Dual PI3K/mTOR Inhibitors Induce Rapid Overactivation of the MEK/ERK Pathway in Human Pancreatic Cancer Cells through Suppression of mTORC2

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Abstract

The PI3K/AKT/mTOR pathway, which is aberrantly stimulated in many cancer cells, has emerged as a target for therapy. However, mTORC1/S6K also mediates negative feedback loops that attenuate upstream signaling. Suppression of these feedback loops opposes the growth-suppressive effects of mTOR inhibitors and leads to drug resistance. Here, we demonstrate that treatment of PANC-1 or MiaPaCa-2 pancreatic ductal adenocarcinoma (PDAC) cells with the dual PI3K/mTOR kinase inhibitor (PI3K/TOR-KI) BEZ235 blocked mTORC1/S6K activation (scored by S6 phosphorylation at Ser²⁴⁰/²⁴⁴), mTORC1/4E-BP1 (assayed by 4E-BP1 phosphorylation at Thr³⁷/⁴⁷), and mTORC2-mediated AKT phosphorylation at Ser⁴⁷³, in a concentration-dependent manner. Strikingly, BEZ235 markedly enhanced the MEK/ERK pathway in a dose-dependent manner. Maximal ERK overactivation coincided with complete inhibition of phosphorylation of AKT and 4E-BP1. ERK overactivation was induced by other PI3K/TOR-KIs, including PKI-587 and GDC-0980. The MEK inhibitors U126 or PD0325901 prevented ERK overactivation induced by PI3K/TOR-KIs. The combination of BEZ235 and PD0325901 caused a more pronounced inhibition of cell growth than that produced by each inhibitor individually. Mechanistic studies assessing PI3K activity in single PDAC cells indicate that PI3K/TOR-KIs act through a PI3K-independent pathway. Doses of PI3K/TOR-KIs that enhanced MEK/ERK activation coincided with those that inhibited mTORC2-mediated AKT phosphorylation on Ser⁴⁷³, suggesting a role of mTORC2. Knockdown of RICTOR via transfection of siRNA markedly attenuated the enhancing effect of BEZ235 on ERK phosphorylation. We propose that dual PI3K/mTOR inhibitors suppress a novel negative feedback loop mediated by mTORC2, thereby leading to enhanced MEK/ERK pathway activity in pancreatic cancer cells.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human diseases. The estimated incidence of PDAC in the United States has increased to 44,000 new cases in 2012 and is now the fourth leading cause of cancer mortality in both men and women (1). Novel targets and strategies for therapeutic intervention in PDAC are urgently needed and will most likely arise from a more detailed understanding of the signaling mechanisms that promote survival, proliferation, and invasiveness and of the complex feedback mechanisms that mediate drug resistance in these cells.

The PI3K/AKT/mTOR pathway, a key module in the regulation of metabolism, migration, survival, autophagy, and growth (2), plays a pivotal role in the pancreas, mediating acinar-to-ductal metaplasia, and PDAC formation (3, 4) and is active in premalignant pancreatic lesions and pancreatic cancer tissues (4–6). The mTOR functions as a catalytic subunit in two distinct multiprotein complexes, mTORC1 and mTORC2 (7). mTORC1, a complex including RAPTOR, phosphorylates and controls at least two regulators of protein synthesis, the 40S ribosomal protein subunit S6 kinase (S6K) and the translational repressor 4E-binding protein 1, referred as 4E-BP1. mTORC2, characterized by RICTOR, phosphorylates several AGC protein kinases, including AKT at Ser⁴⁷³. The PI3K/mTOR pathway functions downstream of RAS (8), which is mutated in 90% of PDACs, and plays a key role in insulin/IGF receptor signaling. PDAC cells express insulin and insulin-like growth factor (IGFI) receptors and overexpress IRS-1 and IRS-2 (9–12) and PDAC (but not normal) tissue expresses activated IGFR (12) and IGFI (13). Mutation of p53, as seen during the progression of 50% to 75% of PDAC, has been recognized to upregulate the insulin/IGF/mTORC1 pathway (14). Recently, individual gene variations in the IGFI signaling system have been associated with worse survival in PDAC (15). Cross-talk between insulin/IGFI receptors and G protein-coupled
Cell transfection

MiaPaCa-2 cells were transfected with the plasmid containing a cDNA encoding a GFP tagged-AKT pleckstrin homology domain (AKT-PH-GFP) from Addgene (pcDNA3-AKT-PH-GFP cat. no. 18836) by using Lipofectamine 2000 (Invitrogen) as suggested by the manufacturer. Analysis of the cells transiently transfected was performed 24 hours after transfection.

Real-time GFP-AKT-PH imaging in single live cells

Single live-cell imaging of the GFP tagged-AKT-PH domain was achieved with a fluorescence microscope. The microscope used was an epifluorescence Zeiss Axioskop and a Zeiss water objective (Achromplan 40/0.75W Carl Zeiss, Inc.). Images were captured as uncompressed 24-bit TIF files with a cooled (−12°C) single CCD color digital camera (Pursuit, Diagnostic Instruments) driven by SPOT version 4.7 software.

Quantitative analysis of the relative change in plasma membrane and cytosol fluorescence intensity of individual cells was performed by importing the TIF images into Zeiss LSM 510 software and performing profile scans with the largest line width. Five equally spaced line profiles were taken for each cell or cell pair. Intensities were background corrected, and the intensities at the membrane were divided by those in the immediately surrounding cytoplasm. We analyzed 30 to 45 cells in each experiment, and each experiment was performed in duplicate. The selected cells displayed in the figures were representative of 90% of the population of positive cells.

Knockdown of rictor levels via siRNA transfection

Silencer Select siRNAs was purchased from Life Technologies and designed to target human RICTOR. Cells were transfected using the reverse transfection method. Either Silencer Select nontargeting negative control or a 10-nmol/L rictor siRNA was mixed with Lipofectamine RNAi MAX (Life Technologies) according to the manufacturer's protocol and added to 35-mm tissue culture plates. MiaPaCa-2 cells were then plated on top of the siRNA/Lipofectamine RNAiMAX complex at a density of 10^3 cells/well in DMEM containing 5 mmol/L glucose and 10% FBS. Three days after transfection, cells were used for experiments and subsequent Western blot analysis.

Assay of cell proliferation

Cells (10^5) were plated on 35-mm tissue culture dishes in DMEM containing 10% FBS. After 24 hours of incubation at 37°C, cultures were incubated with DMEM containing 5% FBS in the absence or presence of BEZ235, PD0325901 (30), or the combination of both drugs. In other experiments, PKI-587 and GDC-0980 were tested instead of BEZ235. The concentrations of PD0325901 used in the experiments reflected that we found MiaPaCa-2 cells more sensitive to this inhibitor than PANC-1 cells. After 72 hours, cell count was determined from a minimum of six dishes per condition using a Coulter counter, after cell clumps were disaggregated by passing the cell suspension 10 times through a 19-gauge, and subsequently, a 21-gauge needle.

For cell colony formation, 300 MiaPaCa-2 cells were plated into 35-mm tissue culture dishes in DMEM containing 10% FBS. After
24 hours of incubation at 37°C, cultures were incubated with DMEM containing 5% FBS either in the absence or presence of 5 μmol/L of BEZ235, 5 μmol/L of PD0325901, or the combination of both drugs. A colony consisted of at least 50 cells (31). Cell colony numbers from three dishes per condition were determined after 8 days of incubation.

Materials
DMEM was obtained from Invitrogen. Neurotensin and insulin were obtained from Sigma Chemical. BEZ235, PKI-578, and GCD-0980 were from Selleck Chemicals. PD0325901 and U0126 were from Tocris BioScience. The structure of these inhibitors is shown in Supplementary Fig. S1. All antibodies were purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were from GE Healthcare Bio-Sciences Corp. All other reagents were of the highest grade available.

Results
BEZ235 causes overactivation of the ERK pathway in human PDAC cells
Initially, we determined the effect of the PI3K/TOR-KI BEZ235 (24, 25) on the activity of mTORC1 and mTORC2 in MiaPaCa-2 cells, an extensively used model of ductal pancreatic adenocarcinoma cells. Serum-starved cultures of MiaPaCa-2 cells were incubated with increasing concentrations of BEZ235 (0.005–1 μmol/L) for 2 hours. Then, the cells were stimulated with a combination of insulin and neurotensin to elicit potent mitogenic cross-talk signaling (16, 17), including phosphorylation of S6K at Thr549, a site directly phosphorylated by mTORC1 and S6 at Ser240/244, a site directly targeted by S6K (Fig. 1A). Treatment with BEZ235, at the lowest concentration tested (0.005 μmol/L), markedly inhibited phosphorylation of both S6K and S6 (Fig. 1A; quantification in Fig. 1B). The phosphorylation of these proteins was completely suppressed by higher doses of BEZ235 (>0.01 μmol/L). BEZ235 also inhibited the phosphorylation of 4E-BP1 at Thr37/46 (Fig. 1A), sites that are sensitive to active-site mTOR inhibitors but not to rapamycin in PDAC cells (23).

Stimulation with neurotensin and insulin also induced phosphorylation of AKT on Ser473, a site directly phosphorylated by mTORC2 and at Thr308, a site phosphorylated by PDK1 in response to PI3K activation. AKT phosphorylation on both Ser473 and Thr308 was markedly decreased at 0.05 μmol/L BEZ235 and it was completely abrogated at higher concentrations. These results indicate that BEZ235 inhibits the S6K arm of mTORC1 signaling at lower doses (<0.01 μmol/L) than those required to

Figure 1.
BEZ235 induces overactivation of ERK phosphorylation in PDAC cells. A, cultures of MiaPaCa-2 cells were incubated in the absence or in the presence of BEZ235 (0.005–1 μmol/L) for 2 hours. Then, the cells were stimulated for 30 minutes with 5 nmol/L neurotensin (NT) and 10 ng/mL insulin (ins) and lysed with SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies illustrated in the figure. B, quantification of phosphorylated ERK at Thr202 and Tyr204, S6 at Ser240/244 and AKT at Ser473 was performed using Multi Gauge V3.0 in three independent experiments similar to Fig 1A. C, cultures of MiaPaCa-2 cells were incubated in the absence or in the presence of 1 μmol/L of BEZ235 for 2 hours and then stimulated with 5 nmol/L neurotensin (NT) and 10 ng/mL insulin for various times and lysed. Immunoblotting was performed as described in A, D, cultures of BxPC-3 and AsPC-1 cells were treated with or without 1 μmol/L of BEZ235 (BEZ) for 2 hours and then stimulated with NT and Ins for 30 minutes and lysed. Immunoblotting was performed as in A. Image editing: irrelevant lanes were removed (indicated by a thin, vertical black line) from the acquired digital images and flanking lanes juxtaposed using Adobe Photoshop.
block mTORC1/4E-BP1, mTORC2, or PI3K/PDK1 (> 0.05 μmol/L) in MiaPaCa-2 cells.

The salient feature in Fig. 1A is that BEZ235 induced a striking and dose-dependent stimulatory effect on ERK activity in MiaPaCa-2 cells, as monitored by ERK phosphorylation on Thr^{202} and Tyr^{204} (quantification in Fig. 1B). The maximal enhancement of ERK activation (3.1 ± 0.2 fold; n = 3) occurred at doses of BEZ235 that inhibited 4E-BP1 and mTORC2 (> 0.05 μmol/L). Treatment with BEZ235 also activated MEK, upstream of ERK, as scored by phosphorylation of Ser^{217/221}, residues in MEK directly phosphorylated by RAF kinases (Fig. 1A).

A potent overactivation of ERK induced in response to BEZ235 was also demonstrated when MiaPaCa-2 cells were stimulated with neurotensin and insulin for 2 hours instead of 30 minutes (Supplementary Fig. S2A). Exposure to BEZ235 markedly enhanced the level of phosphorylated ERK in MiaPaCa-2 cells stimulated with neurotensin and insulin for as little as 15 minutes and persisted for 120 minutes (Fig. 1C). Treatment with BEZ235 also enhanced ERK activation in other PDAC cells, including AsPC-1, BxPC-3 cells (Fig. 1D), and PANC-1 cells, another extensively used model of PDAC cells (Supplementary Fig. S2B). Collectively, these results show that the dual PI3K/mTOR-KI BEZ235 profoundly inhibits mTORC1, mTORC2, and PI3K but induces rapid, striking, and dose-dependent activation of the MEK/ERK pathway in human PDAC cells.

Treatment with MEK inhibitors abolishes overactivation of the ERK pathway induced by BEZ235

We next determined whether cell exposure to MEK inhibitors prevents ERK overactivation in response to PI3K/mTOR inhibition. Treatment of MiaPaCa-2 cells with U0126 (32), a preferential inhibitor of MEK, abrogated ERK overactivation induced by BEZ235 (Fig. 2A). Similarly, enhanced ERK phosphorylation induced by BEZ235 was blunted by U0126 in PANC-1 cells (Fig. 2B). PD0325901, a potent and specific allosteric inhibitor of MEK (30, 33), also abrogated ERK overactivation induced by increasing

**Figure 2.** MEK inhibitors suppress ERK overactivation induced by BEZ235. A and B, cultures of MiaPaCa-2 cells (A) and PANC-1 (B) cells were incubated for 2 hours in the absence or presence of increasing doses of BEZ235 with or without U0126 at 1 or 5 μmol/L. C and D, cultures of MiaPaCa-2 cells (C) and PANC-1 (D) cells were incubated in the presence of increasing doses of BEZ235 with or without PD0325901 (PD) at 1 or 5 μmol/L for 2 hours. Then, for A–D, the cells were stimulated for 2 hours with 5 mmol/L neurotensin and 10 ng/mL insulin and lysed with SDS-PAGE sample buffer. E and F, cultures of MiaPaCa-2 (E) and PANC-1 (F) cells were incubated for 2 hours in the absence or presence of increasing doses of BEZ235 with or without the addition of PD0325901 (PD) at 1 and 5 μmol/L. Then, the cells were stimulated for 2 hours with 2% FBS (serum) and lysed with SDS-PAGE sample buffer. All samples were analyzed by SDS-PAGE and immunoblotting with the antibodies that detect phosphorylated or total proteins, as described in each panel.
doses of BEZ235 (Fig. 2C and D). An inhibitory effect was elicited by PD0325901 at a dose as low as 5 nmol/L (Supplementary Fig. S3).

To extend further these findings, we also examined the effects of BEZ235 without or with PD0325901 in PDAC cells stimulated with fetal bovine serum (FBS). Exposure to BEZ235 over-activated ERK phosphorylation on Thr202 and Tyr204 in serum-stimulated cells, an effect abolished by PD-0325901 (Fig. 2E and 2F). The results indicate that enhanced ERK activation induced by treatment with BEZ235 can be prevented by cotargeting MEK in PDAC cells.

The intensity and duration of ERK activation are tightly regulated by negative feedback loops within the pathway, including inhibitory phosphorylations of SOS and RAF mediated by active ERK (18). Negative feedback regulation of the ERK pathway has been recently shown in cancer cells with RAS mutation (34). Accordingly, treatment with PD0325901 released feedback inhibition as revealed by overphosphorylation of MEK in either MiaPaCa-2 or PANC-1 cells (Supplementary Fig. S4). Interestingly, BEZ235 further augmented MEK phosphorylation in PDAC cells treated with PD0325901, implying that the dual PI3K/mTOR inhibitor enhanced RAF/MEK activity in cells without ERK-mediated negative feedbacks loops.

Enhanced ERK activation is also elicited by the mTOR/PI3K inhibitors PKI-587 and GDC-0980

Reflecting the intense interest in targeting the PI3K/mTOR pathway, a number of dual mTOR/PI3K inhibitors, other than BEZ235, have been developed, including PKI-587 (26, 27) and GDC-0980 (28), the structure of which is displayed in Supplementary Fig. S1. Next, we determined whether PKI-587 and GDC-0980 also enhance ERK activation in PDAC cells. As shown in Fig. 3A, phosphorylation of S6 on Ser240/244 and AKT on Ser473, monitoring mTORC1 and mTORC2 activity, respectively, was inhibited by treatment with 0.1 and 1 μmol/L of PKI-587. Exposure to PKI-587 also caused a striking increase in ERK activation, an effect completely blocked by concomitant exposure to the MEK inhibitor PD-0325901. Similar effects were elicited by PKI-587 and PD0325901 in PANC-1 cells (Fig. 3B).

GDC-0980 has also recently identified as a selective, potent, and orally bioavailable inhibitor of PI3K and mTOR (28). To examine the effects of GDC-0980, MiaPaCa-2 cells were incubated with or without this PI3K/mTOR-KI and then stimulated for various times (Fig. 3C), as shown before with BEZ235 in Fig. 1C. GDC-0980 completely inhibited phosphorylation of S6 on Ser240/244 and AKT on Ser473 but produced a prominent ERK overactivation at all times examined (Fig. 3C). Thus, multiple clinically relevant dual PI3K/mTOR inhibitors induce ERK overactivation in PDAC cells.

Effect of BEZ235, PD0325901, and their combination on PDAC cell proliferation and colony formation

To examine whether the overactivation of the ERK pathway counterbalances the growth-suppressive effect of mTOR/PI3K inhibitors, we determined the proliferation of MiaPaCa-2 cells treated with BEZ235, PD0325901, or a combination of BEZ235 and PD0325901 (Fig. 4A). Each inhibitor reduced cell proliferation but the combination of BEZ235 and PD0325901 produced a further inhibitory effect on MiaPaCa-2 cell proliferation. Importantly, the difference between PD0325901 and the combination of BEZ with PD0325901 was statistically significant. Similar results were obtained using PANC-1 cells (Fig. 4B). Similarly, the inhibitory effect of PKI-587 and GDC-0980 on MiaPaCa-2 proliferation was markedly enhanced by PD0325901 (Fig. 4C). The results indicate that cotargeting the PI3K/mTOR and MEK induces profound inhibition of PDAC cell proliferation. To test further this conclusion, we determined the effect of long exposure to low concentrations of BEZ, PD0325901, or their combination on the
colony-forming ability of MiaPaCa-2 cells. Treatment with either BEZ235 or PD0325901, each at a concentration as low as 5 nmol/L, markedly reduced the number of colonies formed by MiaPaCa-2 cells (Figs. 4D and 4E). Exposure to the combination of BEZ235 and PD0325901 further inhibited the number of colonies formed by MiaPaCa-2 cells (Figs. 4D and 4E).

Dual mTOR/PI3K inhibitors induce ERK overactivation through a PI3K-independent pathway

Having established that dual PI3K/mTOR inhibitors lead to enhanced MEK/ERK activation in PDAC cells, we next examined the mechanism(s) involved. Previous studies with prostate and breast cancer cells identified a feedback loop that mediates ERK overactivation in response to rapamycin analogs through a PI3K-dependent pathway (35). To evaluate PI3K activity, we determined the effect of BEZ235, PKI-587, and GDC-0980 on the PI3K-generated accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP₃) in the plasma membrane of individual PDAC cells. MiaPaCa-2 cells were transiently transfected with a plasmid encoding a fusion protein between GFP and the PH domain of AKT (AKT-PH-GFP), an in vivo reporter of PIP₃ (36, 37). Stimulation with neurotensin and insulin induced a rapid and striking translocation of AKT-PH-GFP to the plasma membrane, indicative of robust PI3K activation (Fig. 5A; quantification in Fig. 5B). Prior exposure to BEZ235, PKI-587, or GDC-0980 completely prevented the translocation of the PIP₃ sensor to the plasma membrane (Fig. 5A; quantification in Fig. 5B). Similar results were obtained after different times of stimulation (Supplementary Fig. S5). The results presented in Fig. 5 and Supplementary Fig. S5 indicate that dual PI3K/mTOR inhibitors induce MEK/ERK activation in PDAC cells through a PI3K-independent pathway.

Figure 4.

Dual PI3K/mTOR kinase inhibitors and PD0325901 inhibit the proliferation of PDAC cells. A, single-cell suspensions of MiaPaCa-2 cells were plated at a density of 10⁵ cells per dish. After 24 hours, the cultures were shifted to media containing FBS with 100 nM BEZ235 (BEZ), 100 nmol/L PD0325901 (PD), or combination of both drugs as indicated. After 72 hours, cell numbers were determined from six plates per condition. Results are presented as mean ± SEM. B, single-cell suspensions of PANC-1 cells were plated at a density of 10⁵ cells per dish. After 24 hours, the cultures were shifted to media containing FBS with 100 nmol/L BEZ235 (BEZ), 500 nmol/L PD0325901 (PD), or combination of both drugs as indicated. After 72 hours, cell numbers were determined from six plates per condition. Results are presented as mean ± SEM. C, single-cell suspensions of MiaPaCa-2 cells were plated at a density of 10⁵ cells per dish. After 24 hours, the cultures were shifted to media containing FBS with 100 nmol/L PKI-587 (PKI), 100 nmol/L of GDC-0980 (GDC), 100 nmol/L PD0325901 (PD), and combinations of either PKI-587 or GDC-0980 with PD0325901, as indicated. After 72 hours, cell numbers were determined from six plates per condition. Results are presented as mean ± SEM. D, cell colony formation was performed as described in the Materials and Methods section. MiaPaCa-2 cells were incubated for 8 days with 5 nmol/L of BEZ235 (BEZ), 5 nmol/L PD0325901 (PD), or with a combination of both drugs. E, the bars represent the number of colonies (mean ± SEM; n = 3 dishes per condition). * t test P values comparing the indicated two groups were < 0.001.
BEZ235 enhances ERK activation independently of EGFR, HER2, insulin receptor, and IGFIR. Chronic suppression of PI3K/mTORC1 stimulates FOXO-dependent expression of several tyrosine kinase receptors, including, IGF/insulin receptors and HER3 in tumor cells, thereby enhancing ERK activity (38, 39). This mechanism is unlikely to explain our results, given the rapidity of the effects shown here with PDAC cells. To test this possibility directly, we determined whether inhibitors of EGFR (AG1438), EGFR and HER2 (lapatinib) or insulin/IGFI receptors (OSI-906) prevent enhanced ERK activation in response to BEZ235. As a control, we verified that the inhibitors, at the concentrations used, abrogated ERK activation induced by EGF or IGFI in MiaPaCa-2 cells (Supplementary Fig. S6A). Neither AG1438 (EGFR tyrosine kinase inhibitor) nor lapatinib (inhibitor of EGFR and HER2) prevented enhanced ERK activation by BEZ235 (Fig. 5C and D; quantification in Supplementary Fig. S6B and S6C).

We also examined the involvement of the insulin/IGFI receptors in mediating ERK activation in response to BEZ235. Exposure to the insulin/IGFI receptor inhibitor OSI-906 reduced baseline levels of ERK phosphorylation but did not prevent the ERK activation induced by BEZ235 (Fig. 5E). Indeed, the dual PI3K/mTORC1 inhibitor induced a similar relative enhancement of ERK phosphorylation either in the absence or presence of OSI-906 (Supplementary Fig. S6D). Thus, BEZ235 enhances ERK activation through a pathway that does not require EGFR, HER2, or insulin/IGFI receptors.

Knockdown of RICTOR prevents enhancement of ERK activation by BEZ235 independently of AKT

As shown throughout this study, the doses of BEZ235 that enhanced MEK/ERK activation coincided with those that inhibited AKT phosphorylation on Ser473, prompting us to hypothesize that BEZ235 suppresses a negative feedback mediated by mTORC2. To test this possibility, we used RNAi to silence RICTOR, an essential and specific component of mTORC2. Transfection of MiaPaCa-2 cells with siRNA-targeting RICTOR caused a striking decrease in the expression of RICTOR protein (Fig. 6A). Interestingly, knockdown of RICTOR markedly increased baseline levels of ERK phosphorylation and treatment with BEZ235 failed to produce a significant further enhancement of ERK activation (Fig. 6A; quantification in Fig. 6B). As expected, knockdown of RICTOR did not prevent mTORC1/S6K activation, scored by S6 phosphorylation but abolished AKT phosphorylation on Ser473, a function mediated by mTORC2. Surprisingly, knockdown of RICTOR increased baseline levels of ERK phosphorylation and treatment with BEZ235 failed to produce a significant further enhancement of ERK activation (Fig. 6A; quantification of three independent experiments shown in Fig. 6C), whereas BEZ235 enhanced ERK activation in cells transfected with nontargeting siRNA. Transfection with a siRNA directed to a different region of RICTOR also attenuated the enhancement of ERK activity induced by BEZ235.

AKT has been proposed to inhibit RAF-1 activity by direct phosphorylation at Ser259 (40), but this mechanism of negative cross-talk was disputed in subsequent studies (41). Here, we tested whether the enhancement of ERK activation induced by
BEZ235 is mediated by downregulation of AKT-mediated RAF-1 phosphorylation at Ser\(^{259}\). As shown in Fig. 6D, exposure of MiaPaCa-2 cells to BEZ235 did not produce any detectable decrease in the high level of RAF-1 phosphorylation at Ser\(^{259}\), even at concentrations that produced robust enhancement of ERK activation. Furthermore, treatment with allosteric (MK-2206) or active-site (GDC-0068) inhibitors of AKT did not replicate the increase in ERK activation produced by BEZ235 (Fig. 6E). These results indicate that treatment with the dual PI3K/mTOR inhibitor suppresses a novel negative feedback loop mediated by mTORC2, thereby leading to enhanced MEK/ERK pathway activity in pancreatic cancer cells.

**Discussion**

Although augmented PI3K/AKT activity in response to mTORC1/S6K inhibition by rapamycin and its analogs is well documented in a variety of cell types (18–21), including PDAC (23), overactivation of the MEK/ERK pathway by mTOR inhibitors has been less explored (18). Recently, we reported that active-site mTOR inhibitors (KU63794 and PP242) induce a marked increase of MEK/ERK pathway activity in PDAC cells (23). Here, we demonstrate that the structurally unrelated dual PI3K/mTOR inhibitors BEZ235 (24, 25), PKI-587 (26, 27), and GDC-0980 (28) promote a striking, dose-dependent increase in ERK activation in PDAC cells stimulated with cross-talking mitogens such as insulin and neurotensin or serum factors. The dual PI3K/mTOR inhibitors also induced MEK overactivation and MEK inhibitors, including U126 and PD0325901, abrogated the overactivation of MEK. Our findings show, for the first time, that dual PI3K/mTOR inhibitors induce rapid overactivation of the MEK/ERK pathway, a pivotal pathway in PDAC cells and other malignancies.

To understand the mechanism by which dual PI3K/mTOR inhibitors promoted ERK activation, we determined the role of a feedback loop involving mTORC1/S6K/PI3K/ERK, proposed to mediate ERK activation in prostate and breast cancer cells in response to rapamycin analogs (35). In detailed dose–response studies, we found that low doses of BEZ235 profoundly reduced mTORC1/S6K activity but produced small enhancement of the
ERK pathway. Accordingly, neither rapamycin nor everolimus, at concentrations that completely blocked the mTORC1/S6K axis, produced any detectable enhancement of ERK activation in PDAC cells (23). These results indicate that ERK overactivation in response to dual PI3K/mTOR inhibitors can be dissociated from feedback loops mediated through the mTORC1/S6K axis in PDAC cells.

Further evidence supporting that PI3K/TOR-KIs enhance ERK overactivation through a PI3K-independent feedback loop was obtained by showing that these agents suppressed PI3K activity at concentrations that enhanced ERK. Specifically, we evaluated the effect of BEZ235, PKI-587, or GDC-0980 on PI3K activity in single cells, as monitored by the distribution of AKT-PH-GFP, an in vivo reporter of PI3K. We found that dual PI3K/mTOR inhibitors blunted the translocation of AKT-PH-GFP from the cytosol to the plasma membrane, indicating that these agents prevented PI3K’s accumulation at the plasma membrane. Collectively, the results with dual PI3K/mTOR catalytic kinase inhibitors identify a novel PI3K-independent feedback mechanism that restrains the activity of the MEK/ERK pathway, which is different from the loop previously identified with rapamycin (35).

Treatment of a variety of tumor cells with inhibitors that block the PI3K/AKT/mTOR pathway induces a transcriptional response mediated, at least in part by FoxO family members that lead to the overexpression of tyrosine kinase receptors or adaptor proteins, including insulin/IGF1 receptor and HER3, thereby leading to enhancement of ERK (36, 37–45). This expression loop should be distinguished from the effects induced by dual PI3K/mTOR inhibitors preventing PI3K’s accumulation at the plasma membrane. Collectively, the results with dual PI3K/mTOR catalytic kinase inhibitors identify a novel PI3K-independent feedback mechanism that restrains the activity of the MEK/ERK pathway, which is different from the loop previously identified with rapamycin (35).

We found that dual PI3K/mTOR inhibitors suppress a novel negative feedback loop mediated by mTORC2, thereby leading to enhanced MEK/ERK pathway activity in pancreatic cancer cells. We also show that the negative loop mediated by mTORC2 is through an Akt-independent pathway.

Given the role of the RAS/MEK/ERK pathway in PDAC initiation, development, and maintenance (48), we hypothesized that inhibition of overactivated MEK/ERK should increase the growth-suppressive effects of dual PI3K/mTOR inhibitors in these cells. In line with this hypothesis, we found that the potent and highly specific MEK1/2 inhibitor PD1823901 suppressed enhanced ERK activation induced by PI3K/TOR-KIs and enhanced the growth-suppressive effects of these agents in PDAC cells. Given that dual PI3K/mTOR inhibitors are increasingly considered for clinical use, the findings presented here suggest that suppression of cell-specific feedback loops by these inhibitors leading to MEK/ERK overactivation should be considered in their potential use for therapy of PDAC and other malignancies.

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

Authors’ Contributions
Conception and design: H.P. Soares, E. Rozengurt
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.P. Soares, M. Ming, S.H. Young
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