Induction of Therapeutic Senescence in Vemurafenib-Resistant Melanoma by Extended Inhibition of CDK4/6

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Abstract

Dysregulation of the p16–cyclin D1–CDK4/6–Rb pathway occurs frequently in melanoma; however, the therapeutic efficacy of CDK4/6 inhibition remains to be critically evaluated. We demonstrate that CDK4/6 inhibition inhibits melanoma progression through induction of senescence. Palbociclib, a specific CDK4/6 inhibitor, rapidly induces cell cycle arrest within 24 hours and continued exposure for 8 days or longer induces senescence. The induction of senescence correlates with inhibition of mTOR and more specifically mTORC1 signaling. Vemurafenib, a specific BRAFV600E inhibitor, has significant clinical efficacy in BRAFV600E-positive melanomas, but its impact is hampered by a rapid acquisition of resistance. Strikingly, we found that vemurafenib-resistant tumors remain sensitive to palbociclib, suggesting that initial treatment with vemurafenib followed by palbociclib with or without mTOR inhibitors might provide an avenue to overcome recurrence of vemurafenib-resistant metastatic disease. Taken together, these results support palbociclib as a promising therapeutic for treatment of melanoma. Cancer Res; 76(10); 2990–3002. ©2016 AACR.

Introduction

The most frequent mutation that occurs in melanoma occurs in Braf at amino acid 600 (BrafV600E). However BrafV600E alone elicits antitumor effects with BrafV600E melanoma, but not Braf WT tumors. Vemurafenib, an ATP-competitive small-molecule BrafV600E inhibitor, has been approved by the FDA, and has marked antitumor effects with BrafV600E melanoma, but not Braf WT melanoma (14). Initial treatment with vemurafenib results in tumor shrinkage lasting 3 to 6 months in clinical trials; unfortunately, however, tumors relapse due to the acquired resistance to vemurafenib and overall survival is only extended several months (15). Current efforts are focused on the development of second-line therapies that have significant clinical impact on the resistant tumors.

Senescence, initially identified by Hayflick and Moorhead, is induced by a number of biologic stresses such as DNA damage accumulation, acute oncogene activation, reactive oxygen species (ROS), and expression of INK4A/ARF locus in over passages primary cells in a p53-dependent or independent manner (16). Critically, senescence is defined as a permanent cell-cycle arrest and considered as one of the defense mechanisms against cancers (17).

PD0332991, commonly referred to as palbociclib, is an FDA-approved orally available, highly selective ATP-competitive inhibitor of CDK4 (IC50: 0.011 μmol/L) and CDK6 (IC50: 0.016 μmol/L; ref. 18) with clinical evidence of growth inhibitory activity. Palbociclib is being tested in more than 17 clinical trials for malignancies such as mantle cell lymphoma, breast cancer, and colorectal cancer (18–21). However, the utility of the drug has not yet been evaluated in melanoma, a cancer that when metastatic remains largely intractable. We have investigated the efficacy of CDK4/6 inhibition in vemurafenib-resistant melanoma and have begun to dissect the molecular mechanism of acquired resistance to palbociclib.

Materials and Methods

Cell culture and viral infection

Authenticated human melanoma cell lines (1205Lu, WM983B, WM983BR, WM451Lu, WM239A, WM3918) were obtained from M.409 (HEC) 293T cells were purchased from ATCC in 2014 and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. For synchronization in G0–G1, G1–S, and G2–M phase, cells were incubated in medium...
containing 0.5% FBS for 48 hours at high density on the plates (contact inhibition) for G0-containing 0.5% FBS for 48 hours at high density on the plates. For viral production, viral expression plasmids were transfected into 293T cells with lipofectamine together with packaging plasmid. Virus supernatants harvested 48 to 72 hours after transfection were used to infect melanoma cells with polybrene (10 μg/mL).

**Flow cytometric analysis**

Cells were trypsinized and stained with 10 μg/mL propidium iodide (PI) containing 100 μg/mL RNase A and 0.5% FBS followed by fixation with 70% ethanol. For the bromodeoxyuridine (BrdUrd) incorporation, cells were incubated in medium containing 10 μmol/L BrdUrd for 45 minutes before harvest and then fixed with 70% ethanol, treated with 1.5 N HCl, stained with anti-BrdUrd mouse mAb, and subsequently FITC-linked mouse IgG staining. Cytotoxicity for palbociclib was assayed by Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions. For ELISA, melanoma cells were cultured with or without palbociclib. SASP expression such as IL6, IL8 (D80000C), CXCL1/GROα (DGR00) in supernatants were analyzed with ELISA Kits (R&D Systems) according to the manufacturer's protocol.

**Western blot analysis**

Cells were lysed in EBC buffer (50 mmol/L Tris, pH 8.0, 120 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40) containing 1 μmol/L phenylmethylsulfonyl fluoride, 20 U/mL aprotinin, 1 μmol/L leupeptin, 1 μmol/L dithiothreitol, 0.1 mmol/L NaF, 0.1 mmol/L sodium orthovanadate, and 10 mmol/L β-glycerophosphate. Proteins were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted with appropriate antibodies. pRb (S780), p15 Ink4a (M-167), p62 (D-3), and MDM2 (C-18) were obtained from Santa Cruz Biotechnology. p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-mTOR (Ser2448), mTOR, p-Akt (S473), pan-Akt (11E7), p-GSK3β (Ser9), p-S6K (Thr389), S6K (49D7), and p-4E-BP1 (Thr37/46) from Cell Signaling Technology. Raptor and Rictor were purchased from Bethyl laboratories. Cyclin D1 (Ab-3) was obtained from BD Pharmingen. Signals were visualized using ECL (PerkinElmer).

**qRT-PCR analysis**

Total RNA was extracted using the RNeasy Micro Kit (Qiagen), and reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. qRT-PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and the data were normalized by GAPDH.

**IHC and immunohistoﬂuorescence**

Tissues were fixed in 4% paraformaldehyde, deparaffinized, and rehydrated in gradient ethanol. Sections were blocked with 10% goat serum and incubated with the primary antibodies against the following: p-S6 (S235/236), p-Akt (S473), Ki-67, and pRb (S780). For IHC, sections were incubated with biotinylated rabbit antibodies and developed with ABC substrate kit (Vector Laboratories) followed by DAB reaction (Vector Laboratories), and counterstained with hematoxylin (Thermo Scientific). For immunofluorescence, sections were incubated with Alex Fluor 488 or Alexa Fluor 546–conjugated goat anti-mouse/rabbit antibodies (Life Technologies), and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen).

**Senescence assays**

Cells, 2.5×10⁵/60-mm plate, were treated with 1 μmol/L palbociclib with fresh palbociclib added daily for 8 days. Cells were cultured for an additional 14 days following removal of drug, in complete growth media and colonies were visualized by Giemsa stain. For senescence-associated β-galactosidase activity (SA-βGal), cultured melanoma cells or frozen tissue samples in Tissue-Tek OCT compounds (Sakura Finetek) were fixed and incubated with X-gal staining solution (Sigma) according to the manufacturer's instructions. For ELISA, melanoma cells were cultured with or without palbociclib. SASP expression such as IL6, IL8 (D80000C), CXCL1/GROα (DGR00) in supernatants were analyzed with ELISA Kits (R&D Systems) according to the manufacturer's protocol.

**Xenografts**

A total of 2×10⁶ melanoma cells were subcutaneously injected into 6-week-old SCID mice with Matrigel. Mice were treated with DMSO (control) or 90 mg/kg of palbociclib every day when tumors developed 2 mm after injection. Tumor volumes were measured by caliper every 2 or 3 days and calculated by the following formula V = (Length x width x height)/2. Mice were sacrificed and the tumor size was measured when tumors reached 10 mm. Care of experimental animals was done in accordance with the institutional guidelines.

**Results**

**CDK4/6 inhibition induces senescence in melanoma-derived cells**

The dysregulation of p16–CDK4–cyclin D1–Rb pathway in a majority of melanomas suggests that small-molecule inhibition of the cyclin D1/CDK4 kinase might be an effective therapeutic strategy for limiting melanoma progression. To test this, we treated 1205Lu melanoma cells with palbociclib, a specific CDK4/6 inhibitor (18). Palbociclib treatment triggered G1–S arrest that was associated with dephosphorylation of Rb at serine-780 (S780), with no increase in p19INK4d or p21 noted (Fig. 1A and B). Palbociclib-dependent reduction in Rb levels has been observed previously and while it has been associated with reduced stability, the mechanism remains unclear (23). qPCR analysis revealed an approximate 30% to 40% reduction in Rb expression (Fig. 1C). Rb expression could be maintained by proteasome inhibitor treatment revealing coordinate regulation of both expression and protein stability (Fig. 1D). Although CDK4/6 inhibition is widely known to trigger reversible quiescence, prolonged inhibition of cyclin D1-CDK4/6 has also been associated with senescence or permanent growth arrest (19, 24, 25). To address the acquisition of senescence, 1205Lu cells were exposed to palbociclib for varying intervals and senescence was addressed through several distinct criteria: (i) SA-β-gal; (ii) senescence-associated secretory phenotype (SASP) such as IL6, IL8, and CXCL1; (iii) senescence-associated heterochromatic foci (SAHF) formation; and (iv) ATR-X foci formation (23). An increase in all senescence-associated marks was noted at day 4 with maximal induction noted by day 8 by all criteria (Fig. 1E–H).
Figure 1.
Palbociclib treatment induces senescence in melanoma-derived cells. A, FACS analysis of 1205Lu cells treated with palbociclib. B, Western blot analysis of the samples from A. *, nonspecific band. C, qPCR analysis of samples from A. D, Western blot analysis of 1205Lu cells with or without MG132 following palbociclib exposure for 8 days. E, SA-β-gal staining of SAHF (left) and ATRX staining (right) in 1205Lu cells. F, quantification of SA-β-gal-positive WM451Lu, WM983B, WM239A, WM3918, and 1205Lu cells following treatment with or without palbociclib for 8 days (*, P < 0.001).

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Because senescence is defined as permanent or irreversible cell-cycle arrest, whereas quiescence is a reversible cell-cycle exit, we examined reversibility of palbociclib-induced senescence using a clonogenic cell survival assay. Consistent with senescence, 8 days of palbociclib reduced colony formation by 80% (Fig. 1I). We exposed several additional melanoma cell lines to extended palbociclib treatment and observed markers of senescence including SA-β-gal positivity (Fig. 1J).

To independently evaluate the impact of CDK4/6 inhibition, we utilized an INK4 family protein, p19INK4d that binds and specifically inhibits CDK4/6 in Rb-positive cells (26, 27). We utilized two distinct melanoma cell lines. 1205Lu, which in addition to harboring a BRAFV600E mutation, also has a CDK4-R24C mutation; the R24C mutation falls within the INK4-binding interface making this CDK4 allele resistant to INK4-mediated inhibition (28, 29). We also used 983B cells that express wild-type CDK4 and CDK6, as well as BRAFV600E. Expression of p19INK4d induced G0 arrest and Rb dephosphorylation in 983B within the first 36 hours (Fig. 2A and B), while 1205Lu cells were refractory. Consistent with persistent CDK4/6 inhibition inducing a switch from quiescence (G0) to senescence, we noted a striking increase in SA-β-gal positivity 8 days after infection of 983B cells (Fig 2C). To assess whether senescence depends on p53–p21 signaling, we expressed the HPV E6 to inactivate p53 (Fig. 2D). Palbociclib treatment triggered G0-G1 arrest at 1 day and senescence by 8 days in cells expressing E6 (Fig. 2E and F). This is consistent with the observation that p21 is not induced by palbociclib exposure (Fig. 1B).

**Palbociclib-induced senescence abrogates vemurafenib-dependent cytotoxicity**

To assess the effect of combining vemurafenib with palbociclib, 1205Lu cells were treated with vemurafenib, palbociclib, or both. Vemurafenib treatment resulted in 80% of cells undergoing apoptosis, whereas palbociclib treatment promoted only cell-cycle arrest (Fig 3A). Strikingly, palbociclib substantially inhibited
Palbociclib induces senescence in vemurafenib-resistant melanoma

983BR is a vemurafenib-resistant cell line derived from 983B cultured under conditions of increasing concentrations of vemurafenib to acquire the resistance (Supplementary Fig. S1). Palbociclib induced G0 arrest in both parental 983B (Fig. 1I) and 983BR cells (Fig. 4A), with markedly reduced BrdUrd incorporation (Fig. 4B). Arrest was associated with dephosphorylation of Rb, with no increase in CDK inhibitors such as p19INK4d and p21 (Figs. 1B and 4C). Eight days of palbociclib or p19INK4d introduction significantly reduced sensitivity to vemurafenib-dependent killing (Fig. 3B), suggesting acquisition of senescence-conferred resistance. To further test this hypothesis, 1205Lu cells were arrested by serum starvation/contact inhibition [G0/quiescence, (Fig. 3E)], hydroxyurea (G1–S) or nocodazole (G2–M) and then exposed to vemurafenib for 48 hours. Vemurafenib efficiently triggered apoptosis under all conditions (Fig. 3D) demonstrating that cell-cycle arrest per se did not prevent vemurafenib-dependent cytotoxicity. Immunoblot analysis revealed that palbociclib rapidly reduced Rb phosphorylation; strikingly, vemurafenib treatment was associated with reduced Erk activation only in cells exposed to palbociclib for 2 days (Fig. 3C). Palbociclib treatment for 3 or more days reduced sensitivity to vemurafenib (Fig. 3C).

To evaluate Rb function in palbociclib-induced senescence, E7 was introduced into 1205Lu cells. E7 expression inhibited palbociclib-induced senescence (Fig. 3F–H). E7 also resensitized cells to vemurafenib-dependent cytotoxicity (Fig. 3F). As E7 interacts with p107 and p130, we utilized two distinct shRNAs to knockdown Rb. Rb knockdown cells were refractory to palbociclib-induced senescence (Fig. 3I–K), and were resensitized to vemurafenib-induced cell death (Fig. 3F), consistent with CDK4/6-dependent senescence being both Rb-dependent and antagonizing vemurafenib-dependent cell death (30).

Palbociclib induces senescence in vivo

To address the ability of palbociclib to inhibit melanoma progression in vivo, 983B or 983BR cells were injected into SCID mice. Palbociclib efficiently inhibited growth of 983B and 983BR cells (Fig. 5A–C). Palbociclib reduced Ki-67, Rb phosphorylation (Fig. 5D), and residual exhibited strong SA-β-gal positivity (Fig. 5E). Analysis of palbociclib action in vivo suggested that 8 days of exposure was sufficient to induce senescence. To determine whether 8 days were sufficient for senescence in vivo, we conducted a second trial wherein tumor-burdened mice were administered palbociclib daily for 3 or 8 days. Tumors in the 3-day treatment group developed as fast as controls, whereas mice treated for 8 days exhibited stable disease (Fig. 5F). At later time points, some tumors escaped and reinitiated growth. Cell lines derived from these tumors were found to be resistant to palbociclib, suggesting acquired resistance (see Fig. 6).

Inhibition of mTOR signaling pathway is critical for palbociclib-induced senescence in melanoma

Recent work provided evidence that activation of mTOR signaling can bypass senescence in mouse models of melanoma (31). Highlighting a role for mTOR, decreased mTOR phosphorylation occurred by 8 days of palbociclib administration (Fig. 6A). Decreased phosphorylation of Akt, GSK3β, S6K, and 4E-BP1, downstream of targets of mTOR, was observed demonstrating mTOR inhibition (Fig. 6A). In contrast, rapamycin reduced mTORC1 signaling within the first 24 hours. Palbociclib with rapamycin accelerated downregulation of mTOR signaling pathway. On the basis of these data, we considered whether inhibition of mTOR signaling was critical for the transition of senescence. We first assessed whether palbociclib treatment also correlated with mTOR inhibition in vivo. Consistently, IHC of palbociclib-treated tumors revealed reduced p-S6K and p-Akt (S473; Fig. 6B). The downregulation of mTOR signaling is associated with reduced protein stability of mTOR, Raptor, and Rictor (Fig. 6C) following palbociclib treatment as MG132, 26S rescued expression levels while their mRNA levels were unchanged (Supplementary Fig. S2A).

We subsequently evaluated cell-cycle progression following single versus simultaneous exposure for 8 days. Both rapamycin and palbociclib induced G0 arrest with palbociclib exhibiting better potency (Fig. 6D). Combining rapamycin with palbociclib induced G0 arrest in greater than 95% of cells, with no discernable apoptosis; Rb knockdown inhibited palbociclib-induced senescence and resensitized the cells to vemurafenib-dependent cytotoxicity demonstrating Rb dependence (Figs. 3F and 6D). Importantly, clonogenic assays revealed that the combination of palbociclib plus rapamycin synergistically inhibited colony formation relative to either compound alone (Fig. 6E), with 3 days of the combination being as effective as palbociclib only treatment for 8 days in vemurafenib-resistant melanoma cells (Supplementary Fig. S2B), indicating that mTOR signaling inhibition cooperates with palbociclib to induce senescence on melanoma.
Palbociclib efficacy in vemurafenib-resistant melanoma cells. A, FACS analysis of 983BR vemurafenib-resistant melanoma cells treated with palbociclib for 8 days. B, 983BR cells were subjected to BrdUrd incorporation for 45 minutes after palbociclib treatment for 8 days. BrdUrd and DNA content were determined by anti-BrdUrd (y-axis) and PI (x-axis), respectively, and analyzed by FACS. C, Western blot analysis of 983BR cells treated with palbociclib for 1, 2, 3, 4, and 8 days. D, SA-β-gal staining of 983BR cells treated with or without palbociclib for 8 days. E, SA-β-gal staining of 983BR cells infected with retrovirus encoding GFP or p19INK4d. F, clonogenic assay of 983BR cells treated with or without palbociclib for 8 days. G, SA-β-gal staining of 983B (vemurafenib sensitive) cells and 983BR (vemurafenib resistant) cells following palbociclib treatment at different concentrations (0.01, 0.03, 0.1, 0.3, and 1 μmol/L) for 8 days. H, quantification of SA-β-gal–positive cells from G. I, 983B cells and 983BR cells were subjected to BrdUrd incorporation for 45 minutes after palbociclib treatment (0.01, 0.03, 0.1, 0.3, and 1 μmol/L) for 24 hours. BrdUrd and DNA content determined by FACS. J, quantification of BrdUrd-positive cells from I.
Between 10% and 20% of cells treated with palbociclib escape senescence, suggesting either intrinsic or acquired resistance to palbociclib-induced senescence. We therefore conducted additional experiments to address the potential contribution of mTOR signaling to resistance to palbociclib-induced senescence. We isolated palbociclib-resistant melanoma cells by long-term culture in increasing concentrations of drug (PR1-3) or by isolation of single colonies that grew out in cell survival assays following exposure to palbociclib (PR6-7); their resistance to palbociclib-induced cell-cycle arrest was confirmed by flow cytometric analysis (FACS; Supplementary Fig. S2C). We subsequently examined mTOR signaling in palbociclib-resistant clones. Intriguingly, mTOR inhibitors rescued palbociclib-resistant melanoma cells from senescence in vitro (Fig. 5B, C), consistent with previous reports of CDK4 and CDK6 inhibition promoting senescence in other contexts (15).

Figure 5. Palbociclib induces senescence in melanoma in vivo. A total of $2 \times 10^5$ of 983B (vemurafenib sensitive) cells and 983BR (vemurafenib resistant) cells were subcutaneously injected into 6-week-old SCID mice. Mice were treated with palbociclib every day after 3 days after injection for 14 days. A, tumor volume (*, P < 0.001). B, tumor weight (*, P < 0.001). C, representative images of the tumors from B. D, immunofluorescence using sections from tumors treated with either vehicle or palbociclib. E, SA-β-gal staining of SubQ-injected 983B and 983BR cells treated with either vehicle or palbociclib. F, $2 \times 10^5$ of 983BR (vemurafenib resistant) cells were subcutaneously injected into 6-week-old SCID mice. Treatments were initiated when tumors reached $2 \text{mm}^3$ for either 3 or 8 days as indicated with arrows. Tumor volumes were assessed every 2 to 3 days (*, P < 0.001).
Figure 6.
Inhibition of mTOR signaling pathway is critical for palbociclib-induced senescence in melanoma. A, Western blot analysis of 1205Lu cells treated with rapamycin or palbociclib or both for 1, 4, or 8 days. B, IHC of tumors derived from C6 palbociclib. C, Western blot analysis of 1205Lu cells infected with GFP (top) or ShRb1 (middle and bottom) treated with either rapamycin or palbociclib, or both followed by vemurafenib + palbociclib for 2 days (bottom). E, clonogenic assay of 1205Lu cells treated with rapamycin, palbociclib, or rapamycin + palbociclib for 3 days (top) or 8 days (bottom). F, immunoblot analysis of palbociclib-resistant 1205Lu cells.
mTOR signaling pathway was completely recovered and refractory to palbociclib treatment in the palbociclib-resistant cells (PR1, 2, 3, 6, and 7) compared with untreated cells or palbociclib-sensitive cells treated with palbociclib for 8 days (Fig. 6F). We note specifically that Raptor and Rictor protein levels are lost in naïve cells, but recovered in resistant clones; however, their mRNA levels remain stable in the palbociclib-resistant cells (PR1, 2, 3, 6, and 7; Supplementary Fig. S2A). Collectively, our data suggest that inhibition of mTOR signaling following long-term CDK4/6 inhibition contributes to senescence and that acquisition of palbociclib resistance results in re-activation of mTORC1 signaling via induction of Raptor.

Overexpression of mTOR signaling overrides palbociclib-induced senescence in melanoma

mTOR is a serine/threonine kinase that serves as the common catalytic component of two distinct complexes commonly referred to as mTORC1 and mTORC2 (32–34). Although these complexes share a number of components, they are distinguished by certain unique components including, Raptor for mTORC1 complex and Rictor for mTORC2 complex. Although mTORC1 complex is a master regulator of cell proliferation and metabolism through phosphorylation of S6K and 4EBP1, mTORC2 has a more direct impact on cell proliferation, survival, and metabolism through the regulation of AKT (34). Because palbociclib treatment induces Raptor and Rictor loss (and resistant cell reacquire their expression), we chose to interrogate the contribution of mTORC1 and mTORC2 signaling pathways in context of palbociclib-induced senescence in melanoma. Raptor or Rictor were knocked down in 1205Lu melanoma cells with validated shRNAs (Fig. 7B; ref. 35). Raptor knockdown was associated with cell-cycle arrest, whereas Rictor knockdown had little impact (Fig. 7A; top). Concurrent palbociclib treatment with Raptor knockdown for 24 hours induced complete G0 arrest. Extended palbociclib treatment of Raptor knockdown cells for 8 days eliminated colony outgrowth compared with palbociclib alone where approximately 20% of cells exhibited resistance to senescence and the combination of palbociclib and shRictor was similar to palbociclib alone (Fig. 7A, bottom). Similar results were obtained in 983BR (vemurafenib resistant) cells as well as 1205Lu cells, suggesting that mTORC1 and mTORC2 complexes, especially...
mTORC1 complex, are critical for cell viability and proliferation in melanoma cells, and contribute to palbociclib-induced senescence.

We next determined whether we could bypass palbociclib-dependent senescence by driving mTORC1 with Raptor overexpression or as a control myristoylated Akt (Myr-Akt; Fig. 7D). Palbociclib treatment for 1 day induced C5 arrest in control cells. Cells overexpressing Raptor were refractory to palbociclib, whereas Myr-Akt overexpression had no impact (Fig. 7C). In addition, clonogenic assays revealed that Raptor expression inhibited palbociclib-induced senescence; in contrast, overexpression of Akt or S6K had little impact on senescence (Fig. 7E). Similar results were obtained in 983BR vemurafenib-resistant cells (Supplementary Fig. S3). These results indicate that inhibition of mTORC1 is essential for palbociclib-induced senescence.

Discussion

Multiple genetic alterations are reported to directly contribute to the genesis of metastatic melanomas. Among these, BRAFV600E mutation is the most frequently mutated gene in melanoma where the BRAFVE600 mutation is observed in approximately 66% of melanoma (36). Vemurafenib is a specific BRAFV600 inhibitor (14); it has significant clinical impact on BRAFVE600-positive tumors, but has limited long-term potential due to the rapid acquisition of resistance. Thus, the development of secondary treatment strategies for the treatment of vemurafenib-resistant melanoma is of unquestionable importance. On the basis of the results described here, we propose the use of vemurafenib to reduce tumor volume/bulk followed by treatment with a CDK4/6 inhibitor to induce senescence in the residual tumor cells that are resistant to vemurafenib.

The CDK4–Rb pathway is disrupted in a majority of melanomas and mechanisms include cyclin D1 amplification (37), activating mutations in CDK4 (29), inactivation of Fbxo4, an E3 ligase that regulates degradation of cyclin D1, and thereby functions as a tumor suppressor (13), and deletion of gene encoding p16Ink4a (8). On the basis of these and additional observations, the cyclin D1–CDK4/6–Rb axis is considered a major driver of melanomagenesis. Importantly, the observation that Rb, a direct substrate for the cyclin D1/CDK4/6 kinase, is generally wild-type in melanoma (>95%) suggests that mTORC1 function will be required for ongoing melanoma cell proliferation and as such inhibition of CDK4/6 should result in Rb-dependent cell-cycle arrest. Surprisingly, the therapeutic efficacy of the CDK4/6 inhibition in melanoma remains to be examined. Our work demonstrates that melanoma-derived cells are indeed responsive to CDK4/6 inhibition. Treatment of cells with either palbociclib or enforced expression of an Ink4 family member induces rapid Rb-dependent G0 arrest. Prolonged CDK4/6 inhibition induced a switch from quiescence to senescence, "geronconversion" (Fig. 1). Geronconversion was noted to be time dependent, was maximal at 8 days, and exhibited key characteristics of senescence, of which "permanent growth arrest" is the most clinically relevant. In a preclinical model, we noted that palbociclib treatment for 8 days was as effective as continuous exposure with regard to tumor progression. Although the potential translation of this concept to the clinic remains to be carefully evaluated, xenograft tumors were characterized by reduced phospho-Rb, reduced proliferation, and stained positivity for SA-β-Gal consistent with on-target effects of palbociclib. The robust impact of 8 days of palbociclib treatment could have important clinical implications. Clinically, reduced time of treatment will reduce side toxicities associated with CDK inhibition as well as reduce patient cost. More importantly, given the prevalence of acquired resistance to precision medicine, perhaps reduced time of patient exposure could also reduce acquisition of resistance.

Although palbociclib recently received FDA approval (38), there is little data on tumor-intrinsic or acquired resistance. However, intrinsic resistance could be predicted based upon Rb status (39). Importantly, Rb is rarely lost in melanoma. We did note that inactivation of Rb through knockdown or expression of HPV-E7 in melanoma cells, (vemurafenib sensitive or resistant), confers complete resistance to either palbociclib treatment or mTOR expression. With regard to intrinsic and acquired resistance, resistance of a small number of clones that grew out during clonogenic cell survival assays was observed. Consistently, we also noted mice treated with palbociclib eventually succumbed to melanoma several weeks after drug removal (data not shown). Cultured cells from either source revealed resistance to palbociclib-induced growth arrest and senescence. Whether the resistance of cell lines is "acquired" or represents an intrinsic property of a certain population of cells remains to be established. We do note that resistant cell lines are both stably resistant and 100% of cells are resistant; thus, they do not appear to represent outgrowth from a small fraction of stem/repopulating cells that give rise once again to 80% sensitive and 20% resistant. With regard to the mechanism of resistance, two events are worth noting. First, Rb loss was observed in some resistant cell lines (PR1, 2, and 3; Supplementary Fig. S4A) generated in culture, while Raptor overexpression and constitutive mTOR signaling were observed in all resistant cell lines (Fig. 6F), suggesting that mTOR signaling directly contributes to resistance. In addition, Rb suppression is not stable (Supplementary Fig. S4B), whereas resistance to palbociclib and Raptor overexpression are both stably maintained in palbociclib-resistant cells supporting a hypothesis where reduced mTOR/mTORC1 signaling with palbociclib is necessary for senescence.

Whereas the molecular mechanism, whereby inhibition of CDK4/6 impacts Raptor and mTORC1 signaling, remains to be further investigated, we observed that concurrent treatment of melanoma cells with palbociclib and rapamycin (or Raptor knockdown) was much more effective in triggering permanent growth arrest. Raptor was also strongly induced in palbociclib-resistant clones suggesting that mTOR inhibition is critical for senescence induction with palbociclib. Finally, concurrent palbociclib + rapamycin increased melanoma sensitivity to palbociclib and coordinated CDK4/6 and mTOR inhibition both increased palbociclib-resistant senescence in 983BR cells (Supplementary Fig. S2B), suggesting that perhaps reducing the acquisition of cellular resistance. Interestingly, rapamycin was suggested to suppress palbociclib-induced senescence in ME110 cells (40). The basis of this result is unclear as all melanoma-derived cell lines tested herein exhibited increased sensitivity. Importantly, there is likely to be tissue specificity that will determine the impact of combination therapy. Indeed, we noted that rapamycin exposure opposed palbociclib-induced senescence in esophageal cancer cell lines (Supplementary Fig. S5). Collectively, these data reveal the clinically relevant concept that concomitant treatment of palbociclib and rapamycin has a durable effect on senescence and inhibits acquired resistance to palbociclib in melanoma.

CDK4/6 inhibition is showing significant therapeutic promise in ER(+) / HER2(−) advanced breast cancer (41); we suggest it should also have significant clinical value in vemurafenib-
resistant metastatic melanoma. The ability to induce permanent growth arrest would reduce time of patient exposure to drug, which may in turn reduce side toxicities and potentially acquired resistance. The development of a treatment strategy, however, requires detailed molecular understanding of how CDK4/6 inhibition generates a senescent phenotype and in turn when resistance emerges, the precise underlying mechanism.

In this regard, one might first consider known substrates for the CDK4/6 kinase, which include Rb, p107, p130, FOXM1 (19), PRMT5 (42), and GCN5 (43) and how any of these intersect with mTOR signaling, given that mTORC1 suppression is necessary for senescence. How and whether any of these molecules contribute mTOR/mTORC1 signaling/inhibition following CDK4/6 inhibition remains to be established. Importantly, our data support a model where CDK4/6 inhibition triggers reduced expression of mTOR and the mTORC1-specific protein Raptor at the posttranslational level.

A second clinically important consideration in developing a treatment strategy for the use of palbociclib emerges from the data on the concurrent use of vemurafenib and palbociclib treatment. Surprisingly, concurrent treatment is not synergistic; in contrast, palbociclib inhibits the cytotoxicity of vemurafenib. Such an impact in vivo would prevent the efficacious debulking impact of vemurafenib treatment. At a mechanistic level, this is reflected in a failure of vemurafenib to inhibit ERK/MEK activation. This result is not a simple matter of cell-cycle arrest, as vemurafenib was effective in cells arrested via growth factor restriction (G1), nocodazole (G2-M), and hydroxyurea (G1-S). Rather, resistance to vemurafenib correlates directly with geroncconversion. From a clinical point of view, our data support the concept of palbociclib as a second-line therapy that could limit melanoma patient relapse following tumor debulking with a BRAF inhibitor such as vemurafenib (Supplementary Fig. S6). Finally, concurrent use of palbociclib with an mTOR inhibitor may have increased efficacy in the induction of bona fide senescence and also limit the acquisition of palbociclib resistance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: A. Yoshida, E.K. Lee, J.A. Diehl
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