Dual Inhibition of CDK4 and CDK2 via Targeting p27 Tyrosine Phosphorylation Induces a Potent and Durable Response in Breast Cancer Cells

Priyank Patel¹, Vladislav Tsiperson², Susan R.S. Gottesman³, Jonathan Somma³, and Stacy W. Blain²

Abstract

Cyclin-dependent kinase 4/6 (CDK4/6)-specific inhibitors, such as palbociclib, have shown clinical efficacy, but primary or secondary resistance has emerged as a problem. To develop more effective therapeutic approaches, investigation is needed into the mechanisms of resistance or adaption. Here, it is demonstrated that CDK2 compensates for loss of CDK4 activity to rescue palbociclib-arrested breast cancer cells, suggesting that inhibition of both kinases is required to achieve durable response. In addition, a novel strategy is described to inhibit tyrosine phosphorylation of p27Kip1 (CDKN1B) and simultaneously inhibit both CDK2 and CDK4. p27Kip1 is a required assembly factor for cyclin–CDK4 complexes, but it must be phosphorylated on residue Y88 to open or activate the complex. The Brk-SH3 peptide, ALT, blocks p27 Y88 phosphorylation, inhibiting CDK4. Nonphosphorylated p27 is no longer a target for ubiquitin-mediated degradation and this stabilized p27 now also inhibits CDK2 activity. Thus, ALT induction inhibits both the kinase that drives proliferation (CDK4) and the kinase that mediates resistance (CDK2), causing a potent and long-lasting cell-cycle arrest. ALT arrests growth of all breast cancer subgroups and synergizes with palbociclib to increase cellular senescence and to cause tumor regression in breast cancer xenograft models. The use of ALT demonstrates that both CDK4 and CDK2 need to be inhibited if long-term efficacy is to be achieved and represents a novel modality to inhibit breast cancer cells.

Implications: Modulating tyrosine phosphorylation of p27 impacts both proliferative (CDK4) and resistance (CDK2) mechanisms in breast cancer and suggests that phospho-p27 status may serve as a biomarker for patients that are responsive to CDK4/6 inhibition. Mol Cancer Res; 16(3); 361–77. ©2018 AACR.

Introduction

The G₁–S phase cell-cycle transition is governed by two cyclin–cdk complexes, cyclin D–cdk4/6 and cyclin E–cdk2. Cyclin D–cdk4 (hereafter D-K4) phosphorylates the G₁ gatekeeper Rb, causing the release of S-phase–specific transcription factors, such as E2F. E2F causes the transcriptional induction of Cyclin E, which in turn partners with cdk2 to further phosphorylate Rb and irreversibly cause the transition into S-phase (1). Cyclin D1 and cdk4 are overexpressed in a variety of durable cancers, and, in mouse models, loss of either prevents the development of certain oncogene-driven tumors (2). Targeting cdk4 activity has been a long-standing goal in the oncology field and, because D-K4 is downstream of most oncogenic signaling pathways, targeting this kinase might prevent the resistance that frequently occurs when cell surface or upstream signal transducers are inhibited. The advent of cdk4-specific inhibitors (cdk4i), such as palbociclib (PD 0332991, hereafter PD), Abemaciclib, or ribociclib, has demonstrated that cdk4 is a promising target (3). In combination with letrozole, PD extended median progression-free survival (PFS) for metastatic breast cancer patients from 10.2 to 20.2 months (PALOMA trial; ref. 4). However, the overall survival (OS) of patients treated with PD mirrored that seen in patients treated with letrozole alone, suggesting that resistance to this combination therapy occurs. In tissue culture lines, PD- or ribociclib-mediated arrest did not appear durable either (5–8). While loss of Rb appears to distinguish primary PD nonresponsiveness in cell lines, differences in Ki67, cyclin D, cdk4, and p16 do not appear able to stratify responsive and nonresponsive subgroups (9, 10).

Cyclin D is a transcriptional target of the MAPK pathway, but after cyclin D partners with cdk4, the dimer is unstable, and rapidly dissociates back into the monomeric forms, unless a third protein, p27Kip1 or p21Cip1, holds the complex together (11). However, p27 binds to D-K4 in two different conformations: a closed and inactivating conformation OR alternatively, an open and activating form. This transition is mediated by the tyrosine (Y) phosphorylation of p27 on residues Y88 (or Y89), which causes a conformational change, opening the D-K4–p27 ternary complex, thus rendering it able to phosphorylate substrates such as Rb (12, 13). Nonphosphorylated p27–D-K4 complexes are catalytically inactive because the associated
Blocking p27 Phosphorylation Potently Arrests Cell Cycle

p27 both blocks the ATP-binding site on cdk4 and prevents the required Cdk activating kinase (CAK) phosphorylation of the cdk4 domain itself (14). p27 Y88 is phosphorylated by the Y kinase Brk (breast tumor related kinase or PTK6, protein tyrosine kinase 6), and interaction between Brk and p27 is mediated though Brk’s SH3 domain and a proline-rich binding site in p27. Addition of Brk SH3-containing peptides in vitro blocks this interaction, preventing p27 Y88 phosphorylation, which in turn causes inhibition of cdk4. Overexpression of a naturally occurring ALTernatively-spliced form of Brk (ALT), which contains Brk’s SH3 domain, but lacks the SH1 domain, also inhibits Brk’s phosphorylation of p27, inhibits cdk4, and causes growth arrest, suggesting that inhibition of p27 Y88 phosphorylation might be an alternative way to target cdk4-dependent tumors (15, 16).

In contrast to cdk4, cdk2 does not require p27 to stabilize the interaction with its cyclin; actually cdk2’s phosphorylation of RB is inhibited whenever p27, phosphorylated or not phosphorylated, is associated with the complex. But, even when unable to phosphorylate RB, Y-phosphorylated p27–cyclin E–cdk2 complexes are able to phosphorylate p27 on residue T187, which in vivo, results in decreased p27 stability as it becomes a target for ubiquitin-mediated degradation, reducing p27 association with cdk2, and indirectly activating the cyclin E–cdk2 complex (17). Thus, blocking pY88 might have the added bene

Materials and Methods

Antibodies and reagents

Antibodies against Cdk2 (sc-163), Cdk2 Thr 160 (sc-101656), Cdk6 (sc-53638), Cyclin E (sc-481), Cdk4 (sc-260), Rb (sc-102), Rb S780 (sc-12901-R), and Rb S807/811 (sc-16670-R) were from Santa Cruz Biotechnology. Anti-Flag (A2220) and actin (A2066) were from BD Biosciences. Cleaved caspase-3 (9661S) was from Cell Signaling Technology. β-Galactosidase (ab203749) was from Abcam. The p27 Y88 antibody and its specificity for p27 Y88 has been described previously (16). K2i (sc-221409) was from Santa Cruz Biotechnology. Skp2i C1 (4817) was from R&D Systems. Palbociclib (PD 0332991; HY-50767A) and letrozole (HY-14248) were from MedChem Express. Brk (531000) was from EMD Millipore. NMS (normal mouse serum; Invitrogen, 31880) and NRS (normal rabbit serum; Invitrogen, 31883) were used as isotype controls for monoclonal or polyclonal antibodies, respectively.

Cell culture

MCF-7 (ATCC, HTB-22, ER/PR+, Her2+) and MDA-MB-231 (ATCC, HTB-26, ER/PR+, Her2+) cell lines were cultured at low passage numbers in MEM-EBSS with 10% FBS, 1% nonessential amino acids, 10 μg/ml insulin solution (Sigma-Aldrich, I2978), and penicillin–streptomycin. T47D (ATCC, HTB-133, ER/PR+, Her2+) and HCC1954 (CRI 2338; ER/PR+, Her2+) were cultured in RPMI1640 with 10% FBS, 1% nonessential amino acids, 10 μg/ml insulin solution (Sigma Aldrich, I2978), and penicillin–streptomycin. Generation of Flag-tagged ALT doxycycline-inducible lines was generated as per the manufacturer’s protocol (Clontech, 631188). T4A (no GOI) cells expressed the tetracycline transactivator in the absence of gene of Interest and served as a negative control.

Cell proliferation assays

Cells were treated with DMSO or drug combinations in 6-well plates. In all cases, drugs (i.e., PD, K2i, doxycycline) were replenished every 2 days. Cell proliferation and viability were measured by visual counting in a Hemocytometer and by counting with 0.1% Trypan blue stain, respectively. To determine IC50 values, cells were treated with increasing concentrations of PD or doxycycline. Cell counts were determined to get a dose–response curve for each cell line and IC50 values were calculated using GraphPad Prism software. All proliferation experiments were N ≥ 3.

In vitro kinase assays

In vitro kinase assays were performed as described previously (13, 16). Densitometry of immunoblots was performed using LICOR Image Studio software to determine Cdk4 activity [[Rb S780/Rb]/cdk4] and Cdk2 activity [[Rb Ser807-811/Rb]/cdk2]].

β-galactosidase staining

Cells were incubated in Fixative Solution (Cell Signaling Technology; 9860S) for 12 minutes followed by incubation with β-galactosidase antibodies overnight at 37°C. β-Gal− cells were counted manually and %-β Gal− cells were determined (from 100 total).

Figure 1.

p27 Y88 correlates with cdk4 activity in breast cancer cells. A, Breast cancer cell lines treated with PD to determine IC50 values. B, Representative immunoblot with cell lysates from cultures without treatment. Quantitation in Supplementary Fig. S5E. C, Endogenous Rb S780/RB ratio determined by densitometry of immunoblots in B, D, Proliferation determined by cell counting. E and F, Cyclin D1 immunoprecipitated from cells, recombinant RB and RB added to perform in vitro cdk4 kinase assays followed by immunoblot analysis. Cdk4 activity [(RbS780/RB ratio)/cdk4] determined by densitometry of immunoblots in E. Activity normalized to that seen in HCC1954 cells. G and H, p27 immunoprecipitated, followed by immunoblot analysis. pY88/p27 ratio determined by densitometry of immunoblots in H, normalized to that seen in HCC1954 cells. As p27 levels vary in the cell lines, different amounts of lysates were used to immunoprecipitate similar levels of p27. I, p27 immunoprecipitated from MCF7 cells ±400 nM/L PD, followed by pY88 immunoblot analysis. J, Relationship between cell proliferation, p27 Y88 phosphorylation and cdk4 activity upon PD treatment (NRS, normal rabbit serum; NMS, normal mouse serum; Data, mean ± SD, all experiments, N ≥ 3).
Knockdown assay

Cells were transfected with p27 siRNA lentiviral (Santa Cruz Biotechnology, sc-29430-V) particles or scrambled control on day 2 in presence of hexadimethrine bromide to enhance transfection efficiency. Growth media were replenished on day 3 and on day 5, cells were harvested for immunoblot analysis with p27 antibodies to confirm KD or drugs were added. KD cells were plated on day 0, doxycycline or PD were added on day 2 and proliferation was measured by cell counting.

In vivo xenograft study

Animal studies have been conducted in accordance with the Institutional Animal Care and Use Committee (IACUC). Four- to 6-week-old female NOD.CB17-Prkdcscid1/SidNcrCrl mice were purchased from Charles River Breeding Laboratories, implanted with β-Estradiol pellets (SE-121, Innovative Research of America) subcutaneously, and allowed to recover for a week. A total of 0.5 × 10⁶ MCF7-ALT cells were injected subcutaneously near the fourth mammary fat pad. Tumor development was monitored using digital calipers and volume calculated as [length × (width)²]/2. When tumors reached approximately 200 mm³ (between 21–31 days postinjection), mice were treated daily with vehicle (50 mmol/L sodium lactate, pH 4), 100 mg/kg PD (PD) orally, 13.3 mg/kg or 40 mg/kg doxycycline dissolved in drinking water with 1% sucrose, or the combination of PD and doxycycline. After day 9, all doxycycline-treated animals were injected with saline to try to prevent dehydration, until day 19 when the study ended. Tumors were harvested at various time points and were fixed in 4% paraformaldehyde for 2 days followed by incubation in 70% ethanol, followed by IHC analysis.

Statistical analysis

Quantification of all immunoblots was performed using the Image Studio Lite software (LI-COR). In Cdk4 and Cdk2 in vitro kinase assays, Cdk4 or Cdk2 activity from day 4 or 10 treated cells was normalized to that seen in day 4 or 10 DMSO-treated controls, respectively. Outliers were detected using the Thompson Tukey test with unequal variance. Mean mouse tumor volume values were determined using SD. A single-factor ANOVA analysis or a two-tailed Student t test with unequal variance was performed to test for statistical significant difference between groups.

Results

p27 pY88 phosphorylation status correlates with cdk4 activity

Finn and colleagues had stratified a panel of breast cancer cell lines for PD sensitivity and had shown that some lines responded well, with low IC₅₀ values, while others were weak responders, with IC₅₀ values in the 1,000 nmol/L range (19). We confirmed PD sensitivity of three representative lines, MCF-7, MDA-MB-231, and HCC1954 corresponding to a high, moderate, or low response, respectively (Fig. 1A), and confirmed that proliferation was inhibited but viability remained constant (Supplementary Fig. S1A–S1D). Immunoblot analysis of untreated cells (Fig. 1B) demonstrated that all three lines had similar levels of cdk4 and cyclin D1, consistent with reports that suggested that expression of these proteins was not a predictor of PD response (quantitation in Supplementary Fig. S1E; refs. 9, 10). Endogenous RB phosphorylation was measured using RB S780 phospho-specific antibodies and normalized to total RB levels (RB S780/RB; Fig. 1C). MCF-7 cells had the highest levels of phosphorylated RB, consistent with the highest proliferation rate of the three lines (Fig. 1D).

To directly examine cdk4/6 activity, we performed in vitro kinase assays, immunoprecipitating cyclin D1–associated complexes from lysates followed by the addition of recombinant RB in the presence of ATP. Immunoprecipitates were subjected to SDS-PAGE electrophoresis and immunoblot analysis with RB S780, Rb, and cdk4 antibodies (Fig. 1F) and densitometry allowed us to calculate the RB S780/RB ratio (Fig. 1E). Immunoprecipitates from HCC1954 cells yielded a cdk4 that phosphorylated RB approximately 2.5 times more than immunoprecipitates from MCF-7 cells and cdk4 from MDA-MB-231 cells was in between. As we had immunoprecipitated a similar level of D-K4 complexes (as detected from associated cdk4 in the cyclin D1 immunoprecipitates; Fig. 1F), this suggested that the specific activity of cdk4 in HCC1954 cells was higher than that in MCF7 cells. More cdk4 activity in HCC1954 cells would require a higher concentration of PD to achieve an IC₅₀ response, suggesting that cdk4 activity, rather than protein levels, might be a marker of sensitivity.

As cdk4 activity is dependent on the presence of pY88 p27 in the ternary complex, we immunoprecipitated p27, followed by immunoblot analysis with a p27 pY88 phospho-specific antibody (Fig. 1G–I; ref. 16). Two amounts of MCF7 lysate were used to demonstrate concentration-dependent specificity of both the p27 and the pY88 antibodies (Fig. 1H). We determined the pY88/p27 ratio, and found that HCC1954 lysates had approximately three times higher pY88/p27 than MCF7 cells and MDA-MB-231 cells had levels in between (Fig. 1G). MCF7 cells were treated with 400 nmol/L PD (2 × IC₅₀), which completely inhibited proliferation (Supplementary Fig. S1A), and pY88 was not detectable (Fig. 1I). The level of pY88/p27 decreased proportionally to PD-mediated decreases in cdk4 activity and cell proliferation, suggesting that pY88 was a surrogate marker for cdk4 activity (Fig. 1I).

Cdk2 activity compensates for PD-dependent loss of cdk4 activity in high responders

The PD high responding line, MCF-7, was treated with 200 nmol/L PD (IC₅₀ concentration) at day 2, drug was refreshed every 2 days, and proliferation was measured by cell counting, normalizing to proliferation seen in DMSO-treated cells (Fig. 2A). Proliferation was arrested by 50% by day 4, but by day 6, cells had adapted to the presence of drug and resumed proliferation.

Downloaded from mcr.aacrjournals.org on March 5, 2018. © 2018 American Association for Cancer Research.
Figure 2.

Cdk2 activity compensates for loss of cdk4 activity in PD-resistant MCF7 cells. A, Proliferation of MCF7 cells treated with DMSO or PD. Media with DMSO or drug is refreshed every two days starting at day 2 (arrowhead). B, Immunoblot analysis with treated MCF7 lysates (n = 3). C, Cdk4 activity [(RbS780/RB ratio)/cdk4] determined by densitometry (bottom) of in vitro kinase assay immunoblots (top; n = 5). D, Cdk2 activity [(RbS780/811/RB ratio)/cdk2] determined by densitometry (bottom) of in vitro kinase assay immunoblots (top; n = 5). E, p27 immunoprecipitated, followed by pY88 immunoblot (top). pY88/p27 ratio determined by densitometry (bottom; n = 3). F, Cdk2 immunoprecipitated, followed by p27 immunoblot (top). p27 association with cdk2 determined by densitometry (bottom; n = 6).
(Fig. 2A, blue). When a higher concentration of PD (2 × IC50) was used, the arrest was more durable, lasting until day 14, but even then cells started proliferating again in the presence of drug (Fig. 2A, red). PD treatment at either concentration did not affect viability (Supplementary Fig. S2A). A similar transient arrest in the presence of PD was seen with another ER+, high responding line, T47D (Supplementary Fig. S2B). Cell lysates were generated from treated MCF-7 cells at different timepoints and used in immunoblot analysis (Fig. 2B). PD treatment caused loss of endogenous RB S780 phosphorylation at day 4, but it was detected again by day 6 in spite of replenishing drug. PD treatment did not significantly affect cdk4 or cyclin D1 levels (Fig. 2B). We performed cdk4 in vitro kinase assays, immunoprecipitating either cyclin D1-associated or cdk4-associated complexes from MCF7 (Fig. 2C, Supplementary Fig. S2C). A 50% reduction in cdk4 activity was detected in both day 4 and 10 PD-treated lysates (Fig. 2C, lanes 2 and 4), suggesting that PD was still inhibiting cdk4 at day 10, even when endogenous Rb S780 phosphorylation had been restored (Fig. 2B).

In the absence of cdk4 and cdk6, cdk2 or cdk1 can phosphorylate RB S780, suggesting that these kinases might compensate for loss of cdk4 activity in the PD-treated cells (20, 21). To examine cdk2 activity, cdk2 in vitro kinase assays were performed: exogenous RB and ATP was added to cdk2 immunoprecipitates, and phosphorylation was monitored by immunoblot analysis using the RB S807,811 antibody (Fig. 2D). Densitometry was used to calculate the RB807,811/RB ratio as a measure of cdk2 activity. While this activity was reduced in lysates from day 4 PD-treated cells relative to DMSO levels, it was increased in lysates from day 10 cells (Fig. 2D, lanes 2 and 4). In vitro, RB phosphorylation is more ubiquitous and cdk2 can also phosphorylate RB at site S780. Similar results were seen in a cdk2 in vitro kinase assay using RB S780 antibodies (Supplementary Fig. S2D, lanes 1, 2, 4, and 5). This suggests that an increase in cdk2 activity could be responsible for the increase in endogenous RB S780 phosphorylation seen in Fig. 2B (lanes 5–8) and the subsequent reentry into S phase (Fig. 2A). Cdk2 immunoprecipitates were not pull-down-associated cyclin D1 (Supplementary Fig. S2E) as had been reported by others (5).

Immunoblot analysis demonstrated that while neither cdk2 nor cyclin E increased in the presence of PD (Fig. 2B), p27 levels increased in day 4 PD-treated lysates relative to those in DMSO lysates, but decreased from day 6–10 (Fig. 2B, lanes 5–8). We immunoprecipitated p27, followed by immunoblot analysis with pY88 antibodies (Fig. 2E), to determine the pY88/p27 ratio (Fig. 2E, bottom) and found that pY88/p27 decreased by approximately 50% in the PD-treated lysates relative to levels in DMSO treated cells at day 4, consistent with approximately 50% decrease in proliferation. As cells began to proliferate again at day 10, pY88 increased as well (Fig. 2E, lanes 2 and 4). It has been shown that pY88 phosphorylated p27 associates with cdk2 as well, but in this context p27 becomes a substrate for cdk2-dependent phosphorylation on residue T187. p27 phosphorylation on T187 targets it for ubiquitin-mediated degradation (22) and this might explain the decrease in p27 (Fig. 2B, lanes 5–8). Cdk2 immunoprecipitates were probed by immunoblot analysis for associated p27 (Fig. 2F). While p27 association with cdk2 was increased at day 4 (Fig. 2F, lanes 1 and 2), its levels stabilized by day 10 (Fig. 2F, lanes 3 and 4). As p27 is always an inhibitor of cdk2, these data suggest that p27 reduced association at day 10 corresponds to an increase in active, inhibitor-free cdk2, which would be able to compensate for loss of cdk4 activity, phosphorylating RB and restoring proliferation.

**PD inhibits both cdk4 and cdk2 in MDA-MB-231 to cause durable cell-cycle arrest**

The IC50 concentration for MDA MB231 cells is 400 nmol/L, or approximately 2 × the concentration needed to inhibit MCF7 cells. When either 400 nmol/L or 800 nmol/L PD was added to MDA MB231 cells at day 2, and replenished every 2 days, a 50% or 90% arrest was achieved, respectively (Fig. 3A), while viability was unchanged (Supplementary Fig. S3A). But, at both doses, this arrest was durable and persisted for 10 or 26 days, respectively (Fig. 3A). This suggested that while higher concentrations of PD were required to see response, the arrest achieved was more longstanding than that seen in MCF7 cells.

Lysates were generated from MDA-MB-231 cells treated with 400 nmol/L PD at different time points and subjected to immunoblot analysis (Fig. 3B). Loss of endogenous Rb S780 phosphorylation was seen at days 4–10, consistent with the more durable PD arrest seen in these cells (Fig. 3B, lanes 5–8). Cdk4 and cyclin D1 levels increased in the presence of this high concentration of PD, as had been reported by others (23). Despite this increase, the IC50 concentration of PD inhibited cdk4 activity by 50% as seen in the in vitro kinase assay, and this inhibition was also seen at day 10, consistent with the durability of this response (Fig. 3C, lanes 2 and 4). When Cdk2 activity was examined in cdk2 in vitro kinase assays, we found that PD treatment reduced cdk2 activity by approximately 40% at day 4 and it remained inhibited at day 10 (Fig. 3D, lanes 2 and 4). This suggested that PD treatment was durable in MDA-MB-231 cells because cdk2 activity was inhibited, and was unable to compensate for the reduction in cdk4 activity.

Immunoblot analysis of MDA MB231 lysates demonstrated that neither cdk2 nor cyclin E levels changed in the presence of PD, but unlike the situation in MCF7 cells, p27 levels remained high through day 10 of treatment (Fig. 3B, lanes 5–8). p27 association with cdk2 increased in day 4 treated lysates relative to DMSO-treated controls, and this association continued to increase in day 10 lysates (Fig. 3E, lanes 2 and 4). The stable association of p27 with cdk2 would result in the continued cdk2 inhibition seen at day 10 (Fig. 3D). This data suggest that the durability of the PD response seen in MDA-MB-231 cells was due to the fact that at this concentration, both cdk4 and cdk2 were inhibited and remained inhibited during treatment. Palbociclib at >10 μmol/L is required to inhibit cdk2 (24), suggesting that these concentrations were too low to inhibit kinase activity directly and instead the association with p27 was responsible.

**ALT prevents p27 pY88 and inhibits both cdk4 and cdk2**

In MCF-7 cells, a lower concentration of PD was able to inhibit cdk4, but arrest was not durable because cdk2 was able to compensate for loss of cdk4 activity, while in MDA MB231 cells, a higher concentration of PD was needed to inhibit cdk4, but this resulted in the stabilization of p27 and inhibition of cdk2 activity, creating a long-lasting cell-cycle arrest. Thus, an inhibitor that blocks both cdk4 and cdk2 in MCF7 cells might produce a more durable response. We had previously shown that blocking p27 phosphorylation inhibited cdk4 activity, by converting the p27–D-K4 complex into a closed, inactive conformation (12). However, others had shown that blocking pY88 also prevents p27 degradation, permitting it to inhibit cdk2 as well (22). Overexpression of an ALT Brk (ALT), which contains only Brk's SH3
Figure 3.
Inhibition of cdk4 and cdk2 arrests proliferation durably in the moderate responder MDA-MB-231. A, Proliferation of MDA MB231 cells treated with fresh DMSO or PD every two days starting at day 2 (arrowhead). B, Immunoblot analysis with treated lysates. C, Cdk4 activity ([RbS780/RB ratio]/cdk4] determined by densitometry (bottom) of in vitro kinase assay immunoblots (top; n = 4). Lane 5, PD/C3: 0.4 μmol/L PD was exogenously added to the cdk4 immunoprecipitates from DMSO lysates while performing the kinase assay. Lane 6: no Rb added to reaction. D, Cdk2 activity ([RbS780/RB ratio]/cdk2] determined by densitometry (bottom) of in vitro kinase assay immunoblots (top; n = 4). Lane 5: no Rb added to the reaction. E, Cdk2 immunoprecipitated, followed by p27 immunoblot (top). p27 association with cdk2 determined by densitometry (bottom; n = 3).
domain, caused growth arrest (15) and we hypothesized that this might result from direct cdk4 inhibition but also indirect cdk2 inhibition (Fig. 4A).

A stable cell line of MCF7 that inducibly expressed Flag-tagged ALT in a dose-dependent manner was generated (MCF7-ALT). In presence of doxycycline in the media, Flag-ALT was induced in MCF7 cells as detected by immunoblot analysis with Flag antibodies (Fig. 4B, top). Dose–response curves were generated to determine the concentration of doxycycline needed to achieve a 50% reduction in proliferation after 48 hours of treatment (IC50) and viability was constant (Supplementary Fig. S4A and S4E).

Immunoblot analysis demonstrated consistent induction of ALT across different experiments (Supplementary Fig. S4J, top) and ALT expression was dependent on doxycycline dose (Supplementary Fig. S4J, bottom). p27 immunoprecipitates followed by immunoblot (top). pY88/p27 ratio determined by densitometry (bottom; D, PD, IC50). MCF7-ALT cells were treated with doxycycline to induce ALT (Fig. 4D top, lane 3). MCF7-ALT cells were treated with 200 nmol/L PD (IC50, 43.24 μmol/L doxycycline (IC50), or a combination every two days, and proliferation was monitored by cell counting (Fig. 4C). While MCF7-ALT cells adapted quickly to PD treatment alone, doxycycline treatment (ALT induction) inhibited proliferation durably up to day 10 (Fig. 4C, orange line). When cells were treated with doxycycline + PD, a synergistic arrest resulted (with a combination index (CI) below 1 (Fig. 4C, black line, Supplementary Fig. S4M). MCF7-TtA cells, which only contained the tetracycline transactivator and lacked the ALT transgene, were treated with doxycycline to demonstrate that doxycycline treatment did not have any effect on proliferation (Fig. 4C, red).

Lysates from MCF7-ALT cells treated with the DMSO, IC50 PD, IC50 doxycycline, or doxycycline + PD were harvested at day 4 and 10, and probed by immunoblot analysis (Fig. 4D; Supplementary Fig. S4K). While PD treatment reduced endogenous Rb S780 phosphorylation at day 4 (Fig. 4D top, lane 2), it was restored at day 10 (Fig. 4D, top, lane 6). However, when cells were treated with doxycycline to induce ALT (Fig. 4D top, lanes 3 and 7) or treated with doxycycline + PD (Fig. 4D top, lanes 4 and 8), RB phosphorylation was not detected at day 4 or 10. While ALT expression did not increase either cyclin D or cdk4 levels (Supplementary Fig. S4K), it did affect cdk4 and ck2 activity as measured directly in vitro kinase assays (Fig. 4E and F). PD and doxycycline alone reduced ck2 kinase activity by approximately 50% at day 4 (Fig. 4E, lanes 2 and 3), and combination treatment reduced cdk4 activity even further (~70%; Fig. 4E, lane 4). Cdk4 was still inhibited to the same extent in all treatment conditions at day 10 (Fig. 4E, lanes 6–8 as compared with lanes 2–4). However, while PD treatment reduced cdk2 kinase activity by approximately 30% at day 4 (Fig. 4E, lane 2), by day 10 it had increased to the level seen in the DMSO-treated control cells (Fig. 4F, lane 6). However, doxycycline treatment (ALT induction) reduced cdk2 kinase activity by approximately 50% at day 4 and this reduction was maintained at day 10 (Fig. 4F, lanes 3 and 7). Dual doxycycline + PD treatment further reduced cdk2 activity at day 4, and was also durable at day 10 (Fig. 4F, lanes 4 and 8).

Cyclin E and cdk2 levels did not change under the four treatment conditions (Supplementary Fig. S4K). However, in the PD-treated lysates at day 10, p27 levels decreased, consistent with the increase in cdk2 activity seen at this timepoint (Fig. 4D, bottom, lane 6), but in the doxycycline or doxycycline + PD-treated cells, p27 levels were stabilized and did not decrease (Fig. 4D, bottom, lanes 7 and 8). When ck2 immunoprecipitates from PD-treated lysates were probed for p27 by immunoblot analysis, p27 was associated with cdk2 in day 4 PD-treated cells, but then was lost from the complex at day 10 (Fig. 4G, lanes 2, 6). However, p27 remained associated with cdk2 at both day 4 and day 10 in both doxycycline alone or doxycycline + PD-treated lysates (Fig. 4G, lanes 3–4 and 7–8). The ratio of pY88/p27 was decreased relative to DMSO levels at day 4 in PD-treated lysates, as seen before (Fig. 11), but as cells became resistant to PD treatment at day 10, the ratio of pY88/p27 increased (Fig. 4H, lanes 2 and 6). Doxycycline or doxycycline + PD dual treatment, however, caused approximately 50% reduction in pY88 levels in both timepoints (Fig. 4H, lanes 3, 4, 7, and 8), consistent with ALT-mediated that persisted to day 10. These data suggest that when ALT was induced, it physically associated with p27 (Fig. 4B) and blocked Y88 phosphorylation (Fig. 4H). As unphosphorylated p27 binds cdk2 in a ‘closed’ conformation, it can no longer be phosphorylated on residue T187, is not targeted for degradation, and can remain associated with and continue to inhibit cdk2 activity, resulting in a durable cell-cycle arrest in MCF7 cells.

Although there is cell-cycle arrest, both 43.24 μmol/L (IC50) and 86.48 μmol/L (2 × the IC50) doxycycline-treated MCF7-ALT cells were viable (Supplementary Fig. S4A). Frequently, cdk2 inhibitors also inhibit cdk1, due to the high degree of active-site homology between these two kinases (25). Lysates from DMSO, 2 × IC50 PD, 2 × IC50 doxycycline, and doxycycline + PD-treated cells at day 4 were probed with anti-cdk1 and T161cdk1, a phospho-specific antibody that recognizes the active form of cdk1 and is routinely used as a surrogate marker.
for cdk1 activity (ref. 26; Fig. 4I). Cdk1 was still active in PD, doxycycline, or doxycycline + PD-treated cells. As a control, cells were treated with a cdk2 inhibitor, K2i, and this inhibited T161 phosphorylation by 90% (Fig. 4I). This suggested that ALT-mediated arrest was due to cdk4- and cdk2-specific inhibition, while cdk1 was still active.

To demonstrate that the ALT-mediated arrest was dependent on p27, siRNA was used in the MCF7-ALT cells to knockdown endogenous p27, which was confirmed by immunoblot analysis using p27 antibodies (Fig. 4J, inset). p27 KD cells were treated with DMSO, IC150 PD, IC50 doxycycline, or doxycycline + PD and proliferation was monitored by cell counting (Fig. 4J). Neither PD nor doxycycline treatment of the p27 KD cells affected viability (Supplementary Fig. S4I) or inhibited proliferation, which was indistinguishable from that seen in the DMSO-treated p27 KD cells (Fig. 4J, blue, gray lines). Dual doxycycline + PD treatment did retard proliferation slightly from day 2-4, but cells resumed proliferation by day 6 (Fig. 4I, red). These results demonstrated that ALT-mediated and PD-mediated arrest required p27. As a control, doxycycline + PD dual treatment of parent MCF7-ALT cells that had not received the p27 siRNA were included (Fig. 4J, black line).

**Durable arrest requires cdk2 and cdk4 inhibition**

Our data suggest that while cdk4 inhibition is required to cause arrest, to achieve durability, cdk2 would also have to be inhibited to prevent compensation and resistance. To test this model directly, we treated MCF7-ALT cells with K2i, a cdk2-specific inhibitor, alone or in combination with PD (Fig. 5A). K2i also inhibits cdk1 (Fig. 4I), but does not inhibit cdk4 (27). Doxycycline + PD-treated cells served as a control for durability, demonstrating stable arrest for 10 days (A, black). When MCF7-ALT cells were treated with 0.1 or 1 μmol/L K2i alone, proliferation was only inhibited by approximately 10 or 20%, respectively, from day 4 to 10 (Fig. 5A, orange and purple lines), but when combined with PD, approximately 40 or 50% inhibition, respectively, was observed for almost 10 days (Fig. 5A, red and brown lines), suggesting an additive or synergistic effect. 100% of the K2i treated cells were viable at these concentrations (Supplementary Fig. S5A). Lysates from day 4 treated cells were used in immunoblot analysis and probed with cdk2 and T160cdk2 phospho-specific antibodies, to detect active cdk2 and confirm that K2i had inhibited its target (Fig. 5B). While cdk2 levels were unchanged, T160cdk2 was reduced in the K2i+PD-treated cells (Fig. 5B, lanes 5 and 6). PD and K2i treatment each increased p27 levels relative to DMSO controls (Fig. 5B, lanes 3-4), and K2i+PD increased p27 expression to an even greater extent (Fig. 5B, lanes 5 and 6), consistent with the role of cdk2 as a regulator of p27 stability (22, 28). Thus, the inhibition of cdk2 at day 4 likely resulted both from inhibition of the cdk2-active site by K2i and increased p27.

As it appeared that a decrease in p27 levels was responsible for the cdk2-dependent rescue of the PD-mediated arrest seen MCF7- cells (Fig. 2B), we hypothesized that use of a Skp2 inhibitor (Skp2i; 29, 30), which should inhibit the ubiquitin ligase required for p27 degradation, would stabilize p27 and inhibit cdk2, and combined Skp2i and PD treatment might phenocopy ALT-mediated inhibition. MCF7-ALT cells were treated with Skp2i, alone or in combination with PD (Fig. 5C). One-hundred percent of cells were viable in 0.1 and 1 μmol/L Skp2i (Supplementary Fig. S5B). Both Skp2i treatments reduced proliferation by approximately 20% (Fig. 5C, purple, orange lines), while the combination of Skp2i+PD reduced proliferation by approximately 50% (Fig. 5C, red, brown lines). Skp2i (0.1 μmol/L) + PD only inhibited cells up to day 4, and by day 6, proliferation had resumed (Fig. 5C, red), while 1 μmol/L Skp2i+PD-arrested cells up to day 8, but by day 10, these cells too had begun to escape arrest (Fig. 5C, brown).

Immunoblot analysis of lysates from day 4 treated cells demonstrated that Skp2i did stabilize p27 levels to a similar extent as that seen with PD monotheraput (Fig. 5D). Dual Skp2i + PD treatment stabilized p27 slightly more (Fig. 5D, lanes 5 and 6). Both Skp2i mono and Skp2i + PD dual treatment reduced cdk2 activity as measured by cdk2T160 immunoblot (Fig. 5D, lanes 3-6).

Letrozole (hereafter PD) in combination with PD is currently a first-line therapy for women with metastatic ER+ breast cancer (31). Letrozole is a aromatase inhibitor, which reduces estrogen levels, and is thought to reduce Cyclin D1 levels, enhancing the efficacy of PD. We treated MCF7-ALT cells every two days with 0.1 or 1 nmol/L LET, PD, or the combination (Fig. 5E). Either concentration of LET alone reduced proliferation by approximately 20% (Fig. 5E, orange, purple), while the combination of either 0.1 or 1 nmol/L LET with PD arrested proliferation by approximately 50% by day 4 (Fig. 5E, red, brown). The arrest seen with 0.1 nmol/L LET + PD was not durable and cells resumed proliferation by day 6 (Fig. 5E, red), while the arrest with 1 nmol/L LET + PD was durable to day 8 (Fig. 5E, brown). We performed immunoblot analysis to examine its effect on cell-cycle proteins (Fig. 5F). We did not see an effect on cyclin D expression (Supplementary Fig. S5D), but while PD treatment stabilized p27, LET alone did not have any effect (Fig. 5F, lanes 3 and 4). However, at both concentrations of LET+PD, p27 levels were reutilized (Fig. 5F, lanes 5 and 6), and this corresponded to a decrease in cdk2 activity. LET alone did not have an effect on cdk2 activity as measured by T160cdk2 detection, but the combination of LET+PD significantly reduced cdk2 activity (Fig. 5F, lanes 5 and 6).

To determine whether ALT would also arrest cell lines that responded to PD with moderate or poor arrest, additional stable lines that inducibly expressed FLAG-ALT in the presence of doxycycline were generated (MDA MB231-ALT and HCC1954-ALT) (Fig. 5G and I). A similar induction of ALT caused a 50% inhibition and this arrest was durable for 10 days (Fig. 5G and I). This was very striking in HCC1954 cells where 5x more PD (1,000 nmol/L) is required to inhibit proliferation by 50%, but a similar level of ALT induction is just as effective and this is induced with half the amount of doxycycline used for MCF7 (Fig. 5I, blue and orange). A synergistic arrest (CI < 1) was seen with doxycycline + PD dual treatment of MDA MB231 cells (Fig. 5G, black line; Supplementary Fig. S4M), and cdk4 and cdk2 in vitro kinase assays showed that both cdk4 and cdk2 activity was inhibited more in the dual-treated cells (Fig. 5J and K). While the IC50 concentration of PD blocked pY88 by approximately 50%, doxycycline + PD treatment reduced pY88 even more (Supplementary Fig. S5H and S5I). To demonstrate that ALT-mediated arrest was dependent on p27, siRNA was used in the MDA-MB-231-ALT cells to knockdown endogenous p27 and was confirmed by immunoblot analysis using p27 antibodies (Fig. 5L, right). p27 KD did not alter viability (Supplementary Fig. S5K). P27 KD cells were treated
Figure 5.
Cdk2 compensates for cdk4 activity to cause cell resistance. Proliferation of MCF7-ALT cells treated every 2 d. with K2i (A) Skp2i (C), LET (E) alone or in combination with 200 nmol/L PD followed by immunoblot analysis on day 4 (B, D, F).

G and I, Proliferation of MDA MB231-ALT (G) and HCC1954-ALT (I) treated with DMSO, PD, doxycycline, or doxycycline + PD. Proliferation of TtA (no GOI) in doxycycline as control. Statistical significance relative to PD treatment.

H, Cells treated with doxycycline to induce ALT and probed by immunoblot with Flag antibodies.

J and K, Treated MDA-MB-231-ALT cells used in the cdk4 (J) and cdk2 (K) in vitro kinase assays (n = 4). L, Left, proliferation of MDA-MB-231-ALT p27 KD cells with DMSO, PD (0.4 μmol/L), doxycycline (21.62 μmol/L) or the combination of doxycycline and PD. (Inset) Immunoblot analysis to confirm p27 KD. M and N, Proliferation of MDA MB231-ALT cells with DMSO, IC25 PD (0.2 μmol/L), doxycycline (21.62 μmol/L) or doxycycline + PD (M) followed by immunoblot analysis (N).
with DMSO, the IC_{50} concentration of PD or doxycycline, or the combination and proliferation was monitored (Fig. 5L). PD or doxycycline treatment of the p27 KD cells only inhibited proliferation by 20% as compared with the 50% inhibition seen with PD or doxycycline treatment in the parent MDA-MB-231-ALT cells (Fig. 5L vs. Supplementary Fig. 5G). Dual doxycycline + PD treatment did retard proliferation of p27 KD cells by 50% at day 6 (Fig. 5L), but was not as effective as doxycycline + PD treatment in the p27^+ cells (Fig. 5G, black line) where 80% inhibition was detected, suggesting that p27 was required for ALT-mediated inhibition in MDA-MB-231 cells. Knockdown using scrambled siRNA did not alter either PD or doxycycline-mediated arrest (Fig. 5K and L).

We hypothesized that ALT induction might permit a lower, more pharmacologic concentration of PD to be used to elicit arrest. MDA-MB-231-ALT cells were treated with doxycycline, 200 nmol/L PD (IC_{50}) or doxycycline + PD, and proliferation was monitored by cell counting (Fig. 5M). When 200 nmol/L PD (IC_{50}) used, only a 30% inhibition was seen at day 4, and by day 6, proliferation had resumed (Fig. 5M blue). Immunoblot analysis of treated lysates demonstrated that at this lower concentration, PD treatment was unable to block endogenous RB S780 phosphorylation and cdk2 was still active as measured by phospho-cdk2 (Fig. 5N, lane 2). Doxycycline treatment inhibited proliferation by 50% and endogenous RB S780 phosphorylation was reduced (Fig. 5M and N, lanes 3 and 7). However, in doxycycline + PD (200 nmol/L) treated cells, proliferation continued to decrease up to day 10 (~70%), suggesting a smaller fraction of the cells became of this low, nonproliferating population of PD (Fig. 5M, black). Immunoblot analysis of doxycycline + PD-treated lysates harvested at day 4 and 10 demonstrated that endogenous RB S780 phosphorylation and cdk2 activity was inhibited as well (Fig. 5N, lanes 4 and 8).

ALT + PD combine to increase cellular senescence

To determine how sustainable ALT-mediated arrest was, cells were treated with a higher concentration of doxycycline (2 × IC_{50}), which induced more ALT (Supplementary Fig. 4K), a higher concentration of PD (2 × IC_{50}) or the combination (Fig. 6A, D, and G). In the presence of this increased concentration of PD, proliferation was arrested at approximately 10%–20% in all cell lines. In the MCF-7 line, cells became resistant to PD at day 14 (Fig. 6A, blue), while MDA-MB-231 and HCC1954 cells were stably arrested for >26 days (Fig. 6D and G, blue). In the presence of increased doxycycline alone, MCF7 cells were arrested to day 14, although at these extended time points escape eventually occurs (Fig. 6A, orange). MDA-MB-231 cells escaped doxycycline-mediated arrest around day 14, while HCC1954 cells were arrested for >26 days (Fig. 6D and G, orange). However, for all cell lines, doxycycline + PD treatment arrested cells for 30 days (Fig. 6A, D, G, and black). Cells remained viable during this time (Supplementary Fig. 5A–5D).

Cells that had been treated with these different combinations (DMSO, doxycycline, PD or doxycycline + PD) for 6 days and were arrested (Fig. 6A, D, and G), were then trypsinized and replated in fresh, drug-free media (designated DMSO, doxycycline^−, PD^− or doxycycline + PD^−); Fig. 6B, C, E, F, H, and I). Proliferation was monitored by cell counting (Fig. 6B, E, and H) and viability of these pretreated cells was measured by Trypan blue staining (Fig. 6C, F, and I). Upon replating in drug-free media, PD^−-treated MCF7 cells resumed proliferation, demonstrating that PD arrest was reversible (Fig. 6B, green and blue). Reentry back into cycle was delayed in the doxycycline^-treated (ALT induced) cells (Fig. 6B, orange), but the replated doxycycline + PD^− dual treated cells were unable to resume proliferation even after 10 days (Fig. 6B, black). Viability was reduced in the doxycycline + PD^−-treated cells, but approximately 50% of the cells were alive and remained nonproliferative (Fig. 6C). MCF7 cells arrested for 6 days were stained for β-galactosidase and an increase in β-gal^+ cells was detected in the doxycycline + PD^−-treated cells (Fig. 6I), suggesting that these nonproliferative cells were actually senescent and could not reenter cell cycle. Similar results were seen with another ER^− breast cancer cell line, T47D, suggesting that ALT+PD dual therapy induced senescence (Supplementary Fig. 6D–6G; β-gal data not shown). HCC1954-ALT and MDA MB231-ALT cells were assayed for their ability to proliferate in the replating assay. Doxycycline^- and doxycycline + PD^-treated HCC1954 cells did not regain proliferation postreplating, even though the cells remained viable (Fig. 6H and I). All of the drug treatment groups in the MDA-MB-231-ALT cells were retarded in their ability to reestablish proliferation, but did eventually reenter cycle (Fig. 6E). The replated MDA-MB-231 cells were viable (Fig. 6F), and when β-gal expression was analyzed, we found that PD treatment alone produced a significant number of β-gal^- cells, which was not increased significantly in the presence of ALT (Fig. 6K). Together, data suggest that PD treatment was reversible in MCF-7 and HCC1954 cells, but the combination of ALT+PD rendered these cells unable to recover from arrest. As shown in the MCF-7 line, the inability to reenter cycle could be attributed to the induction of senescence (Fig. 6I). In the MDA-MB-231 cells, all treatments impaired, but did not abrogate the ability to escape from arrest.

ALT + PD reduces tumors in xenograft models

Because ALT+PD dual treatment induced senescence in MCF-7 cells, we hypothesized that instead of just stopping tumor growth, dual treatment might cause tumor regression in a breast cancer animal model (Fig. 7A). We injected MCF-7-ALT cells into the fourth mammary gland of NOD/SCID female mice that had an estrogen release pellet subcutaneously implanted, tumors were observed within 5 weeks, and treatment was started when each animal's tumor reached 200 mm^3 (day 1). Some animals were dosed with either 13.33 mg/kg doxycycline (day 1). Tumor growth was monitored every other day using digital calipers and plotted as the change in tumor volume (Supplementary Fig. S4K). Animals were treated with 100 mg/kg PD as a monotherapy or in combination with the doxycycline-containing water. Tumor growth was monitored every other day using digital calipers and plotted as the change in tumor volume (Supplementary Fig. 5A). Tumor growth continued in the vehicle-treated animals and most animals had tumors >3,000 mm^3 by day 17 of treatment, and were removed from the experiment (Fig. 7A, green). Tumor growth in the PD-treated animals was slowed compared with vehicle-treated animals (not statistically significant), but no tumor regression was seen in any treated animals, and tumors >2,500 mm^3 were detected by day 17, when animals were removed from the experiment (Fig. 7A, blue).

Tumor growth was significantly reduced at day 11 in the animals fed the two concentrations of doxycycline (Fig. 7A, orange, red, *) . In the animals receiving doxycycline incapable tumor regression was observed for the first 5 days of treatment, before starting to...
Figure 6.
ALT+PD treatment induces durable arrest and increases senescence. Proliferation of MCF7-ALT (A), MDA MB231-ALT (D), or HCC1954-ALT (G) cells treated with DMSO, 2\textsuperscript{nd} ICD50 PD, 2\textsuperscript{nd} ICD50 doxycycline, or doxycycline + PD, MCF7-ALT (B), MDA MB231-ALT (E), or HCC1954 (H) cells were treated with DMSO or drugs for 6 days, trypsinized and replated in fresh drug-free media (DMSO, PD, etc. denotes the prior treatment conditions). Replated cells were counted. (C, F, and I) % viability determined by Trypan blue stain. MCF7-ALT (J) and MDA MB231-ALT (K) cells treated for 6 days with DMSO or drugs were stained with β-galactosidase.
increase in size again at day 7 (Fig. 7A, red). Tumor regression was not seen in the doxycycline low-treated animals, and in both cases by day 17 of treatment, doxycycline-treated animals had tumors approximately 1,500 mm$^3$ (Fig. 7A, orange, red, /C3/C3/). Tumors in the dual treated animals (doxycycline high $+$ PD and doxycycline low $+$ PD), however, showed actual tumor regression within 2 days of treatment, which continued up to 19 days for doxycycline low $+$ PD animals (Fig. 7A, purple, black). Doxycycline high $+$ PD animals became dehydrated around day 9 due to the doxycycline in the drinking water and had to be euthanized (Fig. 7A, black).

These data demonstrated that dual treatment produced a unique result: instead of just slowing tumor growth, tumors underwent regression.

Tumors were excised from animals treated for 9–11 days, fixed, embedded in paraffin and subjected to IHC (Fig. 7B–G, Supplementary Fig. S7C and S7D). Histologic examination demonstrated that tumors from the doxycycline low, doxycycline high and doxycycline $+$ PD animals all had induced FLAG-ALT as seen both by the presence of bright pink cells and a distinct pink blush in all viable tumor cells as compared with surrounding mouse tissue, while the vehicle and PD-treated samples were FLAG negative (Fig. 7B and C). The most significant histologic finding was that most tumors in all groups had large areas of necrosis, with only patches of viable tumor cells (H&E, data not shown). The total viable cells (as determined from viability in H&E X total tumor volume) E, % of pY88$^+$ viable tumor cells determined from (F, pY88 single stain, green) and (G, pY88/p27 dual stain, red/brown).

...
tumor was cleared away in the doxycycline or doxycycline + PD–treated animals.

To determine whether ALT induction in the xenograft model blocked pY88, we stained sections with pY88 antibodies either as a single stain (Fig. 7F, Supplementary Fig. S7, green) or as part of a dual pY88/p27 double staining assay (Fig. 7G, pink/brown). p27 (brown) was not detectably different in the different treatment conditions. We calculated the % of viable cells that were pY88 and found that while 20% of cells in the vehicle were pY88+, almost no detectable staining was seen in the doxycyclinehigh or doxycycline + PD–treated animals, suggesting that ALT expression had reduced pY88 (Fig. 7E). Interestingly, a larger percentage of viable cells in the PD and doxycycline-low-treated animals stained positive for pY88 than was seen in vehicle-treated animals.

Discussion

We have shown that while ER+ breast cancer cell lines are inhibited by PD in culture, they quickly adapt because they permit the degradation of p27 and a subsequent increase in cdk2 activity, allowing compensatory phosphorylation of Rb and passage into S phase. Using in vitro Rb kinase assays, we demonstrate the effect of PD on cdk4 and cdk2 activity, as opposed to its effect on just expression. While arrest might be dependent on a drug’s ability to inhibit cdk4, durability is dependent on the ability to also inhibit cdk2. Inhibition of cdk2 alone was insufficient but any combination that inhibited both cdk4 and cdk2, including use of K2i, Skp2i, or LET in combination with PD, caused arrest and increased durability. While we could have verified the role of cdk2 in PD-mediated durability by manipulation of cdk2 directly via knock-out or knockdown, we feared that this would alter the balance of p27 association and hinder interpretation. In all cases, the limiting factor in achieving a long-lasting arrest was cdk2 inhibition, supporting the idea that cdk2-specific inhibitors in combination with PD would have the most clinical efficacy. In moderately responding cells like MDA-MB-231, which require higher concentrations to achieve an IC50 arrest, PD stabilized p27 and inhibited cdk2 on its own, causing a durable arrest. To date, most cdk2 inhibitors also inhibit cdk1, which in turn causes loss of viability and associated toxicity. We have demonstrated the feasibility of targeting p27 pY88 as a nontoxic modality to inhibit both cdk4 and cdk2. We have shown that ALT binds to p27 in vitro and blocks Y88 phosphorylation, locking the p27–cyclin D–cdk4 ternary complex into the closed, off conformation. But ALT expression also stabilized p27 itself, which in turn caused cdk2 inhibition, resulting in a durable growth arrest, demonstrating that this may represent a new approach to deal with the drug resistance that occurs when only cdk4 is inhibited.

The stabilization of p27 seen in the presence of ALT inhibits cdk2, but cdk1 remains active. This discrimination distinguishes p1 blocking therapy from most cdk2is, which do not show this preference and for which toxicity has prevented their clinical use.

Cells become resistant to ALT monotherapy at extended time frames (>14 days), and it is unclear whether at these time points, cdk1 eventually compensates for loss of cdk4 and cdk2 and permits progression. Increased concentrations of ALT might have resulted in even longer-term stable arrest. Several other studies have looked at resistance in ER− lines developing in the presence of long-term, very high, less clinically relevant PD concentrations, and in these conditions, amplification of cdk6 or cyclin E or loss of Rb was detected (5, 7). Cyclin E amplification has also been shown to be responsible for PD-mediated resistance in pancreatic ductal adenocarcinoma lines, which a priori one might have felt would be responsive to PD given their near universal loss of p16 and overexpression of Ras (33). However, given the short time course of our experiments and the fact that we used lower, more clinically relevant concentrations of PD in MCF7 and T47D cells, it is unlikely that we are tracking genetic changes, but rather epigenetic modification. This is significant because any ability to escape PD-mediated arrest at the onset of treatment would set the stage for additional proliferation and the subsequent acquisition of future genetic changes to lock in the adaptation, converting the tumor ultimately to full resistance. Patients treated with PD are on treatment for three weeks, and then removed for one week, which could allow expansion of small populations of adapted clones. This suggests that inhibiting both the kinase that drives tumor progression (cdk4) as well as the kinase that will permit escape (cdk2) at the onset would provide advantages.

While ALT expression reduces both cdk4 and cdk2 activity, the ALT+PD combination reduces both activities even more and causes a synergistic arrest, increases senescence, and prevents cells from recovering when drugs were removed. While this may be a result of cdk4 and cdk2 combined inhibition, we cannot exclude the possibility that this synergy may be mediated via other molecular mechanisms. However, arrest was not seen in the p27 KD cells, and inhibition of cdk2 induces senescence by directly blocking the function of the transcription factor c-Myc (34). Interestingly, PD-mediated arrest was not detected in the p27/KD cells either. While p27 KD cells (data not shown) demonstrated p27−cyclin D1–cdk4 ternary complex, removing PD’s target, it would also release cdk2, which has been shown competent to drive proliferation in the absence of cdk4 (21).

Senescent cells irreversibly exit the cell cycle and become targets for removal by the host immune system. When we induced ALT in the xenograft model, we saw tumor reduction, which lasted >17 days, even in the setting of a compromised immune system. This suggests that while PD treatment is cytostatic, slowing down growth kinetics, ALT or ALT+PD causes some degree of cytotoxicity. We did see increased beta-gal and CC expression, but primarily we detected much smaller tumor volumes with large regions of necrosis. ALT induction in large, previously untreated tumors or even PD-resistant tumors (day 17) caused rapid tumor progression within 1 day, suggesting that we may have missed documenting ALT’s direct effect with our histology experiments by using tumors from animals treated >9 days. Our experiments did not measure overall survival as animals were continually removed from the study for histologic analysis or due to dehydration, and additional studies will be required to address this. Recently, several studies have suggested that in order for PD to cause an irreversible senescent exit from the cell cycle, additional targets must be inhibited. Loss of MDM2 or inhibition of ROS and autophagy have all been shown to increase the synergistic and senescence-inducing potential of PD. Our data is consistent with this and suggests that inhibition of cdk2 may be upstream of some of these alternative targets. It also suggests that use of ALT, with its putative one target toxicity, may circumvent differences in genetic background responsible for inducing senescence in the presence of PD (35, 36).

ALT is a naturally occurring molecule, suggesting that nature has already performed its own high-throughput screen and determined that this is a bona fide way to bind to p27 and
inhibit pY88. Use of small peptides spanning the region of p27 that binds to the Brk SH3 domain also acted as competitive inhibitors and blocked pY88 (16). However, SH3 domains show significant homology and use of a p27 peptide might block many other Y kinases. We have shown that ALT had no effect on proliferation when p27 expression was reduced by KD, suggesting that while ALT itself may have other targets, its ability to regulate proliferation occurs via p27. Use of small-molecule Brk inhibitor (Brki) (32) did not produce durable arrest nor cause an irreversible exit from the cell cycle in the replating assay (Supplementary Fig. S6H and S6I). As Brk is upstream in the signaling pathway, it is possible that inhibition of its activity could compensate for its loss, supporting the idea that targeting downstream of Brk could inhibit proliferation. Repression of Brk expression could therefore provide an alternative strategy for inhibiting proliferation in Brk-positive cell types. Indeed, we observed that expression of ALT decreased Brk expression in multiple cell lines, and have shown that lines with low pY88 and high cdk4 activity required lower concentrations of PD, while lines with high pY88 and cdk4 activity required high concentrations of PD. In cell lines, PD treatment inhibits pY88 in a dose-dependent fashion. In the xenograft model, however, we found that animals whose tumors continued to grow in the presence of treatment (PD or ALTmax) regained pY88 and actually had an increase in pY88+ cells, suggesting that this might also correlate with secondary drug resistance. Ki67 was not able to distinguish between these cells, suggesting that pY88 might serve as a surrogate marker for PD or any cdk4-inhibitory therapy’s responsiveness (including ALT).

In our xenograft system, the ALT transgene was induced when animals were fed Dox, and this strategy was a proof-of-concept efficacy study to examine the effect when ALT was expressed in every tumor cell. Clearly, for the peptide ALT to be used as a therapy it needs to be effectively delivered and shown to be nontoxic in vivo. Combination therapy with high levels of PD and several other classes of targeted therapies, including PI3K or autophagy inhibitors (36, 37), have also shown increased efficacy in tissue culture and animal models, but our study with ALT has demonstrated synergy at low, clinically relevant concentrations of PD. Additional work will be required to translate it into a clinical compound, but this proof-of-principle study has shown that ALT as a monotherapy or in combination with PD has several advantageous properties that make it worth investigating. Use of ALT has also clearly demonstrated the requirement to inhibit both cdk4 and cdk2 if long-term efficacy is to be achieved.

Disclosure of Potential Conflicts of Interest
S W. Blain has ownership interest (including patents) in Concarlo Holdings, LLC and is a consultant/advisory board member for Concarlo Holdings, LLC. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: P. Patel, V. Tsiperson, S.W. Blain
Development of methodology: P. Patel, V. Tsiperson, J. Somma, S.W. Blain
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Patel, V. Tsiperson, S.R.S. Gottesman, J. Somma
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Patel, V. Tsiperson, S.R.S. Gottesman, J. Somma, S.W. Blain
Writing, review, and/or revision of the manuscript: P. Patel, V. Tsiperson, S.R.S. Gottesman, J. Somma, S.W. Blain
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Patel, V. Tsiperson
Study supervision: P. Patel, S.W. Blain

Acknowledgments
This work was supported by CCLM Research Fund and NIH R01CA201536. All of the data was independently verified by the SUNY Compliance Management Committee.

The authors thank Safraz Hamid, Khurran Sharique and Mengru Li for expert technical assistance.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 6, 2017; revised December 5, 2017; accepted December 6, 2017; published OnlineFirst January 12, 2018.

References

Molecular Cancer Research
376 Mol Cancer Res; 16(3) March 2018

Downloaded from mcr.aacrjournals.org on March 5, 2018. © 2018 American Association for Cancer Research.
Blocking p27 Phosphorylation Potently Arrests Cell Cycle

Dual Inhibition of CDK4 and CDK2 via Targeting p27 Tyrosine Phosphorylation Induces a Potent and Durable Response in Breast Cancer Cells


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-17-0602

Supplementary Material
Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2018/01/12/1541-7786.MCR-17-0602.DC1

Cited articles
This article cites 37 articles, 12 of which you can access for free at:
http://mcr.aacrjournals.org/content/16/3/361.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/16/3/361.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.