Protein kinase D3 sensitizes RAF inhibitor RAF265 in melanoma cells by preventing reactivation of MAPK signaling

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Abstract

The RAS-RAF signaling cascade plays a central role in the regulation of cell proliferation and survival. Mutations in RAS or BRAF occur in more than 30% of all human cancers, making these proteins very attractive therapeutic targets. However, numerous efforts to inhibit activated RAS with a small molecule have not been successful. As an alternative therapeutic strategy, RAF and MEK inhibitors have been developed to inhibit an oncogenic pathway downstream of RAS. Current pre-clinical and clinical experience has demonstrated that drug resistance frequently occurs in tumors with RAS or BRAF mutations when treated with RAF or MEK inhibitors. In order to better understand the molecular mechanisms of drug resistance, we took a chemosensitization approach to screen for genes that when knocked-down could enhance the cell killing effect of the RAF inhibitor (RAF265) in a BRAF (V600E) mutant melanoma cell line that was resistant to RAF265 treatment. We show that knock down of Protein kinase D3 (PRKD3) enhances cell killing of RAF and MEK inhibitors across multiple melanoma cell lines of various genotypes and sensitivities to RAF265. PRKD3 knock down cooperates with RAF265 to prevent the reactivation of the MAPK signaling pathway, to interrupt cell cycle progression, induce apoptosis, and inhibit colony formation growth. Thus, PRKD3 provides a potential cancer target to develop effective therapeutic strategies to overcome drug resistance encountered with RAF or MEK inhibitors.

Introduction

RAS-RAF mitogen-activated protein kinase (MAPKs) signaling cascade plays a central role in the regulation of cell proliferation and survival, while the deregulation of this pathway frequently occurs in human cancers (1-3). As mutations in RAS or BRAF occur in more than 30% of human cancers, these proteins are very attractive therapeutic targets in many cancer types. Among them, BRAF mutations
occur in about 7% of human cancers, with highest prevalence in melanomas (66%) and thyroid (35-70%) tumors (4, 5). Interestingly, 80% of all BRAF mutations are concentrated on a single substitution of glutamic acid for valine (V600E) within the kinase domain (4). Compared to BRAF, mutations in the two RAF isoforms ARAF and CRAF are rarely found in human cancers, which is likely due to lower basal kinase activities (6, 7). All three RAS isoforms (KRAS, NRAS, and HRAS) are found mutationaly activated in 30% of all human cancers, with highest prevalence in pancreas (90%), colon (50%), thyroid (50%) and lung (30%) cancers (1, 2). Although the RAS oncogene has been studied for more than three decades, there is no drug on the market that sufficiently inhibits RAS, despite extensive efforts to inhibit activated RAS with low molecular weight inhibitors (3, 8). As an alternative therapeutic strategy, RAF and MEK inhibitors have been developed to inhibit the pathway downstream of RAS, and only one RAF inhibitor Sorafineb has been approved by FDA (Food and Drug Administration) and several inhibitors are still undergoing clinical trials (8). However, clinical responses from these reagents are not as effective or durable as expected, drug resistance frequently occurs in tumors treated with RAF or MEK inhibitors (2, 8). PLX4032 appears to be an effective RAF inhibitor in malignant melanoma with an overall response rate of 81%, but responsive time from patients ranged from 2 to more than 18 months and this could limit the long term efficacy of the drug (9). A recent report suggested that up-regulation of N-RAS and other RTK signals such as PDGFRβ are responsible for the acquired resistance to PLX4032 (10). Potential mechanisms of resistant to RAF or MEK inhibitors in RAS or BRAF mutant cancers can be attributed to either co-activation of parallel or downstream survival pathways prior to drug treatments or compensatory activation of alternative survival pathways upon drug administration (11-22). In either situation combinatorial inhibition of multiple survival pathways is required to achieve potent antitumor effects.
RAS signaling pathway is more complex than a linear RAS-RAF-MEK signaling cascade (1). Activated RAS protein can interact with more than 20 effectors including RAF, phosphatidylinositol 3-kinases (PI3K), RAC, RAL and phospholipase C epsilon (PLCe) to regulate cell proliferation, survival and differentiation (2). There are multiple feedback loops known to activate downstream of RAS which are differentially regulated depending on the genetic background and tumor lineage being studied (23, 24). These feedback loops can lead to compensatory activation of parallel survival pathways upon drug treatments, and tumor cells are flexible at utilizing the signal pathways for growth and transformation, resulting in rapid drug resistance. Clinical reports show that activation of the MAPK pathway was induced via S6K-PI3K-RAS signaling in tumor samples from patients treated with RAD001, an inhibitor of PI3K pathway (17). In neuroendocrine tumor cells, inhibitor of RAF strongly induced AKT phosphorylation reflecting an activation of PI3K pathway, and inhibitors of PI3K pathways induced ERK phosphorylation indicating MAPK pathway activation (25). Dual targeting of both PI3K and MAPK signaling pathways showed more potent antitumor effect than single treatment alone (17, 21, 25).

Despite the intensive research efforts on RAS signaling, our understanding of its regulation is still limited. In order to identify potential modulators of RAS signaling pathways and to uncover the molecular mechanisms underlying resistance to RAF or MEK inhibitors, we performed a siRNA screen in combination with a RAF inhibitor (RAF265) to identify genes and/or pathways that sensitize to RAF265 treatment in a BRAF (V600E) mutant melanoma cell line (A2058) which is insensitive to RAF265 induced cell death. Using this approach we identified Protein kinase D3 (PRKD3), that when knocked-down could enhance cell killing by RAF265 in A2058. PRKD3 is one of three members in the Protein kinase D (PKD) family which includes PRKD1 and PRKD2. The physiological functions of PRKD3 are not well understood. Similar to PRKD1 and PRKD2, PRKD3 has been shown to function in protein trafficking and as a HDAC kinase (26, 27). One report demonstrated that PRKD3 enhanced CCK-mediated pancreatic amylase
secretion via MEK/ERK/RSK signaling and this process was activated by GI hormone (28). Another report showed that PRKD3 expression levels were elevated in human prostate cancers compared to normal tissues, PRKD3 over-expression activated AKT and ERK in prostate cancer cell lines, and promoted cell growth and survival (29). PRKD3’s essential role in prostate cancer cells is likely due to regulation of MAPK signaling (32). Using A2058 and A375 (RAF265 sensitive cell line) as melanoma cellular models, we demonstrated that PRKD3 inhibition cooperates with RAF265 to prevent the reactivation of MAPK signaling pathway, induce PARP cleavage and caspase activity, interrupt cell cycle progression, and inhibit colony formation. Finally, we showed that the PRKD3 inhibition sensitizes with multiple RAF and MEK inhibitors in a panel of melanoma cell lines suggesting that PRKD3 functionally interacts with the MAPK signaling pathway. Thus, PRKD3 provides a potential cancer target to develop effective therapeutic strategies to overcome or prevent drug resistance from RAF or MEK inhibitors.

Materials and Methods

Kinome siRNA synthtic lethal screen and data analysis

The kinome siRNA smartpool library directed against 779 kinases was purchased from Dharmacon (cat # G-003500). RNAi screens were performed with two compound doses for 12 plates in A2058 cells (6 plates, in duplicate). Briefly, 4 μl of 206 nM siRNAs in serum-free medium were stamped into each well, 0.03 μl of Dharmafect 1 in 4 μl serum-free medium was mixed and added into each well followed by 30-minute incubation at room temperature to form a lipid/siRNA complex. Then 1500 cells in 25 μl complete medium were loaded on top of siRNA/Lipid complex. Final concentration of siRNA for each reaction was 25 nM. The cells were incubated at 37°C with 5% CO₂. At 24 hours after siRNA transfection, 5 μl of RAF265 was added into each well to make the final concentration of RAF265 at 0.4 μM. Cell viability was analyzed at 72 hours post-RAF265 treatment using CellTiter-Glo (CTG) Assay (Promega) and
data were acquired using an Envision (PerkinElmer). From the primary screen, the CTG value was normalized by one-dimensional (1D) normalization scheme based on plate median of screened 384-well plates. The formula for calculating the 1D normalized value (denoted as \( x_{1D} \)) is 
\[
\frac{x}{\text{median}_{\text{plate}} x_{1D}} = \log x^{1D},
\]
where \( x^{1D} = \frac{\text{CTG}}{\text{median}_{\text{plate}} \text{CTG}} \). The normalized Z score (NZ) was calculated for every \( x_{1D} \). The formula used for calculating \( \text{NZ}_{1D} \) is:
\[
\text{NZ}_{1D} = \frac{x_{1D} - \text{median}(x_{1D})}{\text{MAD}(x_{1D})}.
\]
\( \text{MAD}(x_{1D}) \) is the Median Absolute Deviation of \( x_{1D} \) and is equal to \( 1.4826 \times \text{median} (|x_{1D} - \text{median}(x_{1D})|) \).

Additional Materials and Methods are shown in the supplemental data.

Results

A kinome synthetic lethal siRNA screen with RAF265 in A2058 melanoma cells

RAF265 is an orally bioavailable small molecule that is known to inhibit CRAF, wild type BRAF, mutant BRAF (V600E) and VEGFR-2, and is currently in phase I clinical trials for advanced melanoma (30, 31). As other RAF or MEK inhibitors, RAF265 showed less efficacy in tumors with RAS mutations compared to tumors with a BRAF mutation (30). RAF265 also shows resistance in some tumors bearing the BRAF V600E mutation such as the melanoma cell line A2058. Using A2058 as the cellular model, we performed a synthetic lethal siRNA screen to identify genetic sensitizers of RAF265. The siRNA smartpool kinome library of 779 kinases was used for the screen. We chose to run our screen in the presence of 0.4μM RAF265 where 20% growth inhibition was achieved, leaving a suitable window for additional, siRNA-enhanced cellular toxicity. As positive technical controls, we used siRNAs targeting Polo-like kinases 1 (PLK1), which is a regulator of mitosis and when depleted results in cell lethality (32). BRAF siRNAs were used as the biological positive controls and Luciferase (LUC) siRNAs were used as the negative controls. Duplicates were used for each siRNA and normalized Z-scores (NZ) were calculated. As shown in Figure 1A and 1B, control LUC siRNA centered at NZ of 0 and PLK1 siRNAs have a NZ ranging
from -17 to -28, indicating a robust signal to noise in the screens. We considered a primary hit a gene whose knock-down has a minimal effect on cell growth (NZ<1.5) in the absence of RAF265, but enhances cell killing (NZ> 4.5) in the presence of a low dose of RAF265 (Figure 1C). Twelve primary hits that met our predefined criteria were identified including MGC5601, C9ORF96, ALPK1, CDC7, PRKD3, PDGFRB, PAPSS2, PIK3R4, RAGE, RPS6KB2, DAPK1 and MAPK11 (Figure 1C). To further evaluate the sensitizers, we generated dose curves of RAF265 for all the hits with smartpool siRNAs, and observed significant IC50 shifts with four hits; ALPK1, MAPK11, PRKD3 and PIK3R4 (Figure 1D). The mRNA knock-down efficiencies of the four target genes was confirmed by RT-PCR (Figure 1E).

**PRKD3 inhibition sensitizes RAF265 to kill A2058 melanoma cells**

To prioritize hits to follow-up and to minimize off-target effects when using a smart pool siRNA approach, we tested individual siRNAs of the four hits for sensitization with RAF265. Based on the knock-down levels of the target genes, we selected two potent siRNAs from the four individual siRNAs for ALPK1, MAPK11, PIK3R4 and PRKD3 (left panels, Figure 2A, 2B, 2C, 2D), and measured sensitization over a RAF265 dose response (right panels, Figure 2A, 2B, 2C, 2D). Among them, PRKD3 was the only gene where multiple independent siRNAs resulted in a significant IC50 shift for RAF265 and distinguished itself as the best sensitizer identified from this study (Figure 2D). To confirm the sensitization observed between PRKD3 siRNAs and RAF265, we established three independent stable cell lines with inducible PRKD3 shRNAs expressed in A2058 cells. The sequences of PRKD3 shRNAs were different from the PRKD3 siRNAs used in the screen. The three PRKD3 shRNAs when induced with DOX resulted in 2-8 fold IC50 shifts for RAF265 and the sensitization correlated with knock-down efficiencies of PRKD3 shRNAs (Figure 2E). For the control condition without DOX induction, knock-down of PRKD3 protein levels were not observed, and the sensitization was not significant (Figure 2E). We
demonstrated that knock-down of PRKD3 using multiple siRNAs and shRNAs enhances the cell killing effects of RAF265 in the resistant melanoma cell line A2058.

**PRKD3 inhibition sensitizes RAF265 to prevent reactivation of MAPK signaling, induce PARP cleavage, increase caspase activity, interrupt cell cycle progression, and inhibit colony formation in A2058 cells**

We analyzed pERK and pAKT levels after RAF265 treatment with or without PRKD3 knock-down in A2058 cells. Efficient PRKD3 knock-down was achieved by siRNA transfection as shown by western blot (Figure 3B). RAF265 treatments resulted in the reduction of pERK levels in a dose dependent manner at 2 hours post-RAF265 treatment (Figure 3A). However, at 72 hours post-RAF265 treatment, both pERK and pAKT levels were up-regulated as shown with control siRNA treatments, pERK levels were increased 1.6 to 4.2 fold and pAKT levels were increased more than 2-fold (Figure 3B). After PRKD3 siRNA treatments, pERK levels were reduced to 0.2-0.8, which corresponds to a 2-20-fold change compared to the condition without PRKD3 siRNA transfection (Figure 3B). The pAKT levels were reduced to 0.4-0.6, which corresponds to a 4-6 -fold change compared to the condition without PRKD3 siRNA transfection (Figure 3B). In our system, total ERK and AKT levels were not affected by compound treatment or PRKD3 knock-down (Figure 3B). Similar results were obtained with PRKD3 shRNA transfection (Supplemental Figure 1). Collectively, we demonstrated that PRKD3 knock-down prevented the reactivation of pAKT and pERK induced by RAF265 treatments in A2058 cells.

To further validate PRKD3 in modulating MAPK signaling, pERK and pAKT levels were analyzed after MEK inhibitor U0126 treatment with and without PRKD3 knock-down. Sensitization of the MEK inhibitor U0126 was observed after PRKD3 knock-down (Supplemental Figure 2A). The pERK levels were reduced at 2 hours and were up-regulated at 72 hours post-U0126 treatment (Supplemental Figure 2B, 2C). After PRKD3 siRNA transfection in combination with U0126, the pERK levels were reduced, in
particular at high doses of U0126 (Supplemental Figure 2C). Even though pAKT levels were slightly reduced with PRKD3 siRNA transfection in combination with U0126 treatment, pAKT levels relative to total AKT levels were not affected (Supplemental Figure 2C). We have shown that both RAF and MEK inhibitors tested alone were unable to induce a sustained inhibition of pERK at 72 hours after compound treatment. In contrast, we were able to restore pERK inhibition in combination with PRKD3 siRNAs. Additionally, MEK and pMEK levels were found to be reduced either by PRKD3 siRNA treatment or by RAF265 treatment alone, and further reduced after PRKD3 siRNAs in combination with RAF265 (Figure 3B).

Since elevated CRAF protein levels contributed to resistance to RAF inhibition in a subset of BRAF mutant tumor cells, we tested if CRAF levels were altered by PRKD3 knock-down (15). We observed increased phospho-CRAF (S338) after RAF265 treatments (Figure 3B), which has been reported for other RAF inhibitors (33). However, PRKD3 siRNA mediated knock down had no effect on pCRAF or CRAF levels, indicating PRKD3 affects pERK levels in a CRAF independent manner. We also used genetic approaches to validate the sensitization between PRKD3 inhibition and BRAF but not CRAF. PRKD3 siRNAs in combination with BRAF siRNA treatments resulted in a greater growth inhibition compared to any of the single treatment (Figure 3C). However, neither the combination of PRKD3 and CRAF siRNA treatments nor the combination of BRAF and CRAF siRNAs showed potent growth inhibition (Figure 3C). The knock-down efficiencies of PRKD3, BRAF and CRAF siRNAs were validated by western blots (Figure 3C).

To further understand the mechanism of PRKD3 knock down induced RAF265 sensitization, we analyzed apoptosis markers, PARP cleavage, and caspase activity. RAF265 alone was not able to induce detectable PARP cleavage or caspase activity in A2058 cells (Figure 3B, 4A). Similar observations were reported for MEK inhibitor AZD6244 which was shown to induce cell cycle arrest and growth inhibition
rather than apoptosis in melanoma cell lines and xenograft models (34). PRKD3 siRNA in combination with RAF265 treatments induced significant PARP cleavage and increased caspase activities, indicating PRKD3 knock-down sensitized RAF265 to induce apoptosis (Figure 3B, 4A).

Previous report showed that Cyclin D1, a cell cycle regulator for G1 phase entry, was reduced after RAF265 treatments (30). Cyclin D1 was analyzed in A2058 cells in the presence or absence of PRKD3 siRNAs. Consistent with previous reports, RAF265 treatments resulted in a reduction of Cyclin D1, and the reduction was dose dependent (Figure 3B). PRKD3 siRNA alone did not affect Cyclin D1 expression, but PRKD3 siRNA in combination with RAF265 enhanced the reduction of Cyclin D1, suggesting an interruption of cell cycle progression (Figure 3B). In consistent with this observation, cell cycle profiles showed that PRKD3 siRNAs or 0.5 μM RAF265 alone partially blocked G2/M progression, but PRKD3 siRNAs in combination with 0.5 μM RAF265 completely blocked G2/M progression, result in a 0% of cells in G2/M (Figure 4B, Supplemental Figure 3). It was shown that PRKD3 was phosphorylated at its activation loops during mitosis, suggesting that PRKD3 activity is regulated in a cell cycle dependent manner (35). Although PRKD3 siRNAs or 0.5 μM RAF265 alone resulted in a reduction of G0/G1 cell numbers, we did not observe cooperative effects in reducing G0/G1 cell numbers (Figure 4B, Supplemental Figure 3). It is possible that the cooperative effects of PRKD3 inhibition and RAF265 in reduction of Cyclin D1 is a consequence from blocking of G2/M entry.

Finally, the sensitization of PRKD3 knock down and RAF265 was tested in a colony formation growth assay. PRKD3 siRNAs or RAF265 (0.5 and 2 μM) alone partially inhibited the colony formation of A2058 cells, but PRKD3 siRNAs in combination with 0.5 μM or 2 μM RAF265 completely inhibit A2058 cell growth in colonies (Figure 4C). Taken together, we have shown that PRKD3 knock down mediates RAF265 sensitization to prevent the reactivation of MAPK signaling, induce apoptosis markers, reduce
cell cycle progression, and induce tumor cell growth inhibition at high density in plastic and low density colony formation assays in A2058 cells.

**PRKD3 inhibition sensitizes RAF265 to kill the A375 melanoma cells**

We analyzed a RAF265 sensitive melanoma cell line A375, which also harbors the BRAF (V600E) mutation. A375 cells are sensitive to RAF265 with an IC50 of 0.3 μM as shown by control luciferase siRNAs transfected cells (Figure 5A). After PRKD3 siRNA transfection, the IC50 for RAF265 was shifted from 0.3 μM to 0.16μM for both PRKD3 siRNAs (Figure 5A). Next, colony formation assays were performed. PRKD3 siRNAs or RAF265 (0.05 μM) alone only partially inhibited A375 cell growth in colony formation, but PRKD3 siRNAs in combination with 0.05 μM RAF265 completely inhibited cell growth in colony formation (Figure 5B).

Similar to A2058 cells, pERK was reactivated after 72 hour RAF265 treatment and reduced after the combination with PRKD3 knock-down (Figure 5D). pMEK and MEK levels were reduced after RAF265 treatment, and further reduced after combination with PRKD3 knock-down (Figure 5D). In A375, RAF265 resulted in a reduction of Cyclin D1, and the reduction was dose dependent (Figure 5C, D). PRKD3 siRNAs alone did not affect Cyclin D1 expression, but PRKD3 siRNA in combination with RAF265 enhance the reduction of Cylin D1 (Figure 5D). PRKD3 siRNA transfection or RAF265 treatments alone in A375 were not sufficient to induce detectable PARP cleavage, while PRKD3 siRNA transfection in combination with RAF265 treatments resulted in significant induction of PARP cleavage (Figure 5C, D).

We did not see changes on total CRAF after RAF265 treatment in A375 cells (Figure 5D). Instead, we observed decreased phospho-CRAF (S338) after RAF265 treatments (Figure 5D), it is likely that CRAF is an efficacy target of RAF265 in the A375 cell line. PRKD3 siRNA mediated knock down had no effect on pCRAF or CRAF levels, indicating PRKD3 affects pERK levels in a CRAF independent manner. In A375,
PRKD3 siRNAs in combination with BRAF siRNA treatments resulted in a greater growth inhibition compared to any of the single treatments (Figure 5E). However, neither the combination of PRKD3 and CRAF siRNA treatments nor the combination of BRAF and CRAF siRNAs showed additive growth inhibition (Figure 5E). The knock-down efficiencies of PRKD3, BRAF, and CRAF siRNAs were validated by western blots (Figure 5E). The effects on levels of pERK, pMEK, Cyclin D1 and PARP cleavage, as well as colony formation, after PRKD3 knock-down in combination with RAF265 are similar in A375 and A2058 cells, indicating this mechanism is shared by different tumor cells from the same lineage or with the same genetic background BRAF(V600E). This data also suggests that single agent sensitivity to RAF265 is not a requirement for combination PRKD3 knock down induced cell killing.

**PRKD3 inhibition sensitizes with RAF and MEK inhibitors across multiple melanoma cells**

In order to better understand whether the sensitization between PRKD3 inhibition and RAF265 in A2058 and A375 cells is shared with other RAF or MEK inhibitors across cell lines of various lineage and genetic background, we tested additional RAF and MEK inhibitors in a panel of 12 cell lines (Figure 6 and Supplemental Figure 4). In addition to RAF265, we analyzed another RAF inhibitor PLX4032, and two MEK inhibitors U0126 and PD0325901. We tested 12 cell lines including six melanoma cell lines carrying BRAF(V600E) mutation (RPMI7951, IGR39, A2058, A375, SKMEL5, WM115), four cell lines bearing KRAS mutations of various lineages (PANC1, A549, SW620, DU145), one non-melanoma cell line with BRAF(V600E) mutation (SKHEP1, liver cancer), and one cell line which is wild type for both BRAF and RAS (G402, kidney cancer) (Figure 6).

For both RAF inhibitors RAF265 and PLX4032, six out of six melanoma cell lines tested showed sensitization with both PRKD3 siRNAs (Figure 6). Among the lines tested, three cell lines RPMI7951, IGR39, and A2058 were resistant to RAF265 and PLX4032 (Figure 6A, 6C) when 2 μM of compound was
used in combination with RPKD3 siRNAs (Figure 6B, 6D). For three sensitive cell lines A375, SKMEL5, and WM115, 0.3 μM of compound was used in the sensitization assays (Figure 6B, 6D). Among the cell lines tested, the sensitization between PRKD3 knock down and RAF inhibitors was only observed in the melanoma cell lines tested, with only one exception where PRKD3 siRNAs sensitized with RAF265 in colon cancer cell line SW620. It could be that additional genetic lesions outside of the MAPK signaling pathway are necessary for PRKD3 sensitization in non-menlanoma lineages.

For both MEK inhibitors, the sensitization was mainly observed with melanoma cell lines (Supplemental Figure 4). For MEK inhibitor U0126, sensitization with both PRKD3 siRNAs was observed in five out of six melanoma cell lines showed (Supplemental Figure 4B), with one exception that no sensitization was found with SKMEL5. For MEK inhibitor PD0325901, four out of six melanoma cell lines exhibited sensitization with both PRKD3 siRNAs, but sensitization was not found with melanoma cells IGR39 and WM115 (Supplemental Figure 4D). In addition, PRKD3 siRNAs sensitized with PD0325901 in two non-melanoma cell lines SW620 and G402. Collectively, we showed that PRKD3 inhibition sensitizes with RAF and MEK inhibitors in multiple melanoma cell lines. Therefore, it appears that the sensitization between PRKD3 inhibition and RAF or MEK inhibitors is in melanoma lineage selective.

Discussion

Primary and acquired resistance to RAF and MEK inhibitors has been linked to reactivation of the MAPK pathway and PI3K pathway. Rebound of pERK levels has been observed for RAF inhibitor such as PLX4032 and MEK inhibitor like PD0325901 (12, 15, 36). Compensatory activation AKT has been reported both for the RAF inhibitor RAF265 and MEK inhibitor AZD6244 (AstraZeneca) (20, 25, 37, 38). Here, we show that inhibition of PRKD3 sensitizes with RAF265 in a resistant melanoma cell line (A2058) by preventing rebound of pERK and pAKT. Our data support a compound induced resistance mechanism.
where the reactivation of the MAPK pathway and PI3K pathway act coordinately to promote cell survival when RAF and/or MEK is inhibited. We observed that pAKT was reduced when PRKD3 was knocked down together with RAF265 in A2058 cells, but not in A375, a difference that could be attributable to the presence of the PTEN deletion in the A2058 cells (20).

The mechanism of how PRKD3 interacts with pERK and pAKT remains to be clarified. PKCe has been shown to phosphorylate and regulate PRKD3 activity in prostate cancer cells (29). We tested whether PKCe knock-down can phenocopy the PRKD3 knock-down in sensitizing RAF inhibitor. However, we did not observe any sensitization effects (data not shown). Among the three PKD proteins, PRKD1 is the most extensively studied, and has been implicated in a broad range of cellular process and can be activated by a variety of regulatory peptides (39-41). It has been shown that PRKD1 phosphorylates RIN1 and releases it from competing with RAF for binding to RAS, thus resulting in an activation of the RAF-MEK-ERK pathway (42). It will be interesting to investigate whether PRKD3 shares any function with PRKD1 such as RIN1 phosphorylation. We have tested whether PRKD1 or RIN1 siRNAs can sensitize RAF265 in killing A2058. We did not observe any sensitization effects (data not shown). In melanoma cells, PRKD2 protein expression was undetectable and thus it was not tested in our sensitization studies. Even though PRKD members share some cellular functions, they can be differentially expressed and exert different functions (28). Further investigation of PRKD3 specific cellular function(s) will be required to better understand how PRKD3 ablation augments RAF265 activity in melanoma. One limitation of our screen is that RAF265 can inhibit multiple RAF isoforms, a variety kinases, and VEGFR (43). Similar to Sorafenib, the efficacy RAF265 in vivo may not be due exclusively to the activity on BRAF V600E (45). Thus, the sensitization seen with PRKD3 knock down and RAF265 could be due to a combinatorial effect(s) with other targets in addition to RAF isoforms and VEGFR. The lack of selectivity
of RAF265 complicates the interpretation of PRKD3 knock down sensitization and confounds the ability to clearly define a mechanism of action at this time.

This study is the first demonstration that PRKD3 inhibition can sensitize with RAF or MEK inhibitors in BRAF (V600E) melanoma cells. Our current data support a model where PRKD3 could prevent reactivation of MAPK signaling caused by RAF or MEK inhibitors and sensitize with these inhibitors to kill resistant tumor cells. PRKD3 is a potentially druggable kinase since there are known inhibitors of this class of enzymes (44, 45). It is not clear whether the kinase activity or a potential scaffold activity of PRKD3 is required for sensitization with the RAF or MEK inhibitor(s). Although our understanding of how PRKD3 modulates pERK and pAKT in response to RAF265 requires further study, PRKD3 provides a synthetic lethal target opportunity to develop effective combination therapy to overcome drug induced resistance caused by RAF or MEK inhibitors.

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Reference List


RAF/VEGFR2 inhibitor, and RAD001 (Everolimus) in combination. Mol Cancer Ther 2010;9:358-68.


Figure Legends

**Figure 1. Primary synthetic lethal siRNA screens with RAF265 in A2058 cells.** (A) Smart pool siRNA screens without RAF265 or in the presence of 0.4 μM RAF265 (B). The Y- and X- axis refer to the Normalized Z score (NZ) replicate 1 and 2, respectively. C) The primary hits selected from the screen. The Y and X-axis refer to the averaged NZ in the presence of 0 and 0.4 μM of RAF265, respectively. The primary hits are labeled by their gene symbols. For A-C, LUC, BRAF and PLK1 siRNA controls are shown in black stars, diamonds, and triangles, respectively. All experimental Smart pool siRNAs are shown in grey dots. D) Dose curves of RAF265 for selected primary hits in A2058. E) Relative RNA expression for selected primary hits as measured by RTPCR. Error bars are shown and indicate standard deviations calculated from eight independent experiments.

**Figure 2. Validation assays for four primary hits identified from siRNA screens in A2058 cells.** Relative RNA expression and dose curves of RAF265 after transfection with siRNAs against ALPK1(A), MAPK11(B), PIK3R4 (C) and PRKD3 (D) are shown. E) RAF265 dose curves for three stable cell lines expressing PRKD3 shRNAs with (left) or without (right) DOX exposure are shown. Western blots show PRKD3 protein levels with and without DOX induction in three stable cell lines. The numbers below the image blots indicate the quantification of signal intensities normalized against tubulin. Arrow indicates the PRKD3 protein at the predicted molecular weight, an asterix (*) refers to a non-specific band. Error bars indicate the standard deviations for four replicates at each condition.

**Figure 3. PRKD3 inhibition prevents reactivation of pERK and pAKT in A2058 cells.** A) Western blots showing protein levels at 2 hours after RAF265 treatment, with and without PRKD3_01 siRNA transfections. B) Western blots showing protein levels at 72 hours after RAF265 treatment, with and without PRKD3_01 siRNA transfections. Tubulin levels served as a loading control. The numbers below
the image blots indicate the quantification of signal intensities normalized against tubulin. The signal intensity of 0 μM compound in the absence of PRKD3 knock-down was set as reference 1.0. C) Cell viability measured 72 hours after co-transfection with BRAF and PRKD3 siRNAs, CRAF and PRKD3 siRNAs, BRAF and CRAF siRNAs. Western blots showing protein levels of PRKD3, BRAF, and CRAF after siRNA transfections against PRKD3, BRAF, and CRAF, respectively. Arrow indicates the PRKD3 protein at the predicted molecular weight, an asterix (*) refers to a non-specific band. Error bars indicate standard deviations calculated from four independent experiments.

Figure 4. PRKD3 inhibition sensitizes RAF265 to induce caspase activity, inhibit cell cycle progression, and colony formation in A2058 cells. A) Caspase activity as measured at 72 hours after PRKD3 siRNA transfection. Error bars indicate standard deviations calculated from eight independent experiments. B) Cell cycle profiles of A2058 cells after RAF265 treatment, with and without PRKD3 siRNA transfections. G0/G1, S, and G2/M phases are shown separately. C) Colony formation assays in A2058 cells after RAF265 treatment in combination with LUC (luciferase), PRKD3_01 or PRKD3_02 siRNA transfections.

Figure 5. PRKD3 inhibition sensitizes RAF265 to kill A375 melanoma cells. A) PRKD3 knock-down sensitizes with RAF265 over a dose response treatment with RAF265 and PRKD3 siRNAs as compared to control Luciferase siRNAs. Error bars indicate standard deviations calculated from eight independent experiments. B) Colony formation assays in A375 cells after RAF265 treatment, in combination with LUC (luciferase), PRKD3_01 or PRKD3_02 siRNA transfections. Western blots demonstrating protein levels at 2 hours (C) and 72 hours (D) after RAF265 treatment, with and without PRKD3 siRNA_01 transfection. Tubulin levels served as loading controls. The numbers below the image blots indicate the quantification of signal intensities normalized against tubulin controls. The signal intensity of 0 μM RAF265 in the absence of PRKD3 knock-down was then set as reference 1.0. E) Cell viability measured at 72 hours after co-transfection with BRAF and PRKD3 siRNAs, CRAF and PRKD3 siRNAs, BRAF, and CRAF.
siRNAs. Western blots showing protein levels of PRKD3, BRAF and CRAF after siRNA transfections against PRKD3, BRAF, and CRAF, respectively.

**Figure 6. PRKD3 inhibition sensitizes RAF inhibitor RAF265 and PLX4032 in multiple melanoma cells.** A) GI50s for RAF265 in multiple cell lines, GI50 refers to the concentration of compound that inhibits growth of cells by 50%, not reached up 20 μM. B) Relative cell viabilities after RAF265 treatments, with or without PRKD3 siRNA transfections, are shown for multiple cell lines. C) GI50s for PLX4032 across multiple cell lines. D) Relative cell viabilities after PLX4032 treatments, with or without PRKD3 siRNA transfections, are shown for each cell line. Cell viabilities are normalized to 0 μM compound under each condition. Error bars indicate standard deviations calculated from six independent experiments, an asterix (*) refers to a cell line showing sensitization with both PRKD3 siRNAs compared to the control LUC siRNA condition (P<0.01).
Protein kinase D3 sensitizes RAF inhibitor RAF265 in melanoma cells by preventing reactivation of MAPK signaling

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