Molecular Cancer Therapeutics

Direct CDKN2 Modulation of CDK4 Alters Target Engagement of CDK4 Inhibitor Drugs

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Abstract

The interaction of a drug with its target is critical to achieve drug efficacy. In cases where cellular environment influences target engagement, differences between individuals and cell types present a challenge for *a priori* prediction of drug efficacy. As such, characterization of environments conducive to achieving the desired pharmacologic outcome is warranted. We recently reported that the clinical CDK4/6 inhibitor palbociclib displays cell type–specific target engagement: Palbociclib engaged CDK4 in cells biologically sensitive to the drug, but not in biologically insensitive cells. Here, we report a molecular explanation for this phenomenon. Palbociclib target engagement is determined by the interaction of CDK4 with CDKN2A, a physiologically relevant protein inhibitor of CDK4. Because both the drug and CDKN2A prevent CDK4 kinase activity, discrimination between these modes of inhibition is not possible by traditional kinase assays. Here, we describe a chemo-proteomics approach that demonstrates high CDK4 target engagement by palbociclib in cells without functional CDKN2A and attenuated target engagement when CDKN2A (or related CDKN2/INK4 family proteins) is abundant. Analysis of biological sensitivity in engineered isogenic cells with low or absent CDKN2A and of a panel of previously characterized cell lines indicates that high levels of CDKN2A predict insensitivity to palbociclib, whereas low levels do not correlate with sensitivity. Therefore, high CDKN2A may provide a useful biomarker to exclude patients from CDK4/6 inhibitor therapy. This work exemplifies modulation of kinase target engagement by endogenous proteinaceous regulators and highlights the importance of cellular context in predicting inhibitor efficacy.

Introduction

The eukaryotic cell cycle is tightly regulated to prevent excessive replication or propagation of cells with damaged DNA. Retinoblastoma protein (pRB, commonly referred to as RB), encoded by the gene *RB1*, is a negative regulator that blocks the G_1–S phase transition. Cyclin-dependent kinase 4 (CDK4) and the homologous CDK6, in complex with D-type cyclins (e.g., CCND1), overcome the RB cell-cycle checkpoint by phosphorylating and inactivating RB. Cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as p16 or INK4a) is another negative regulator of the cell cycle, which binds to and inhibits CDK4/6. CDKN2A, CDKN2B (p15/INK4b), CDKN2C (p18/INK4c), and CDKN2D (p19/INK4d) comprise the CDKN2/INK4 family and are structurally and functionally similar, but display different expression patterns (1). Disruptions in the CDKN2A–CCND1–CDK4–RB axis are associated with diverse malignancies (2–4).

Three ATP-competitive CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) are approved by the FDA as therapies for advanced hormone receptor-positive, HER2-negative breast cancer either alone or in combination with hormone therapy. Estrogen receptor (ER) provides a surrogate marker for dependence upon CDKN2A–CCND1–CDK4–RB signaling, and initial evaluations of individual pathway biomarkers (*RB1* expression, *CCND1* overexpression, and *CDKN2A* loss) have not yet further refined predictions of patient response to CDK4/6 inhibitor therapies in this group (2, 3, 5–7). Clinical utility for CDK4/6 inhibitors may extend beyond ER^+ advanced breast cancer and clinical trials are ongoing for a variety of other cancer types, including triple-negative breast cancer ($ER^-/PR^-/HER\text{-}2^-$). In such cases, where ER cannot be used to guide patient selection, additional biomarkers to prospectively identify CDK4/6 inhibitor responsive patients are needed.

We recently demonstrated that palbociclib occupies the active sites of native CDK4 and CDK6 in both cellular lysates and live cells (target engagement) in a cell type–specific manner (8). Although cellular permeability was confirmed in all cells by inhibition of established off-targets, CDK4/6 target engagement was only observed in cell lines that are sensitive to growth-inhibition by palbociclib. Surprisingly, no CDK4 or CDK6 target engagement was observed in palbociclib-insensitive cell lines, suggesting that additional mechanisms for CDK4/6 regulation were likely in these cells. Here, we report that CDKN2/INK4 proteins specifically prevent both nucleotide probe binding to CDK4 and target engagement by small molecule inhibitors. This work provides an example of how regulatory proteins modulate the susceptibility of a protein kinase to small molecule inhibition.

Materials and Methods

Cell culture and proliferation assays

All cells were cultured in media supplemented with 10% FBS and 1% penicillin/streptomycin in a 37°C incubator with 5%

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CO_2. Basal media was EMEM (MCF7, SK-MEL-28), DMEM (MDA-MB-231, MDA-MB-468), or RPMI (DU-145, HCC-1806). The above cell lines were obtained directly from the ATCC and passaged less than 6 months. The HAP-1 WT and HAP-1 DKO cell lines were obtained from Horizon and cultured in Iscoves's Modified Dulbecco's medium (IMDM; Gibco) without phenol red supplemented with 10% charcoal stripped FBS (Gibco) and 1% of penicillin/streptomycin. HAP-1 knockouts were engineered using the CRISPR-Cas9 system to introduce frameshift mutations into the coding sequence of genes of interest. All HAP-1 knockout cell lines were validated by PCR amplification and Sanger Sequencing to confirm the mutation at the genomic level by Horizon.

HAP-1 proliferation assays were conducted in 96-well plates, with 1,500 cells seeded per well. Twenty-four hours after seeding, cells were exposed to vehicle or palbociclib at the indicated concentrations, followed by growth in media for three cycles. Then cells were fixed with 4% glutaraldehyde in PBS, stained with 0.1% of crystal violet in methanol, and solubilized with 10% of acetic acid in PBS. Absorbance of each well was measured at 560 nm. For MCF7 proliferation assays, transfected cells were seeded in 96-well plates at 1,000 cells per well (the day after transfection) and exposed to vehicle or palbociclib at the indicated concentrations. After growth in media for 5 days, the CellTiter-Glo assay was performed (Promega G7570).

Transfections and siRNA

Cells were transfected in 10 cm plates at 80% confluency with 9 μg DNA using the Fugene HD transfection reagent (Promega) according to the manufacturer's instructions. All expression plasmids were purchased from Origene: PS100010 (GFP), RC220937 (CDKN2A), RC204895 (CDKN2B), RC213083 (CDKN2C), RC214065 (CDKN2D), RC201817 (CDKN1A), RC01661 (CDKN1B), and RC209840 (CDKN1C). After brief expansion, transfected cells were divided into vessels for immunoblotting or KiNativ processing and harvested 3 days posttransfection. CDKN2A siRNA (Dharmacon SMARTpool, 1029) or nontargeting control was delivered to MDA-MB-468 cells by nucleofection (Lonza Nucleofector 4D, SE, DS-120).

Immunoblotting

Cells were seeded into six-well plates at 400,000/well in complete media. The following day, cells were rinsed with PBS then scraped directly in 1× sample buffer with BME. Samples were sonicated and boiled immediately prior to SDS-PAGE. The following primary antibodies were used: DDK (Origene, clone OTI4C5, 1:1,000), CDKN2A (Abcam ab108349, 1:1,000), CDK4 (CST 12790, 1:1,000), pRB (CST 9309, 1:1,000), GAPDH (Abcam ab8245, 1:5,000).

Sample preparation

Cell pellets were lysed by sonication in lysis buffer [50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 0.1% Triton X-100, phosphatase inhibitors (Cocktail II AG Scientific #P-1518)]. After lysis, the samples were cleared by centrifugation and $MnCl_2$ was added to a final concentration of 20 mmol/L. Compounds were from Selleckchem (palbociclib-S1116 and abemaciclib-S7158). After clarification, 5 μL of 100× compound or DMSO was added to 445 μL of lysate in duplicate. After 15-minute incubation, 50 μL of a 10× aqueous solution of the desthiobiotin-ADP-acyl phosphate probe (ADP probe) was added to each sample for a final probe concentration of 20 μmol/L and incubated for an additional 15 minutes. Samples were prepared for MS analysis as described previously (9, 10). Briefly, probe-labeled lysates were denatured and reduced (6 M urea, 10 mmol/L DTT, 65°C, 15 minutes), alkylated (40 mmol/L iodoacetamide, 37°C, 15 minutes), and gel filtered (Bio-Rad Econo-Pac 10G) into 2 M urea, 5 mmol/L methionine, 1M Tris. The desalted protein mixture was digested with trypsin (0.015 mg/mL) for 1 hour at 37°C, and desthiobiotinylated peptides were captured using 12.5 μL high-capacity streptavidin resin (ThermoFisher Scientific). Captured peptides were then washed extensively, and probe-labeled peptides eluted from the streptavidin beads using two 35 μL washes of a 50% CH_3CN/water mixture containing 0.1% TFA at 20 to 25°C.

LC/MS-MS

Samples were analyzed by LC/MS-MS as described previously (10). Samples were analyzed on Thermo LTQ ion trap mass spectrometers coupled with Agilent 1100 series micro-HPLC systems with autosamplers, essentially as described (11), using a custom target list comprising 269 unique kinase peptides that had been previously identified during proteome characterization in data-dependent mode (10).

Data analysis

For signal extraction and quantitation, typically up to four ions were selected based on their presence, intensity, and correlation to the reference MS-MS spectrum. The resulting chromatographic peaks from each run were then integrated and the integrated peak areas were used to determine relative probe ratios between treated samples and controls. The number of replicates and statistical tests used are indicated in the figure legends.

Results

CDKN2 proteins reduce nucleotide probe binding of CDK4

Crystal structures of CDKN2A and CDKN2D proteins bound to CDK6 showed that CDKN2 binding induces conformational changes in the kinase that interfere with ATP binding (12, 13). These reports prompted our investigation into the influence of CDKN2 on nucleotide acyl-phosphate probe labeling of CDK4 and CDK6. We used desthiobiotin-linked nucleotide (ATP or ADP) probes to covalently modify active-site lysine residues in protein kinases (9, 10); a method widely used to profile both endogenous kinases and kinase inhibitors in whole cell lysates. The ADP acyl phosphate probe yields improved signal-to-noise ratios for CDK4 and CDK6 compared to the corresponding ATP-acyl-phosphate probe (8) and was used for this study. We expressed *CDKN2A* in MCF7 cells, which are known to harbor homozygous deletion of *CDKN2A* (14), and measured the signals of probe-labeled kinases in lysates derived from transfected cells. In agreement with prior studies using purified proteins (12, 13), CDK4 probe-labeling in lysates was inhibited greater than 99% by *CDKN2A* expression, although total CDK4 protein levels were unchanged (Fig. 1A and B). Notably, *CDKN2A* expression did not alter probe-labeling for any of the 200 other kinases measured (Fig. 1C). CDK6 was not routinely detectable in MCF7 cells by MS or Western blot analysis, therefore subsequent studies were focused on CDK4. We next tested whether of knockdown of *CDKN2A* expression in MDA-MB-468, a cell line with high levels of endogenous *CDKN2A* (Fig. 1D), would show the opposite

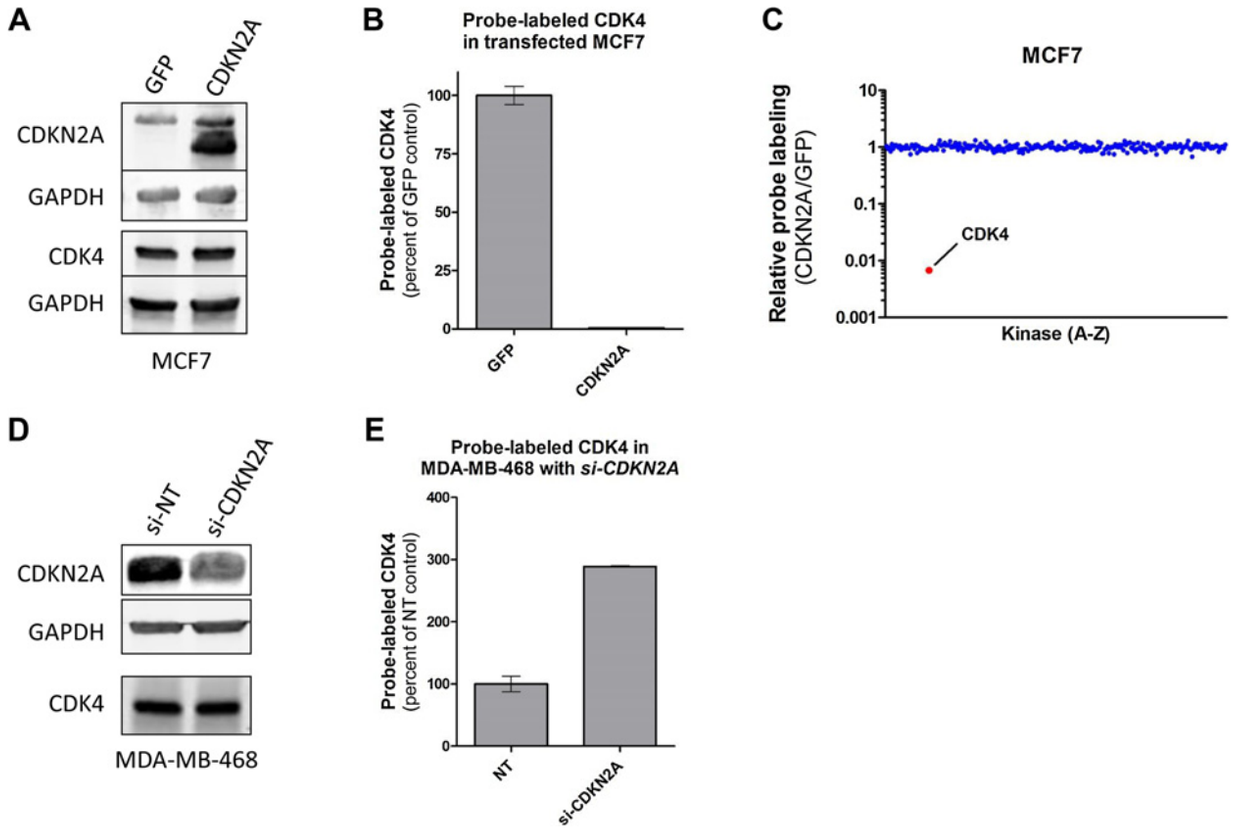

Figure 1.

CDKN2A inhibits binding of CDK4 to nucleotide probe. **A,** Immunoblot measurement of CDK4 and CDKN2A in MCF7 cells transfected with *CDKN2A* or *GFP* as a control. **B,** The CDK4 MS signal is greatly reduced with CDKN2A overexpression. The data reflect the average of duplicate samples; error bars show the range. **C,** Probe-labeled kinase profile of *CDKN2A*-overexpressing MCF7 relative to *GFP*-overexpressing MCF7. CDK4 was inhibited >99%; no other kinases were inhibited. **D,** Immunoblot measurement of CDK4 and CDKN2A in MDA-MB-468 cells transfected with nontargeting (NT) control or *CDKN2A* siRNA. **E,** MS measurement of CDK4 probe labeling in lysates from MDA-MB-468 cells that were transfected with NT control or *CDKN2A* siRNA, normalized to NT.

effect. Indeed, CDK4 probe-labeling was increased roughly threefold upon *CDKN2A* knockdown (Fig. 1E).

The other CDKN2 family members CDKN2B, CDKN2C, and CDKN2D also specifically bind to and inhibit CDK4/6, but display different expression patterns (1). Like *CDKN2A*, each of these *CDKN2* family members strongly inhibited CDK4 probe-labeling when expressed in MCF7 cells (Fig. 2A and B). Interestingly, expression of *CDKN2C* inhibited labeling of the interferon-inducible double-stranded RNA-dependent protein kinase (PKR), and potentially represents a previously undescribed protein–protein interaction between CDKN2C and PKR (Table 1). Further work is warranted to elaborate the nature of this apparent interaction and its impact on PKR catalytic activity.

The CIP/KIP proteins, another class of CDK inhibitor proteins, include CDKN1A (also known as p21/CIP1), CDKN1B (also known as p27/KIP1), and CDKN1C (also known as p57/KIP2). In contrast to CDKN2 proteins, which selectively inhibit CDK4/CDK6, the CIP/KIP proteins bind to and inactivate Cyclin A-, E-CDK2 complexes, and can either activate or inhibit Cyclin D-CDK4/6 complexes (15). CDK4 probe-labeling was not inhibited by expression of any *CDKN1* member (Fig. 2A), although moderate modulation of a few other kinases was observed (Table 1). Inhibition of CDK2 probe labeling upon *CDKN1B* expression is consistent with reported CDKN1B occupancy of the CDK2 catalytic cleft (16). The extent of CDK2 inhibition likely reflects the composite of cyclin levels, posttranslation modifications (e.g., phosphorylation), and other protein–protein interactions (15). That altered CDK2 probe labeling was observed upon CDKN1B expression further demonstrates the capacity for this chemo-proteomics approach to assess the impact of protein–protein interactions on the ATP binding pocket.

CDKN2A–CDK4 interaction determines CDK4 target engagement by small-molecule CDK4/6 inhibitors

To further refine the relationship between CDKN2A status and CDK4 target engagement by small molecule inhibitors, we evaluated target engagement in a small panel of cell lines with genetic backgrounds representing naturally-occurring alterations to the CDK4–CDKN2A interaction (Fig. 3A and B). The reported biological sensitivity (proliferation arrest) of these cell lines to palbociclib is shown in Table 2. The MCF7 cell line, which is biologically sensitive to palbociclib (17, 18), exemplifies an ER^+/$HER2^-$ tumor type that would be a candidate for CDK4/6 inhibitor therapy. In MCF7 lysates, 0.5 μmol/L palbociclib treatment reduced the amount of probe-labeled CDK4 to 18% of corresponding control samples (DMSO-treated MCF7 lysate). The

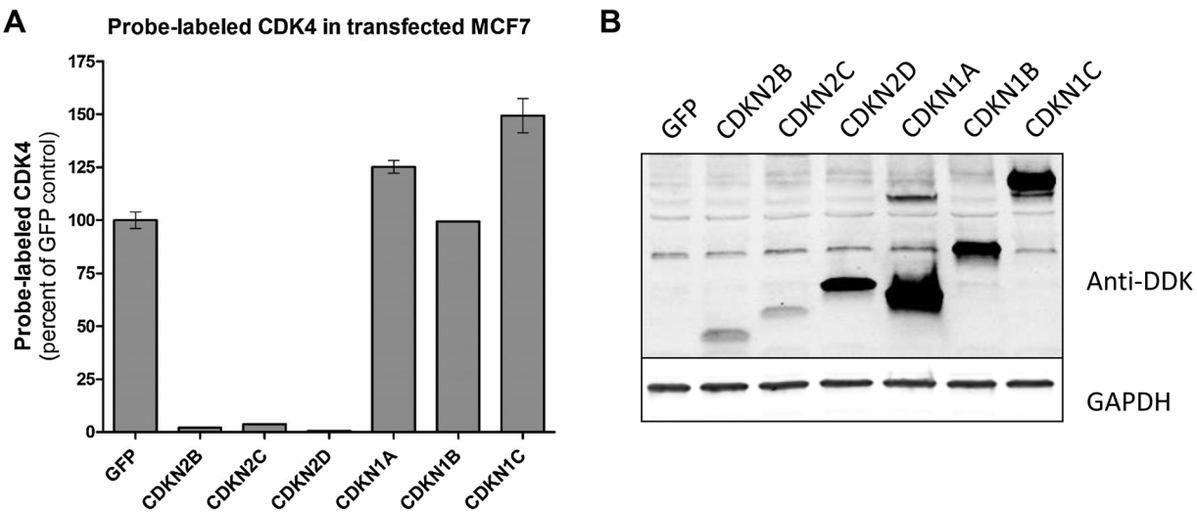

Figure 2.

CDKN2, but not CDKN1, proteins reduce binding of CDK4 to nucleotide probe. **A,** Measurement of CDK4 probe labeling in lysates of MCF7 cells transfected with CDK-inhibitor cDNAs. The data reflect the average of duplicate samples; error bars show the range. **B,** Expression of transfected CDK-inhibitor proteins was confirmed by immunoblot detection of the N-terminal DDK tag.

MDA-MB-231 cell line represents a triple-negative ($ER^-/PR^-/HER2^-$) tumor type that would currently be excluded from CDK4/6 inhibitor treatment, although the cells are in fact biologically sensitive to palbociclib as measured by cell counting (17) or BrdUrd labeling (19). MDA-MB-231 cells are RB-proficient and harbor genomic deletion of *CDKN2A* (14, 20). As expected, based upon cellular response to the drug, CDK4 probe-labeling was effectively reduced to 28% of the DMSO-treated control sample by 0.5 μmol/L palbociclib. In contrast, minimal target engagement was observed in lysates from MDA-MB-468 cells that have high endogenous expression of wild-type *CDKN2A* and are biologically insensitive to palbociclib (7, 18, 21).

Missense mutations in *CDKN2A* that disrupt binding to CDK4/6 have been identified, including at position D84 which is critical for CDK inhibition (22). To examine whether such mutations disrupt the ability of CDKN2A to block CDK4 target engagement, we measured palbociclib-CDK4 target engagement in lysates from the DU-145 cell line, which has similar levels of CDKN2A protein as MDA-MB-468, but carries a D84Y mutation in CDKN2A (23). Probe-labeled CDK4 was effectively reduced, consistent with a requirement for CDKN2A–CDK4 interaction to block target engagement. Thus, absolute levels of CDKN2 proteins alone are insufficient to predict target engagement; mutations in CDKN2 that disrupt the CDK4/6 interaction also modulate inhibitor susceptibility. Notably, despite effective target engagement, DU-145 cells are biologically insensitive to palbociclib due to *RB1* deficiency (21, 24). Resistance mechanisms downstream of CDK4, such as by increased Cyclin E1 activity and CDK2 activation (19, 21, 25–27) have been reported. For example, HCC-1806 cells, which are *RB1* wild type and *CDKN2A*$^-$, are reported to be resistant to palbociclib due to Cyclin E-mediated bypass of CDK4 (19, 21). Consistent with this alternate mechanism of resistance, target engagement was observed in HCC-1806 cells.

The SK-MEL-28 cell line has an R24C mutation in CDK4 that reduces the CDKN2A binding affinity, but does not inherently reduce kinase activity (28). Accordingly, endogenously expressed CDKN2A in SK-MEL-28 cells partially attenuates palbociclib target engagement. CDK4 probe-labeling in SK-MEL-28 lysates was only reduced to 45% of the DMSO-treated control, an intermediate effect significantly different from both the resistant MDA-MB-468 cell line and the remaining cell lines in the panel (MCF-7, MDA-MB-231, DU-145, HCC-1806). The relative sensitivity of each cell line to palbociclib was confirmed with abemaciclib, which demonstrated a similar pattern of CDK4 target engagement (Fig. 3A).

We next compared the palbociclib dose–response for CDK4 target engagement between SK-MEL-28 and MCF-7 ($IC_{50} = 0.49$ μmol/L vs. 0.14 μmol/L; Fig. 3C). The Hill coefficient observed in SK-MEL-28 cells (−0.7) likely reflects negative cooperativity between endogenous CDKN2A and palbociclib resulting from

Table 1. Modulation of kinase labeling by CDKN1 and CDKN2 proteins

			Percent inhibition of probe labeling in MCF7 lysate						
Symbol	**Labeled lysine**	**Region**	**Txn CDKN2A**	**Txn CDKN2B**	**Txn CDKN2C**	**Txn CDKN2D**	**Txn CDKN1A**	**Txn CDKN1B**	**Txn CDKN1C**
CDK4	K142	ATP binding	**98**	**99**	**96**	**99**	−24	5	−47
PKR	K296	ATP binding	18	1	73	39	39	32	22
PKR	K416	ATP binding	13	10	72	38	33	25	19
PKR	K440	Activation Loop	10	1	70	33	32	39	38
CHK2	K349	ATP binding	15	−6	27	19	60	57	60
CDK2	K129	ATP binding	−5	12	7	−5	31	51	6
CDK2	K83	ATP binding	−8	8	6	5	23	51	4

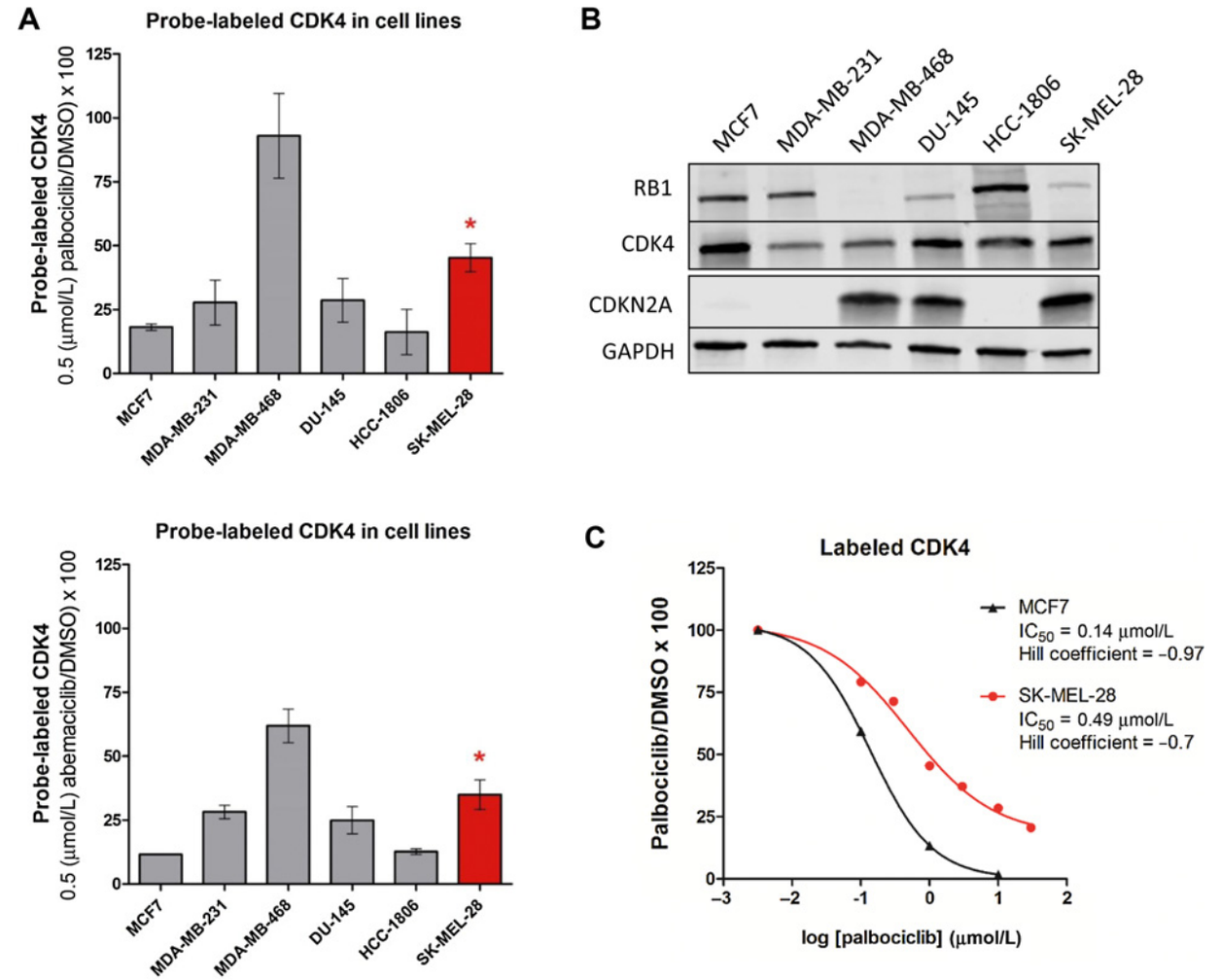

Figure 3.

CDKN2A–CDK4 interaction determines CKD4 target engagement by small molecule CDK4/6 inhibitors. **A,** Inhibition of CDK4 probe labeling by 0.5 μmol/L inhibitor in lysates from a panel of cell lines. The data reflect the mean and SD of six mass-spec samples from two different days (palbociclib) or from four mass-spec samples (abemaciclib), as a percentage of the DMSO-treated controls for each cell line. Asterisk indicates that SK-MEL-28 is significantly different (P <0.05) from every other cell line in panel by one-way ANOVA. **B,** Immunoblots of RB1, CDK4, and CDKN2A. **C,** Dose–response curves of CDK4 target engagement in MCF-7 and SK-MEL-28 lysates.

the R24C mutation. Comparatively, the Hill coefficient of −0.97 in MCF-7 reflects the CDKN2A-independent interaction between palbociclib and CDK4. As described above, *CDKN2A* transfection in MCF-7 cells abrogated probe labeling. Combined, these data demonstrate that nucleotide probe labeling accurately surveys the integrity of the ATP binding pocket in CDK4 and the ability of active site-directed inhibitors to engage this target within a background of protein–protein interactions, genetic mutations, and potentially posttranslational modifications.

Relationship between *CDKN2A* expression and biological sensitivity to CDK4/6 inhibitors

Modulation of CDK4/6 inhibitor target engagement by CDKN2A suggests that CDKN2A would also modulate biological

Table 2. Reported sensitivity of cell lines to growth inhibition by palbociclib

Cell line	Cell-cycle arrest	Palbociclib EC_{50} (μmol/L)	Reference	Mechanism of resistance
MCF7	Sensitive	0.1-0.15	Fin and colleagues, Fry and colleagues	NA
MDA-MB-231	Sensitive	0.432	Fin and colleagues	NA
MDA-MB-468	Resistant	>1, >3, >8	Fin and colleagues, Fry and colleagues, Vijayaraghavan and colleagues	RB deficiency
DU145 (CDKN2A D84Y)	Resistant	>8	Vijayaraghavan and colleagues, Zentella and colleagues	RB deficiency
HCC1806	Resistant	>1, >8	Fin and colleagues, Vijayaraghavan and colleagues	Cyclin E bypass
SK-MEL-28 (CDK4 R24C)	Intermediate	1.8	Mahboub and colleagues	

sensitivity to these drugs. Interrogation of this relationship requires a model with an intact G_1 restriction checkpoint (proficient CDKN2A, CDK4, and RB) that is not bypassed downstream of CDK4 (e.g., by elevated Cyclin E activity), as such bypass would confer independence from CDK4 signaling and preclude measurement of CDK4 inhibition. In addition, cell lines with high levels of wild-type CDKN2A that continue to proliferate in culture are evidently desensitized to its growth inhibitory effects, implicating co-occurrence of either a defect in the CDKN2A–CDK4 interaction, downstream bypass, or *RB1* deficiency. For the above reasons, we selected a cellular model (HAP-1) with low endogenous levels of CDKN2A and obtained an isogenic variant with simultaneous knockout of both *CDKN2A* and *CDKN2B* (double knockout, DKO). CDKN2A expression was validated by immunoblot; CDKN2B was undetectable in either WT or DKO (Fig. 4A). Although both cell lines expressed similar levels of CDK4, probe-labeled CDK4 was increased two-fold in HAP-1 DKO cell lysates compared with HAP-1 WT, suggesting that endogenous CDKN2A masked detection of a pool of CDK4 in HAP-1 WT cells (Fig. 4B). Despite the increase in probe-labeled CDK4 in HAP-1 DKO, the percent of probe-labeled CDK4 remaining after 0.5 μmol/L palbociclib treatment (25% in WT, 26% in DKO) and after 0.5 μmol/L abemaciclib treatment (14% in WT, 10% in DKO) was similar between the two cell lines. Thus, in the HAP-1 cell line, any contribution of CDKN2A to CDK4 target engagement is below the sensitivity of the assay, a result that can be explained by the low levels of CDKN2A expression.

To examine the impact of CDKN2A/B knockout on biological sensitivity to palbociclib, we measured the dose–response of HAP-1 WT and DKO to palbociclib in proliferation assays. We first determined the population doubling times of the WT and DKO cell lines, which were 17 and 19 hours, respectively. That doubling time was not reduced in DKO, but rather showed a slight increase, indicates that CDKN2A/B does not measurably restrict proliferation in HAP-1 cells. Next, we performed proliferation assays over three population doublings with palbociclib treatment. HAP-1 DKO cells were slightly more sensitive to palbociclib than HAP-1 WT (Fig. 4C). Therefore, although probe-labeling indicates that CDKN2A/B inhibits a fraction of CDK4, sufficient free CDK4 remains such that CDKN2A/B removal did not substantially impact the biological sensitivity to palbociclib. In a complementary experiment, we examined the impact *CDKN2A*

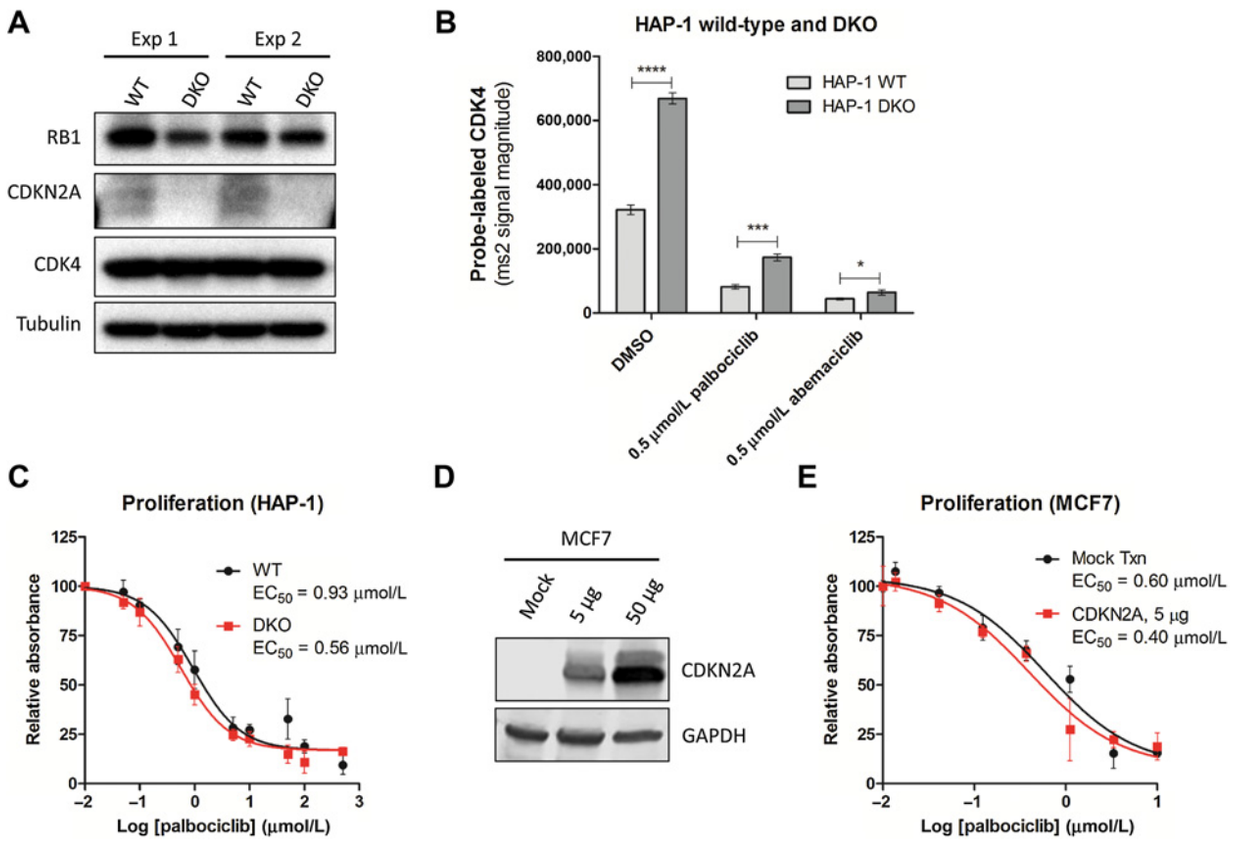

Figure 4.

Effects of CDKN2 knockout on CDK4 probe labeling and biological sensitivity to palbociclib. **A,** Immunoblots of RB1, CDKN2A, CDK4, and Tubulin in HAP-1 WT and HAP-1 DKO, measured from two different lysates. CDKN2B was undetectable. **B,** Measurement of CDK4 probe labeling in lysates from HAP-1 WT and HAP-1 DKO cells treated with 0.5 μmol/L inhibitor. Data reflect the mean and SD from four mass spec samples. Asterisks reflect *P* values of <0.0001 (****), 0.0002 (***), and 0.03 (*) from pairwise *t* tests. **C,** Dose–response curves of HAP-1 WT and HAP-1 DKO proliferation assays measured by crystal violet staining after three population doublings. Data reflect the mean and SEM from three independent experiments. **D,** Immunoblot of CDKN2A and GAPDH from lysates of MCF-7 cells transfected with 5 or 50 μg of a CDKN2A expression plasmid, 5 days, posttransfection. **E,** Proliferation dose–response curves of mock and CDKN2A (5 μg)-transfected MCF7 cells measured by CellTiter-Glo after 5 days of palbociclib treatment. Data reflect the mean and SD.

overexpression on the biological sensitivity of MCF-7 cells to palbociclib. As observed with HAP-1 cells, low levels of CDKN2A did not significantly impact the biological sensitivity of MCF-7 cells to palbociclib (Fig. 4D and E). Transfection with 10-fold more DNA inhibited MCF-7 proliferation thereby precluding measurement of the palbociclib EC_{50}.

Although transient expression of high levels of *CDKN2A* in cells with an intact G_1 restriction checkpoint (MCF-7) induced proliferation arrest, many cell lines exist with naturally-occurring high levels of CDKN2A. As described above, these cell lines have evidently evolved to acquire independence from the G_1 restriction checkpoint. To investigate whether adaptation to high levels of CDKN2A simultaneously confers resistance to CDK4/6 inhibitor drugs, we examined inhibitor sensitivity in a previously reported panel of cell lines with a diversity of naturally-occurring CDKN2A levels (17). The dataset published by Finn and colleagues (2009) includes palbociclib sensitivity and transcriptome profiles for a panel of 33 breast cancer cell lines (17). Grouping the cell lines into two groups based on palbociclib EC_{50} shows that *CDKN2A* levels are significantly higher in the palbociclib-insensitive cell lines (Fig. 5A). Although an inverse correlation between *CDKN2A* expression and palbociclib sensitivity was noted in the original cell line study, *CDKN2A* loss was not predictive of biological sensitivity in the PALOMA-1 trial of patients with advanced ER^+ breast cancer (7). Interestingly, when we excluded ER^- cell lines from the cell line panel (as patients with ER^- tumors were excluded from the clinical trial), every cell line with high *CDKN2A* was also excluded (Fig. 5B) and the remaining ER^+ cell lines presented no correlation between *CDKN2A* expression and palbociclib sensitivity. The comparative palbociclib sensitivity observed between low and negative CDKN2A expression in isogenic cells (HAP-1 and MCF-7, above), therefore, supports a model where high levels of CDKN2A are independently predictive of CDK4/6 inhibitor insensitivity, but normal/low levels are not predictive of sensitivity.

Discussion

CDKN2-mediated reduction of CDK4/6 affinity for ATP was initially reported with the crystal structures of CDK4/6 bound to CDKN2A or CDKN2D (12, 13). Here, we report that the original conclusions based upon purified proteins also apply in the context of a complex, native cellular proteome and that the capacity to influence CDK4/6-nucleotide binding extends to the other CDKN2 family members (CDKN2B and CDKN2C), but not to the CDKN1 family of CDK-inhibitor proteins. We further demonstrate that CDKN2 isoform and levels alter the binding affinity between CDK4/6 and small molecule CDK4/6 inhibitors. This mechanistic insight illuminates how these drugs inhibit the intended targets in a native cellular environment.

CDK4/6 inhibitors are approved for the treatment of advanced/metastatic estrogen receptor (ER)-positive/HER2-negative breast cancer, where ER status serves as a surrogate marker for dependence on CDKN2A–CCND1–CDK4–RB signaling to progress through the cell cycle. Although some patients with RB-proficient/ER^- tumors could potentially benefit from CDK4/6 inhibitor treatment, there are no existing biomarkers to identify such patients. As efforts are underway to extend CDK4/6 inhibitor therapies to ER^- cancer types, it is becoming increasingly clear that assessing dependence on the CDKN2A–CCND1–CDK4–RB signaling axis will likely require a multifactorial evaluation of pathway components and downstream mediators.

The minimal effect of *CDKN2A/B* knockout on the biological response to palbociclib in HAP-1 cells may address an apparent paradox regarding CDKN2A as a clinical biomarker. Although *CDKN2A* expression levels inversely correlated with palbociclib

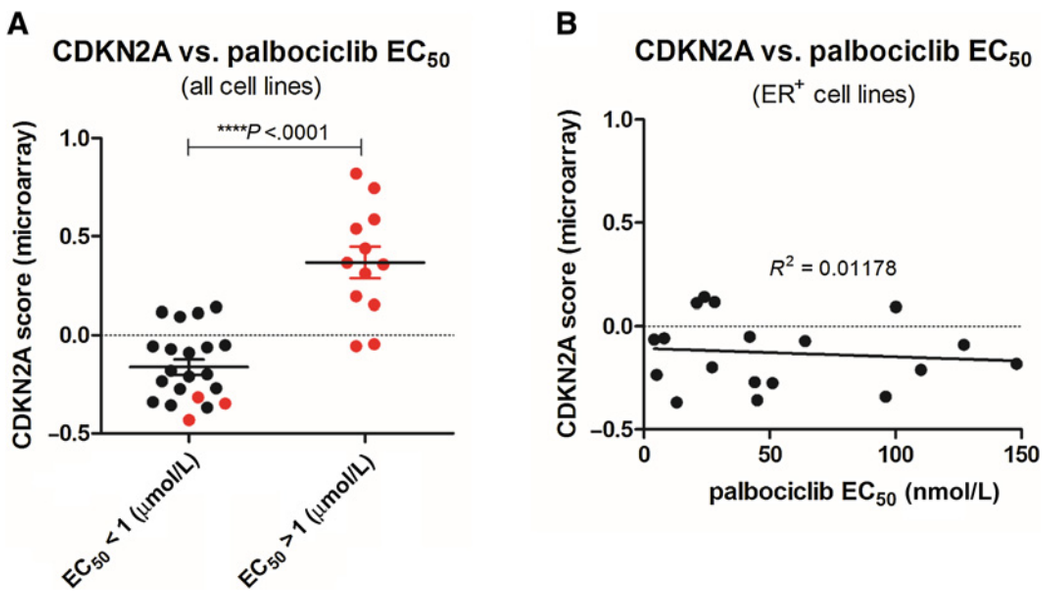

Figure 5.
CDKN2A levels correlate with palbociclib insensitivity in a diverse panel of breast cancer cell lines, but not in the ER^+ subset. Analysis of *CDKN2A* expression (microarray) and palbociclib sensitivity from a panel of 33 breast cancer cell lines published by Finn and colleagues (2009). **A,** *CDKN2A* levels were significantly higher (*t* test) in cell lines insensitive to palbociclib (EC_{50} >1 μmol/L) than in sensitive cell lines. ER^+ cell lines are shown as black circles; ER^- cell lines are shown as red circles. **B,** Palbociclib EC_{50} of ER^+ cell lines is plotted against *CDKN2A* score.

response in a large panel of breast cancer cell lines (17), *CDKN2A* loss did not enrich for responsive patients in the phase II PALOMA-1 clinical trial of palbociclib treatment for advanced ER^+ breast cancer (7). One difference between the cell-based study and the clinical study is that the cell-based study included cell lines with high levels of *CDKN2A*, which is frequently indicative of RB deficiency (29–34). In contrast, the subjects in the clinical study were pre-selected for ER^+ tumors, a population in which RB deficiency is rare (2). Considering the above relationships between ER, RB, and CDKN2A, we speculate that few patients with CDKN2A-high tumors were included in the PALOMA-1 trial. As such, if ER^- cell lines are removed from the cell line panel, reflecting the ER^- patients in the PALOMA-1 trial, the lack of correlation between CDKN2A levels and palbociclib response is consistent between the two studies. Furthermore, as loss of genomic *CDKN2A* was assessed in the PALOMA-1 trial using a FISH assay, patients with elevated *CDKN2A* mRNA or protein would not have been identified (7, 35). Consequently, if *CDKN2A*-high tumors were either not present or not identified in the clinical trial, then a correlation between high CDKN2A levels and palbociclib response could not have been detected. The minimal change in palbociclib sensitivity observed upon *CDKN2A/B* knockout in HAP-1, where endogenous levels were low, supports a hypothesis where only high *CDKN2A* is predictive of palbociclib insensitivity, but normal low levels or loss of *CDKN2A* is not predictive.

It was previously proposed that CDKN2A-high tumors should be insensitive to CDK4/6 inhibitors because (i) CDK4 is presumably already inhibited by CDKN2A and (ii) these tumors are intrinsically resistant to CDK4/6 inhibitors due to *RB1* deficiency or downstream bypass (3, 36, 37). The results presented here bolster the rationale to exclude patients with CDKN2A-high tumors from treatment with CDK4/6 inhibitors by adding: (iii) CDKN2A prevents target engagement by small molecule CDK4/6 inhibitors. Although CDKN2A may not be a critical biomarker for ER^+ breast cancer patients due to the likely exclusion of patients expressing high levels of CDKN2A based on existing selection criteria, our results suggest that high CDKN2A is an independent predictor of palbociclib sensitivity with utility for the selection of patients in cancer types where ER status is not relevant. Although predictions of patient response based solely upon target engagement are confounded by the potential for *RB1* deficiency and/or downstream bypass of CDK4, target engagement is minimally required for response. Therefore, assessing the potential for target engagement may provide an important component of a patient selection strategy. As missense mutations in CDK4 and/or CDKN2A that disrupt CDK4–CDKN2A binding are determined to modulate target engagement, a sequence-based evaluation of these transcripts in addition to measures of protein or transcript abundance would allow for the most accurate predictions of target engagement.

Although not always sufficient, target engagement is an essential component to achieving clinical efficacy for pharmacological agents. As shown here, kinase target engagement becomes dependent on the cellular context when nucleotide binding and affinity for ATP-competitive drugs is modulated by endogenous inhibitors. The CDKN2A example of context-dependent target engagement is likely not unique. Indeed, inhibition of CDK2 probe-labeling by *CDKN1B* expression is another example that agrees well with crystallographic studies regarding these proteins (16). Moreover, inhibition of PKR probe-labeling by *CDKN2C* expression is a potentially novel finding. This work, therefore, provides a foundation for the discovery of protein–protein interactions that alter kinase signaling networks, as well as the potential to screen peptides or allosteric inhibitors that may alter the integrity of the nucleotide binding site. Significantly, the approach described herein could be directly applied to clinical samples. Evaluating the affinity of kinase targets for nucleotide probes and small molecule inhibitors in multiple biologically relevant samples should facilitate predictions of drug response, especially in cases where the compendium of endogenous cellular inhibitors is not fully understood.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. Green, E.S. Okerberg, J. Sejd
Writing, review, and/or revision of the manuscript: J.L. Green, E.S. Okerberg, L. Monserrat, V. Serra, T. Nomanbhoy
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Palafox, L. Monserrat
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