala, Sweden). This is an enzyme-linked immunosorbent-based assay in which BrdU (5-bromo-2'-deoxyuridine) is incorporated into a synthetic DNA strand fixed to the assay plate, with the extent of BrdU incorporation reflecting TK1 activity present in the serum (Fig. 3). In breast cancer, serum TK1 activity was associated with advanced stage, higher grade, tumor necrosis, vascularity, and estrogen receptor and progesterone receptor negativity.⁸² TK1 activity was also elevated in patients with *BRCA1/2* mutations compared with wild-type and was an independent predictor of disease recurrence.⁸² In addition, TK1 activity levels were determined in patients with advanced breast cancer before they underwent combination chemotherapy with either epirubicin/paclitaxel or epirubicin/paclitaxel/capecitabine using the DiviTum assay. Analysis then identified TK1 activity to be predictive for PFS and overall survival, as well as response to treatment.⁸³ Similarly, patients with hormone receptor-positive breast cancer with low baseline levels of serum TK1 activity or an early drop in TK1 activity had improved outcomes in response to endocrine therapy.⁸⁴

Because TK1 expression is E2F-dependent, CDK4/6 inhibitor treatment should result in reduced serum TK1 activity levels. Coupled with its demonstrated prognostic value in early-stage breast cancer, serum TK1 activity may act as a potential biomarker for target engagement and clinical benefit following CDK4/6 inhibitor treatment. This was examined as part of the NeoPalAna trial¹⁴ that examined neoadjuvant palbociclib and anastrozole in early-stage hormone receptor-positive breast cancer, in which serum TK1 activity was serially monitored until the time of surgery using the DiviTum assay.⁸⁵ Patients received an initial 4 weeks of anastrozole, followed by palbociclib on cycle 1, day 1 (C1D1) for four 28-day cycles. Surgery occurred following 3–5 weeks of washout from the last dose of palbociclib, except in a small subset of patients who received palbociclib (cycle 5) continuously until surgery. Serum TK1 activity was determined at baseline, on C1D1, on C1D15, and at time of surgery. Despite a considerable drop in tumor Ki-67 with anastrozole monotherapy, there was no statistically significant change in TK1 activity. However, a striking reduction in TK1 activity was observed 2 weeks after initiation of palbociclib (C1D15), which then rose significantly with palbociclib washout. At C1D15, TK1 activity was below the detection limit in more than 90% of patients, indicating a profound effect of CDK4/6 inhibition. There was high concordance between changes in serum TK1 and tumor Ki-67 and TK1 mRNA levels in the same direction from C1D1 to C1D15 and from C1D15 to surgery time points. Similar findings were demonstrated in the phase I study of palbociclib and the MEK inhibitor PD-0325901, which compared two different palbociclib dosing schedules—continuous and 3 weeks of every 4 weeks (3/1 dosing).⁸⁰ Serial measurements of TK activity during the first treatment cycle demonstrated that serum TK activity mirrored palbociclib administration; in patients who received 3/1 palbociclib dosing

serum, TK1 activity decreased for the 3 weeks of palbociclib exposure, with subsequent increases during the off week. In patients treated with continuous palbociclib, TK activity remained low throughout the cycle. In addition, TK activity profiles also reflected those seen for phospho-RB and Ki-67 staining in keratinocytes.

These preliminary results highlight the potential for serum TK1 activity to act as a noninvasive biomarker for CDK4/6 inhibitor target engagement. This was further examined in 46 patients enrolled in the TREnd study, a phase II trial that tested the activity and safety of single-agent palbociclib against palbociclib combined with the endocrine therapy on which patients had progressed most recently before enrollment. Although baseline TK1 activity was not prognostic, after one cycle of treatment, patients who demonstrated an increase in TK1 activity had a worse outcome compared with those in which activity was decreased or stable, with a median PFS of 3.0 months versus 9.0 months, respectively. TK1 activity was also assayed at the time of resistance; those with activity higher than the median fared worse on poststudy treatment compared with those with lower activity.⁸⁶ This work suggested that an initial rise in TK1 activity correlated with intrinsic drug resistance and that a rise after an initial reduction correlated with acquired resistance that could be stratified prognostically.

Further work will be required to fully define the degree of reduction in TK1 activity necessary to predict response or long-term clinical benefit. Identification of this threshold is particularly important in combination therapy studies in which attenuation of CDK4/6 inhibitor dosing may be required. In addition, further studies with more intensive serial sampling will be required to determine whether a rise in TK1 activity that indicates acquired resistance predates resistance defined radiographically. Finally, to date, most studies using serum TK1 activity have focused on palbociclib, and additional studies of the effects of abemaciclib and ribociclib on this marker are warranted.

FUTURE PROSPECTS

With intense ongoing research, it is expected that more therapeutic combinatorial strategies will emerge from the preclinical arena. Rationally developed clinical trials that incorporate biomarkers for patient selection, assess tumors at the time of resistance, and incorporate pharmacodynamic endpoints will be crucial to fully leverage the promise of targeting the RB pathway in cancers.

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PRACTICAL APPLICATIONS

- Selective CDK4/6 inhibitors typically lead to G1 arrest and/or senescence against solid tumors and have demonstrated meaningful clinical and survival benefit in combination with hormonal treatment in estrogen receptor-positive breast cancer.
- Multiple intrinsic and acquired resistance mechanisms have been described, including loss of RB, elevated CDK6 activity, FGFR pathway activation, and cyclin E-CDK2 activation.
- Analysis of matched pre- and post-progression biopsies among early and late progressors will help define biomarkers of intrinsic and acquired resistance that will ultimately guide development of rational combinations relevant to breast cancer and other tumor types.
- Pharmacodynamic markers of selective CDK4/6 inhibition include reduced phosphorylation of RB, as well as reduced fluorothymidine-positron emission tomography uptake and reduced serum thymidine kinase 1 activity as noninvasive markers of G1 arrest that can confirm target engagement in clinical trials.

FIGURE 1. Canonical G1/S Cell Cycle Control

Activated retinoblastoma (RB) participates in the repression of an E2F-mediated transcriptional program required for S-phase progression. Mitogenic signals stimulate the assembly of cyclin D-CDK4/6 complexes that initiate RB phosphorylation, followed sequentially by cyclin E-CDK2-mediated phosphorylation that ultimately releases phosphorylated RB and allows E2F-mediated transcription. Cyclin D-CDK4/6 complexes may be inhibited by endogenous inhibitors, such as p16INK4A, or by mimetic drugs, including palbociclib, ribociclib, and abemaciclib.

Abbreviation: RB, retinoblastoma.

FIGURE 2. Analysis of Disease Progression

Analysis of disease progression while on treatment in advanced breast cancer, ER-positive/HER-2 negative patients treated with (A) first-line (CDK4/6 inhibitors + aromatase inhibitor) or (B) second-line (CDK4/6 inhibitors + fulvestrant) therapy. The progressors in each treatment group are divided into intrinsic (early) or acquired (intermediate + late) resistance as described in the text.

FIGURE 3. Liquid Biopsy for G1 arrest (Biovica DiviTum Assay)

TK phosphorylates the nucleoside analog BrdU (5-bromo-2'-deoxyuridine) to BrdUMP (5-bromo-2'-deoxyuridine 5'-monophosphate), which is further phosphorylated to BrdUTP (5-bromo-2'-deoxyuridine 5'-triphosphate). BrdUTP is then incorporated into a solid-phase DNA-strand and incorporated BrdU detected using an anti-BrdU monoclonal antibody conjugated to the signal generating enzyme alkaline phosphatase. The level of BrdU incorporated over time is proportional to the level of TK activity in the sample. Reproduced with permission from [Biovica.com/Divitum](https://www.biovica.com/divitum).

Abbreviation: TK, thymidine kinase.

TABLE 1.

Representative Combinations of CDK4/6 Inhibitors With Hormonal Agents, Signal Transduction Inhibitors, and Chemotherapy

Combination	Representative Clinical Studies	NCT Identifier
+ Estrogen inhibition (breast cancer)	FDA-approved	
+ Estrogen inhibition (gyn malignancies)	Abemaciclib With Letrozole in Recurrent or Persistent Endometrial Cancer	NCT03675893
	Ribociclib and Letrozole Treatment in Ovarian Cancer	NCT03673124
	Palbociclib Plus Letrozole Treatment After Progression to Second Line Chemotherapy for Women With ER/PR-positive Ovarian Cancer: (LACOG1018)	NCT03936270
+ Androgen inhibition (prostate cancer)	A Study of Abiraterone Acetate Plus Prednisone With or Without Abemaciclib (LY2835219) in Participants With Prostate Cancer	NCT03706365
	Enzalutamide With and Without Ribociclib for Metastatic, Castrate- Resistant, Chemotherapy-Naive Prostate Cancer That Retains RB Expression	NCT02555189
	A Phase II Study of Androgen Deprivation Therapy With or Without Palbociclib in RB-Positive Metastatic Prostate Cancer	NCT02059213
+ MTOR inhibitor	Ribociclib and Everolimus in Treating Children With Recurrent or Refractory Malignant Brain Tumors	NCT03387020
	Phase II Trial of Ribociclib and Everolimus in Advanced Dedifferentiated Liposarcoma (DDL) and Leiomyosarcoma (LMS)	NCT03114527
	A Study of LEE011 With Everolimus in Patients With Advanced Neuroendocrine Tumors	NCT03070301
+ MEK inhibitor	Study of Safety and Efficacy of Ribociclib and Trametinib in Patients With Metastatic or Advanced Solid Tumors	NCT02703571
	A Phase Ib/II Study of LEE011 in Combination With MEK162 in Patients With NRAS Mutant Melanoma	NCT01781572
	Study of the CDK4/6 Inhibitor Palbociclib (PD-0332991) in Combination With the MEK Inhibitor Binimetinib (MEK162) for Patients With Advanced KRAS Mutant Non-Small Cell Lung Cancer	NCT03170206
+ PI3K inhibitor	Binimetinib and Palbociclib or TAS-102 in Treating Patients With KRAS and NRAS Mutant Metastatic or Unresectable Colorectal Cancer	NCT03981614
	Study of the CDK4/6 Inhibitor Palbociclib (PD-0332991) in Combination With the PI3K/mTOR Inhibitor Gedatolisib (PF-05212384) for Patients With Advanced Squamous Cell Lung, Pancreatic, Head & Neck and Other Solid Tumors	NCT03065062
	PIPA: Combination of PI3 Kinase Inhibitors and Palbociclib (PIPA)	NCT02389842
+ Tyrosine kinase inhibitor	A Study of Abemaciclib in Combination With Sunitinib in Metastatic Renal Cell Carcinoma	NCT03905889
+ ALK inhibitor	Study of Safety and Efficacy of LEE011 and Ceritinib in Patients With ALK- positive Nonsmall Cell Lung Cancer.	NCT02292550
+ EGFR inhibitor	Study of Safety and Efficacy of LEE011 and Ceritinib in Patients With ALK- positive Nonsmall Cell Lung Cancer.	NCT02292550
	Palbociclib and Cetuximab in Metastatic Colorectal Cancer	NCT03446157
	PD 0332991 and Cetuximab in Patients With Incurable SCCN	NCT02101034
+ Taxane	LEE011 (Ribociclib) in Combination With Docetaxel Plus Prednisone in mCRPC	NCT02494921
+ Platinum	Dose-Escalation Study of Palbociclib + Nab-Paclitaxel in mPDAC	NCT02501902
	Palbociclib With Cisplatin or Carboplatin in Advanced Solid Tumors	NCT02897375

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Combination	Representative Clinical Studies	NCT Identifier
	Ribociclib (LEE-011) With Platinum-based Chemotherapy in Recurrent Platinum Sensitive Ovarian Cancer	NCT03056833
	Phase II Trial Evaluating the Efficacy of Palbociclib in Combination With Carboplatin for the Treatment of Unresectable Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma	NCT03194373
+ Anthracycline	Ribociclib and Doxorubicin in Treating Patients With Metastatic or Advanced Soft Tissue Sarcomas That Cannot Be Removed by Surgery	NCT03009201
+ Gemcitabine	Ribociclib and Gemcitabine Hydrochloride in Treating Patients With Advanced Solid Tumors or Lymphoma	NCT02414724
+ Combination chemotherapy	PD-0332991, 5-FU, and Oxaliplatin for Advanced Solid Tumor Malignancies	NCT01522989
	Study of Palbociclib Combined With Chemotherapy in Pediatric Patients With Recurrent/Refractory Solid Tumors	NCT03709680

Abbreviation: FDA, U.S. Food and Drug Administration.

TABLE 2.

Representative Clinical Trials Combining CDK4/6 Inhibition With Immune Checkpoint Blockade or With Inhibitors of Cellular Metabolism

Combination	Representative Clinical Studies	NCT Identifier
+ Immune-checkpoint inhibitor (anti-PD-1/anti-PD-L1)	Abemaciclib and Pembrolizumab in Locally Advanced Unresectable or Metastatic Gastroesophageal Adenocarcinoma: Big Ten Cancer Research Consortium BTCRC-G118-149	NCT03997448
	Clinical Trial of Abemaciclib in Combination With Pembrolizumab in Patients With Metastatic or Recurrent Head and Neck Cancer	NCT03938337
	Phase Ib Study of TNO155 in Combination With Spartalizumab or Ribociclib in Selected Malignancies	NCT04000529
	Avelumab, Cetuximab, and Palbociclib in Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma	NCT03498378
	Pilot Study of Pembrolizumab Combined With Pemetrexed or Abemaciclib for High Grade Glioma	NCT04220892
	Abemaciclib and Nivolumab for Subjects With Hepatocellular Carcinoma	NCT03781960
	Ribociclib and Spartalizumab in R/M HNSCC (RISE-HN)	NCT04213404
	Hydroxychloroquine, Palbociclib, and Letrozole Before Surgery in Treating Participants With Estrogen Receptor Positive, HER2 Negative Breast Cancer	NCT03774472
	A Study of Telaglenastat (CB-839) in Combination With Palbociclib in Patients With Solid Tumors	NCT03965845
+ Metabolic inhibitors		