CDK4/6 and IGF1 Receptor Inhibitors Synergize to Suppress the Growth of p16\textsuperscript{INK4A}-Deficient Pancreatic Cancers

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Abstract

Loss-of-function mutations in p16\textsuperscript{INK4A} (CDKN2A) occur in approximately 80% of sporadic pancreatic ductal adenocarcinoma (PDAC), contributing to its early progression. Although this loss activates the cell-cycle–dependent kinases CDK4/6, which have been considered as drug targets for many years, p16\textsuperscript{INK4A}-deficient PDAC cells are inherently resistant to CDK4/6 inhibitors. This study searched for targeted therapies that might synergize with CDK4/6 inhibition in this setting. We report that the IGF1R/IR inhibitor BMS-754807 cooperated with the CDK4/6 inhibitor PD-0332991 to strongly block proliferation of p16\textsuperscript{INK4A}-deficient PDAC cells \textit{in vitro} and \textit{in vivo}. Sensitivity to this drug combination correlated with reduced activity of the master cell growth regulator mTORC1. Accordingly, replacing the IGF1R/IR inhibitor with the rapalog inhibitor temsirolimus broadened the sensitivity of PDAC cells to CDK4/6 inhibition. Our results establish targeted therapy combinations with robust cytostatic activity in p16\textsuperscript{INK4A}-deficient PDAC cells and possible implications for improving treatment of a broad spectrum of human cancers characterized by p16\textsuperscript{INK4A} loss.

Introduction

The retinoblastoma tumor suppressor (pRB) pathway is a key negative regulator of cell proliferation and functionally inactivated in many cancers (1). During the G\textsubscript{1} phase of the cell cycle, cyclin-dependent kinases phosphorylate pRB, allowing E2F transcription factors to drive S-phase entry and to promote cell-cycle progression (2). The regulation of pRB is frequently compromised in tumor cells by loss of the CDKN2A locus (3). CDKN2A encodes p16\textsuperscript{INK4A}, an inhibitor of CDK4 and CDK6, and p14\textsuperscript{ARF}, an inhibitor of MDM2-mediated p53 degradation (4). Loss of p16\textsuperscript{INK4A} results in hyperactive cyclin D–CDK4/6 kinase complexes and pRB is the best-known member of a plethora of CDK4/6 substrates (5). In normal cells, cyclin D–CDK4/6 kinases integrate mitogenic signals. Elevated CDK4/6 activity promotes tumor growth by counteracting tumor suppressor mechanisms, such as senescence and apoptosis (6–8). Because the great majority of cancers with CDKN2A loss retain intact R\textsubscript{B1}, there is considerable interest in targeting CDK4/6 as a treatment strategy for these tumors, and in identifying drug combinations that potentiate the therapeutic effect.

PD-0332991 (palbociclib) is an orally active CDK4/6-specific inhibitor, which selectively inhibits CDK4 and CDK6 with \textit{in vitro} IC\textsubscript{50} values of 11 and 16 nmol/L, respectively (9). In cells that express intact pRB, PD-0332991 blocks pRB phosphorylation at sites normally targeted by CDK4/6 (including serines S807/S811 and S780) and causes G\textsubscript{1} cell-cycle arrest (9, 10). PD-0332991 has been well tolerated in phase I clinical trials and is effective in mantle cell lymphoma as well as in estrogen receptor (ER)–positive breast cancer (10–13). By contrast, single-agent treatment with PD-0332991 has produced only modest responses in most other malignancies regardless of CDKN2A mutational status.

The mechanisms of resistance to PD-0332991 in tumors predicted to have hyperactive CDK4/6 are poorly understood. Mutant forms of pRB that lack CDK phosphorylation sites give a dominant arrest in tumor cell lines (14). A combination of PD-0332991 and drugs that converge on the pRB pathway might lead to more effective CDK4/6 suppression and more stable pRB reactivation. Indeed, recent preclinical studies have shown CDK4/6 inhibition to cooperate with therapeutics targeting oncogenic drivers of p16\textsuperscript{INK4A}-mutant cancers such as pediatric astrocytoma and malignant melanoma (15, 16).

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the United States and highly resistant to existing treatments. Because CDKN2A inactivation occurs in 80% to 95% of cases and contributes to the early progression of PDAC precursor lesions whereas R\textsubscript{B1} remains intact, CDK4/6 is an attractive target in these tumors (17–21). Here we sought to identify compounds showing synergy with CDK4/6 inhibitors in PDAC. Analysis of a comprehensive screen identifying genomic markers for drug sensitivities in cancer cell lines (22) suggested that mutational status of CDKN2A may correlate with sensitivity to inhibitors.
Figure 1. CDK4/6 and IGF1R/IR inhibitors synergize to reduce viability of PDAC cells with CDKN2A deletion. A, MIA-PaCa-2 cells were treated with BMS-754807 (BMS), PD-0332991 (PD), or their fixed-ratio (1:10) combination (BMS/PD) over a concentration range (μmol/L) for 72 hours and cell viability was measured relative to DMSO-treated controls. (Continued on the following page.)
that selectively target the insulin-like growth factor I receptor (IGF1R) and the related insulin receptor (IR). We found that concurrent targeting of CDK4/6 and IGF1R/IR resulted in synergistic effects on proliferation of CDKN2A-mutant PDAC cell lines in vitro and potent suppression of tumor growth in vivo. pRB depletion or activation of the key IGF1R/IR down-stream effector mTORC1 was sufficient to render cells resistant to the synergistic drug combination, suggesting that alterations in these pathways converge to promote the growth of CDKN2A-deleted PDAC cells.

Materials and Methods

Cell lines and drugs

All cell lines were provided and authenticated by The Center of Molecular Therapeutics (Massachusetts General Hospital Cancer Center) and cultured in RPMI medium containing 5% fetal bovine serum (FBS), except for YAPC, HPAC, HPAF-II, and Panc1, which were grown in DMEM with 10% FBS, and MIA PaCa-2, which was grown in DMEM/F12 with 5% FBS. Cell media were supplemented with penicillin/streptavidin and glutamine, and cells were maintained at 37°C and 5% CO2. BMS-754807, OSI-906, and PD-0332991 were purchased from ChemieTek, temsirolimus, and AZD8055 were from Selleck Chemicals. The compounds used to treat cells were dissolved in DMSO at 10 mmol/L and stored at −80°C.

Cell viability and synergy assays

Cells were seeded in 96-well plates at 2,000 to 4,000 cells/well depending on the growth characteristic of the cell line so that each was in growth phase at the end of the assay (60%-80% confluency). The following day, cells were treated in duplicate with single agents and their fixed-ratio combination for 72 hours over an 8-point, 128-fold concentration range, which was centered on the single-agent concentrations that inhibited viability by 50% (IC50). Cell viability was measured by staining the cells for 4 hours with 100 μg/mL resazurin (Sigma) whose conversion was detected at wavelengths of 510/595 nm using a SpectraMax M5 plate reader (Molecular Devices). The R software package mixlow (23) was applied to derive Loewe synergy indexes from the sensitivities to the single agents and their combination.

Mouse treatment study

One million YAPC cells in 100 μL PBS were injected subcutaneously into each flank of 10-week-old female CB17/Prkdcscid/lcrCrl mice (Charles River Laboratories). After 1 week, tumor volumes were determined using electronic calipers to measure the length (L) and width (W) and calculated according to the formula (L × W2)/2. The mice were separated into four groups matched for tumor volume (50-60 mm3), which were randomly assigned to treatment arms. For oral administration, BMS-754807 was dissolved in sterile polyethylene glycol 400 (PEG400)/water (4:1, v/v) and PD-0332991 was dissolved in sterile 50 mmol/L sodium lactate (pH 4). The drugs or their vehicles were administered by gastric gavage every other day starting from day 8 after injection, with PD-0332991 at 75 mg/kg being fed in the morning and BMS-754807 at 15 mg/kg in the evening (minimum of 6 hours between PD-0332991 and BMS-754807). Tumor volumes were assessed twice weekly as described above. For pharmacodynamic evaluation at the study endpoint, tumor tissue was harvested 3 hours after the final BMS-754807 dose and frozen in liquid nitrogen or fixed in 10% formalin. All mouse studies were conducted through Institutional Animal Care and Use Committee (IACUC #2005N000148)-approved animal protocols in accordance with institutional guidelines.

Additional Materials and Methods are described in the Supplementary Methods.

Results

CDK4/6 and IGF1R/IR inhibitors synergize in CDKN2A-deleted PDAC lines

To identify targeted therapies that sensitize cancer cells to the CDK4/6 inhibitor PD-0332991, we examined drug sensitivity data from a comprehensive cancer cell line screen (22). As expected, sensitivity to PD-0332991 was associated with CDKN2A loss and lack of R11 mutations. We noted that CDKN2A was the cancer gene whose mutational status most frequently correlated with differential drug response (Supplementary Fig. S4 of ref. 22). The correlations with CDKN2A status were particularly evident in PDAC cell lines where 32 drugs showed a trend toward decreased efficacy in the context of mutant CDKN2A versus wild-type CDKN2A. However, the number of CDKN2A wild-type PDAC lines included in the screen was small (N = 3), thus any hypothesis coming from this data needed to be verified experimentally. We reasoned that if the difference in drug sensitivity was indeed associated with inactivation of CDKN2A then it should be possible to recapitulate this effect by combining these drugs with PD-0332991 in CDKN2A-mutant PDAC cells, because this would mimic functional p16INK4A (Supplementary Fig. S1).

The drugs with decreased sensitivities in CDKN2A-mutant PDAC cells included three different small molecule IGF1R/IR kinase inhibitors (Supplementary Table S1 and Fig. S2). BMS-

(Continued)
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754807, with in vitro IC_{50} value of less than 2 nmol/L for IGF1R/IR, is the most potent of these compounds (24). IGFIR and IR are receptor protein tyrosine kinases that upon ligand binding phosphorylate IR substrate (IRS) proteins, which activate the PI3K–Akt–mTOR as well as the RAS-MAPK signaling axes (25), key effectors in oncocgenic KRAS-driven PDAC development (26).

To test whether targeting of CDK4/6 sensitizes PDAC cells to IGF1R/IR inhibitors, we used a Loewe synergy assay, which allows for testing of drug combinations in fixed ratios (23). PDAC cells were treated with PD-0332991, BMS-754807, and their combination over an 8-point, 128-fold concentration range for 72 hours, and viability was measured by resazurin. The combination of PD-0332991 and BMS-754807 reduced viability of the PDAC cell line MIA-PaCa-2 significantly at doses that had comparatively minor effects in the single-agent treatments (Fig. 1A). The combination of PD-0332991 with a second IGFIR/IR inhibitor (OSI-906) resulted in similar sensitivities (Fig. 1B), suggesting that the effects of the drug combination are because of on-target IGF1R/IR inhibition (Supplementary Fig. S5), suggesting that CDKN2A loss is the primary requirement for the drug synergy. Of note, depletion of CDK4 and CDK6, but did not affect cells treated with control siRNA or CDK6 siRNA. These data suggest that targeting of CDK4/6 sensitizes PDAC cells to IGF1R/IR inhibition and thus cooperates to reduce PDAC cell viability.

Next we carried out a systematic assessment of drug synergy across a panel of PDAC cell lines. Loewe synergy indexes were derived from the sensitivities to the single agents and their combination using the R software package mixlow (23) in 14 PDAC cell lines, including lines with homozygous CDKN2A deletion, CDKN2A point mutations, and wild-type CDKN2A (Fig. 1D and Supplementary Fig. S4). BMS-754807 (15–500 nmol/L) and PD-0332991 (0.15–5.0 μmol/L) synergized significantly to reduce viability up to 70% to 80% in the cell lines MIA-PaCa-2, PSN1, HuP-T3, and YAPC and showed synergy limited to a part of the effect range in the HPAC and AsPC-1 lines (Fig. 1E). No synergy was observed at high doses (PD-0332991 > 10 μmol/L), most likely because of off-target drug effects.

All cell lines susceptible to the drug synergism harbor deletions that span the CDKN2A locus and frequently affect adjacent genes (Sanger COSMIC Cell Line Project). To assess the potential role of these codeleted genes, the drug synergy status of the cell lines was compared with their CDKN2A deletion, CDKN2A point mutations, and wild-type CDKN2A (Fig. 1D and Supplementary Fig. S4). BMS-754807 (15–500 nmol/L) and PD-0332991 (0.15–5.0 μmol/L) synergized significantly to reduce viability up to 70% to 80% in the cell lines MIA-PaCa-2, PSN1, HuP-T3, and YAPC and showed synergy limited to a part of the effect range in the HPAC and AsPC-1 lines (Fig. 1E). No synergy was observed at high doses (PD-0332991 > 10 μmol/L), most likely because of off-target drug effects.

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sufficient to generate sensitivity to the drug combination. Because p16INK4A is frequently lost by means other than genetic deletion, we used siRNA against CDK4/6 to verify that the proliferation of 2 sensitive lines depends on CDK4/6, whereas the proliferation of 2 CDKNA2 wild-type lines does not (Fig. 1G). In conclusion, we have identified a drug combination that synergistically inhibits viability in more than half (4/7) of the CDKNA2-deleted PDAC cell lines tested.

**Concurrent inhibition of CDK4/6 and IGF1R/IR enhances G1 arrest and senescence**

Cyclin D–CDK4/6 kinase complexes drive G1–S cell-cycle progression. We performed cell-cycle analysis to examine how the CDK4/6 inhibitor affects viability when combined with IGF1R/IR inhibitor. Treatment of MiaPaCa-2 cells with PD-0332991 for 24 hours resulted in an increased G1–G0 population (from 41% to 61%) and reductions in the proportion of cells in S-phase (45% to 30%) and G2–M (14% to 8%; Fig. 2A). In combination with BMS-754807, G1–G0 arrest was markedly augmented (15% S, 77% G1–G0, and 7% G2–M), whereas BMS-754807 alone did not significantly affect cell-cycle distribution (Fig. 2A). To determine the timing of this enhanced G1–G0 arrest, we investigated shorter treatment durations. PD-0332991 reduced the S-phase population more potently to 13% and 10% after 8 and 16 hours, respectively (Fig. 2B). BMS-754807 addition decreased this fraction further to 4% at 16 hours, but had no added effect at 8 hours. These data suggest that IGF1R/IR inhibition synergizes with CDK4/6 inhibition by enhancing the PD-0332991 induced G1–G0 arrest starting from 16 hours after addition of the drugs and by maintaining the G1–S block more efficiently. The enhanced G1–G0 arrest at 24 hours was confirmed for the sensitive PSN1, HuP-T3, and YAPC lines (Fig. 2C).

Prolonged G1–G0 arrest can lead to senescence and PD-0332991 has previously been shown to cause senescence in solid tumors (6). Correspondingly, staining for senescence-associated β-galactosidase (SA-β-gal; ref. 27) revealed that combination treatment induced high levels of senescence compared with the single agents (combination treatment 61% SA-β-gal positive cells vs. BMS-754807 13% and PD-0332991 42%; Fig. 2D). Comparable results were obtained for HuP-T3 and YAPC cells, in which the drug combination induced a significant increase of SA-β-gal. These findings were further corroborated by counting population doublings of HuP-T3 and YAPC cells cultured in the presence of the inhibitors and passaged every 4 days. Combination treatment prevented population doubling in HuP-T3 cells and strongly slowed it in YAPC cells treated with half the drug doses compared with the HuP-T3 experiments, consistent with induction of a cytostatic senescence response, whereas BMS-754807 had no significant effect on cell proliferation and PD-0332991 showed an intermediate effect (Fig. 2E).

**Combined CDK4/6 and IGF1R/IR inhibition is synergistic in vivo**

We evaluated whether combined CDK4/6 and IGF1R/IR inhibition is synergistic in vivo and could be a potential therapeutic approach against PDAC. Xenograft tumors were generated with YAPC cells, which are sensitive to the drug combination in vitro and form tumors within a week after subcutaneous injection into SCID mice. Once tumors reached 50 mm³ in volume, the mice were grouped and treatment was initiated with submaximal tolerated doses of each inhibitor. Administration of the single agents had no significant effect on the tumor volume, paralleling the in vitro results (Fig. 3A). In contrast, the combination therapy slowed tumor growth approximately 2.5-fold and significantly reduced tumor burden as compared with both the vehicle and the monotherapy cohorts (Fig. 3A), suggesting that concurrent targeting of CDK4/6 and IGF1R/IR is synergistic in vivo. This efficacy was achieved in the absence of any apparent adverse effects. Consistent with the in vitro findings, we observed increased staining for the senescence marker trimethylated lysine 9 histone H3 in PD-0332991 treated tumors, which was further enhanced by the drug combination (Supplementary Fig. S6).

To confirm that the drugs inhibited their respective targets in vivo, we analyzed the tumors at the study endpoint for CDK4/6 and IGF1R/IR signaling. BMS-754807 alone or in combination with PD-0332991 reduced tyrosine autophosphorylation of IGF1R/IR as well as phosphorylation of the IGF1R/IR downstream effector AKT (Fig. 3B). Single-agent PD-0332991 did not affect these phosphorylation events. To read out the activity of mechanistic target of rapamycin complex 1 (mTORC1) downstream of IGF1R/IR-AKT, we detected S6K1 phosphorylation at the mTORC1 site T389. Although BMS-754807 alone had only a slight effect, the drug combination abolished S6K1 phosphorylation, suggesting that IGF1R/IR-AKT inhibition only translated to effective mTORC1 suppression in the presence of the CDK4/6 inhibitor, which did not decrease S6K1 activation by itself. To evaluate CDK4/6 activity in the tumors, we detected pRB phosphorylation at the CDK4/6 sites S807/S811. The drug combination notably reduced phospho-pRB levels, whereas either single-agent treatment led to a minimal decrease in pRB phosphorylation. In accordance with a previous study noting that PD-0332991 stabilizes cyclin D1 (28), we observed elevated cyclin D1 levels in tumors that had been treated with the CDK4/6 inhibitor alone or in combination, indicating that PD-0332991 targeted CDK4/6 in both regimes. In vitro experiments confirmed that BMS-754807 enhances PD-0332991-mediated inhibition of pRB phosphorylation in YAPC cells even under conditions optimal for drug delivery (Supplementary Fig. S7). Together, these data indicate that the single agents affected their respective targets, but did not suppress tumor growth. In contrast, the drug combination elicited a tumoricidal response corresponding with enhanced S6K1 inhibition and pRB reactivation.

**The drug combination specifically reduces S6K1 phosphorylation**

To elucidate the mechanistic basis for the drug synergy, we investigated the phosphorylation of the CDK4/6 substrate pRB and of IGF1R/IR effectors after addition of BMS-754807, PD-0332991, and their combination. PSN1 cells were treated with PD-0332991, which abrogated pRB phosphorylation 16 hours after drug addition (Fig. 4A). This effect was not present at time points as early as 2 hours, most likely because of the fact that asynchronously cycling cell populations were used. CDK4/6...
6 inhibition was combined with low doses of BMS-754807 that dampened AKT activation in a dose-dependent manner (Fig. 4A). As observed in the xenograft tumors, BMS-754807–mediated inhibition of S6K1 T389 phosphorylation was enhanced by PD-0332991 (Fig. 4A). Thus, these compounds cooperate to inactivate mTORC1 in PDAC cells \textit{in vitro} and \textit{in vivo}.

To address whether this effect may be responsible for the drug synergy, we compared the effects on S6K1 phosphorylation in two lines that displayed the synergy with two lines that did not show the synergistic effects. Although concurrent treatment with BMS-754807 and PD-0332991 significantly reduced S6K1 phosphorylation in the sensitive MIA-PaCa-2 and HuP-T3 cell lines (Fig. 4B), it had no effect in PANC-03-27 or CFPAC-1 cells, in which the inhibitors do not synergize. The inhibitors did not affect ERK1/2 activity, suggesting that the signaling alterations were specific to the PI3K-mTOR pathway (Fig. 4B).

**Cyclin D1 or CDK4/6 depletion sensitizes PDAC cells to S6K1 inhibition**

The cooperation of CDK4/6 inhibitor with IGF1R/IR targeting on the level of mTORC1 raised the question whether this effect of PD-0332991 was through inhibition of CDK4/6 or off-target. To distinguish between these possibilities, cyclin D1 or CDK4/6 were depleted with siRNA followed by BMS-754807 treatment for 2 hours. Cyclin D1 knockdown sensitized MIA-PaCa-2 and YAPC cells to BMS-754807–mediated inhibition of S6K1 phosphorylation (Fig. 5A). Identical results were obtained in YAPC cells after CDK4/6 depletion. CDK4/6 knockdown in MIA-PaCa-2 cells lead to increased phospho-S6K1 that was strongly reduced by BMS-754807, which had no effect on control siRNA–transfected cells (Fig. 5B). These findings indicate that the PD-0332991–mediated sensitization to mTORC1 inhibition is specific and could thus be shared by other CDK4/6 inhibitors.

**CDK4/6 and mTOR inhibitors synergize in CDKN2A-deleted PDAC lines**

The observed effect on phosphorylation of S6K1, a primary substrate of mTORC1, raised the question whether mTORC1 inhibition would suffice to synergize with CDK4/6 inhibition. Indeed, PD-0332991 synergized with temsirolimus, an allosteric mTOR inhibitor that specifically targets mTORC1, to reduce
viability of not only the PDAC lines sensitive to BMS-754807/PD-0332991, but also the additional lines PANC-03-27, BxPC-3, and PANC-10-05 (Fig. 6A and Supplementary Fig. S8). IGF1R/IR signaling may not be required for mTORC1 activity in these lines. Remarkably, combined CDK4/6 and mTORC1 inhibition synergized, at least partially, in all of the CDKN2A-deleted PDAC lines tested (Fig. 6B).

Catalytic mTOR kinase inhibitors, such as AZD8055, have recently been developed in order to target both mTOR complexes and abolish mTORC1 activities more effectively than rapalogs. AZD8055, which is two orders of magnitude more potent than temsirolimus in HuP-T3 cells, synergized with PD-0332991 (Fig. 6C), indicating that the effects of these compounds can be further enhanced by concurrent CDK4/6 inhibition. This result excludes any catalytic off-target effects of PD-0332991 on mTOR kinase itself, as the combination of two catalytic mTOR inhibitors would have been expected to be additive, rather than synergistic.

**TSC2 or pRB depletion restores growth in the presence of CDK4/6 and IGF1R/IR inhibitors**

To evaluate the role of mTORC1 in mediating the synergistic drug effects, we performed sensitivity assays with cells after knockdown of tuberous sclerosis 2 (TSC2). TSC2 is an integral part of the tuberous sclerosis complex and incorporates signaling inputs from AKT, RSK, nutrient, and stress signaling to restrain mTORC1 activity. TSC2 depletion in three different cell lines (PSN1, MIA-PaCa-2, and HuP-T3) restored cell proliferation in the presence of PD-0332991 and BMS-754807/PD-0332991 (Fig. 7A), suggesting that mTORC1 activation is sufficient to render cells resistant to the CDK4/6 inhibitor and the combination therapy. This effect was specific to the combination of CDK4/6 and IGF1R/IR inhibitors, as TSC2 depletion did not completely prevent growth inhibition in the presence of PD-0332991 and temsirolimus, which blocks mTORC1 downstream of TSC2 (Fig. 7A).

The best-established cyclin D–CDK4/6 substrates are the pRB family members. Because pRB fulfills a unique role among the pocket proteins in mediating oncogene-induced senescence, we tested whether loss of pRB would render cells insensitive to the synergistic drug combination. We made stable MIA-PaCa-2 and HuP-T3 lines, in which pRB was depleted with two different shRNAs, and determined their sensitivities to BMS-754807, PD-0332991, and their combination. The pRB-depleted cell pools were less sensitive to either drug alone as well as to the drug combination (Fig. 7B), with the shRNA construct that gave the stronger reduction in pRB levels (shRB1-2) conferring a higher degree of resistance. These data suggest that both IGF1R/IR and CDK4/6 inhibitors engage the pRB tumor suppressor pathway and that pRB loss can alleviate the synergistic effects of the drug combination.

**Discussion**

Here we identify inherent resistance of CDKN2A-mutant PDAC cells to CDK4/6 inhibitor and describe drug combinations that efficiently reactivate the pRB tumor suppressor pathway to trigger cell-cycle arrest and senescence. CDKN2A loss is frequent in PDAC and contributes to PDAC progression by bypassing oncogenic RAS-induced senescence (29–31). Although several CDKN2A-mutant PDAC cell lines indeed depend on CDK4/6, only 1 of 13 cell lines with CDKN2A mutations is sensitive to PD-0332991 (AsPC-1 IC50 0.25 μmol/L; Supplementary Table S1). Our data suggest that CDKN2A-deleted PDAC cells are intrinsically resistant to CDK4/6 inhibition alone, because PD-0332991 failed to induce sustained growth arrest and senescence at concentrations that initially block CDK4/6 activity as evidenced by decreased pRB phosphorylation. This inherent resistance to PD-0332991 is likely because of elevated P38K-mTOR activity. KRAS-driven PDAC formation and maintenance has been demonstrated to depend on active P38K signaling (26) that suppresses oncogenic RAS-induced senescence in pancreatic cancer (30). The results described here show that targeting of IGF1R/IR, which...
potently inhibited the PI3K effector AKT, in combination with CDK4/6 inhibition resulted in prolonged cell-cycle arrest and increased SA-β-gal activity in vitro as well as tumor stasis in vivo, in accordance with the drug combination reinstating oncogene-induced senescence.

Despite the evidence for IGF1R being an important target in PDAC, clinical trials with IGF1R-blocking antibodies have been disappointing (32). Our study provides evidence that CDKN2A-mutant PDAC cells are inherently insensitive to IGF1R/IR inhibition alone. CDK4/6 has long been known for its role to promote growth in model organisms and its constitutive activation following p16INK4A loss may provide growth signals in the absence of IGF1R/IR signaling. Although the IGF1R/IR inhibitor BMS-754807 repressed AKT activation at low concentrations...
nanomolar doses, it failed to efficiently inhibit the mTORC1 substrate S6K1 at these concentrations and impacted viability only at doses that were 2 orders of magnitude higher. Persistent activation of mTOR has recently been described to correlate with resistance of breast cancers to PI3K inhibitor (33) as well as with resistance of melanoma to RAF or MEK inhibitor (34). Similarly, here we found that the response to concurrent CDK4/6 and IGF1R/IR inhibition correlated well with decreased mTORC1 activity. PD-0332991–mediated sensitization to mTORC1 inhibition could be recapitulated with siRNA against either cyclin D1 or CDK4/6, suggesting that other CDK4/6 inhibitors could share this activity. Overexpressed cyclin D has been previously shown to bind the mTORC1 negative regulator TSC2 (35), providing evidence of a direct physical connection between cyclin D1–CDK4/6 and regulation of mTORC1. To date, we have been unable to confirm this interaction between the endogenous proteins. Our results do, however, suggest that TSC2 likely plays a critical role in this synergistic drug response, as its depletion rendered cells resistant to the combination of CDK4/6 and IGF1R/IR inhibitors. Furthermore, PD-0332991 synergized with the rapalog temsirolimus. This drug combination may be more easily translatable, because it expanded the number of sensitive CDKN2A-mutant lines and rapalogs have been well tolerated in clinical settings.

The synergistic effects seen by combining PD-0332991 with other treatments apply to other cancer types. A recent drug synergy screen and network modeling study suggested that hyperactive CDK4 and IGF1R signaling also cooperate in liposarcoma (36). Targeting additional cancer types may require the identification of context-specific oncogenic drivers that cooperate with CDK4/6 deregulation (37). Apart from the clinical benefit of a combination of PD-0332991 and letrozole seen in ER-positive breast cancer, several preclinical studies have shown PD-0332991 to be effective in combination with drugs targeting mitogenic drivers in pediatric astrocytoma, melanoma, and prostate cancer (15, 16, 28). In conclusion, our work suggests that CDKN2A loss in PDAC can be exploited by concurrent targeting of CDK4/6 and the IGF1R/IR-mTOR axis in order to efficiently reactivate pRB tumor suppressor functions and suppress growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Figure 7. TSC2 or pRB depletion restores growth in the presence of CDK4/6 and IGF1R/IR inhibitors. A, relative viability of PSN1, MIA-PaCa-2, or HuP-T3 cells treated with 0.25 μmol/L BMS-754807 (BMS), 2.5 μmol/L temsirolimus (Tem), 2.5 μmol/L PD-0332991 (PD), or their combinations (BMS/PD and Tem/PD) after knockdown of TSC2 (siscbl, control siRNA). B, MIA-PaCa-2 and HuP-T3 cells were infected with two different shRNAs targeting RB1 (shRB1-1, shRB1-2) or control shRNA (shscbl). After puromycin selection, cells were treated for 72 hours with 0.25 μmol/L BMS-754807, 2.5 μmol/L PD-0332991, or their combination. Error bars represent SEM for all experiments.
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Emerging landscape of oncogenic signatures across human cancers.
CDK4/6 and IGF1 Receptor Inhibitors Synergize to Suppress the Growth of p16INK4A-Deficient Pancreatic Cancers

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