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Los Angeles

Targeting mTOR in Pancreatic Cancer

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Molecular Biology

By

Heloisa Prado Soares

2015

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ABSTRACT OF THE DISSERTATION

Targeting mTOR in Pancreatic Cancer

by

Heloisa Prado Soares

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2015

Professor Juan Enrique Rozengurt, Chair

Pancreatic ductal adenocarcinoma (PDAC), which comprises 90% of all human pancreatic cancers, is a devastating disease with overall 5-year survival rate of only 5%. A major challenge is to identify novel targets and develop strategies for its treatment. New approaches will most likely arise from a detailed understanding of the molecular signaling pathways that stimulate the unrestrained proliferation of these cells. The PI3K/AKT/mTOR pathway plays a pivotal role in pancreatic cancer. Several drugs, including mTOR kinase inhibitors, are in development to target this pathway. In addition to growth-promoting signaling, the mTORC1/S6K axis also mediates negative feedback loops that restrict signaling via insulin/IGF receptor and other tyrosine kinase receptors and can lead to drug resistance. In this dissertation, I describe studies performed in PDAC cell lines using different inhibitors of the mTOR pathway, including: a) rapamycyn, an allosteric mTOR inhibitor; b) PP242 and KU63794, active-site mTOR inhibitors; c) NVP-BEZ235, GDC-0980 and PKI-587, dual PI3K/mTOR inhibitors, d) metformin and berberine, two anti-diabetics drugs with emerging

promising anti-cancer properties. We show that active-site mTOR and dual PI3K/mTOR inhibitors induce an unexpected increase in the activity of the ERK pathway in PDAC cells. Additionally, we demonstrate that ERK over-activation can be abrogated by the use of MEK inhibitors. We also show that metformin and berberine are capable of inhibiting mTOR signaling without ERK over-activation. Our mechanistic studies demonstrate that dual PI3K/mTOR inhibitors suppress a novel PI3K-independent negative feedback loop mediated by mTORC2 thereby leading to enhancement of MEK/ERK pathway activity in pancreatic cancer cells. Finally, we review negative feedback mechanisms that restrain signaling via upstream elements of the PI3K/AKT/mTOR pathway as well as mechanisms leading to the compensatory activation of other pro-oncogenic pathways, including MEK/ERK. Taken together, the data presented in this dissertation have important translational applications and provide a rationale for the study of combinatory target therapy in pancreatic cancer.

The dissertation of Heloisa Prado Soares is approved.

John J Colicelli

Samuel Wheeler French

Guido Eibl

Francis Charles Brunicardi

Juan Enrique Rozengurt, Committee Chair

University of California, Los Angeles

2015

To my husband, David

Our daughter, Isabella Grace

My parents, Ruy and Salete

And the patients that I treated with this unkind disease, pancreatic cancer

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VITA

1997-2002	Medical Doctor (MD), ABC Foundation School of Medicine Santo Andre, Brazil
2002-2007	Research Associate, Cancer Control Department H. Lee Moffitt Cancer Center and Research Institute Tampa, FL
2007-2010	Resident, Internal Medicine Mount Sinai Medical Center Miami Beach, FL
2010-2014	Fellow, Division of Hematology-Oncology David Geffen School of Medicine University of California, Los Angeles Los Angeles, CA
2014-2015	Health Sciences Clinical Instructor, Division of Hematology-Oncology David Geffen School of Medicine University of California, Los Angeles Los Angeles, CA

PUBLICATIONS

Selected peer-reviewed publications:

Wainberg Z, **Soares HP,** Patel R, DiCarlo B, et al: Phase II Trial of Everolimus in Patients with Refractory Metastatic Adenocarcinoma of the Esophagus, Gastroesophageal Junction and Stomach: Possible role for predictive biomarkers**.** Cancer Chemother Pharmacol. *In press*

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Chapter I.

INTRODUCTION

Epidemiology of pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is a neoplasm of the cells of the exocrine pancreas that represent 90% of all pancreas cancer. According to the American Cancer Society, adenocarcinoma of the pancreas (simply called pancreatic cancer) is expected to affect more than 48,000 thousand people in the United States of America during the year of 2015 and more than around 40,000 are expected to die of this disease annually. In fact, it the fourth leading cause of cancer death in this country. (1) (2) The overall 5-year survival rate is a dismal 5%. Patients with advanced disease have a median survival of less than 1 year despite the use of the best available standard chemotherapy regimens. (3) Even when patients are diagnosed with early stage disease and undergo primary tumor resection, their 5 years overall survival is less than 25% as the tumor typically relapses.

The median age of pancreatic cancer patients at the time of the diagnosis is 71 years, typically ranging from 40 to 80 years old. As the population in the US ages, we expect more cases. By 2030, this malignancy is expected to be the second leading cause of cancer-death in US just behind lung cancer. (4)

Predisposition factors

In addition to age, several other risk factors are known to contribute to the development of pancreatic cancer including smoking, obesity, chronic pancreatitis and race. Additionally, family history plays an important role in the development of this disease. In fact, 5 to 10% of patients are thought to have a known genetic syndrome or family history. (5, 6) Unfortunately, an individual with a first-degree relative with this disease has a 7-9 fold

increase chance in developing it as well in the future. (7). The main known germlime mutations associated with increased risk are BRCA 1, BRCA 2, p16, PALB2 among others. Despite these known predisposition factors, we still do not have established guidelines for screening for several reasons including the fact that we do not have appropriate screening tools. (8)

Diagnosis of pancreatic cancer

Usually pancreatic cancer does not manifest symptoms until its more advanced stages. The typical signs and symptoms are weight loss, abdominal pain, bloating and diarrhea. If the tumor is localized in the head of the pancreas, patient will often present with painless jaundice. The work-up for a suspected tumor routinely includes high resolution abdominal computed tomography (CT) scans. But tissue sample is required for confirmatory pathological diagnosis. The tissue can be obtained via surgery in the case of small, resectable and suspicious masses. In other cases, a CT or ultrasonography (US) guided biopsy can be done from the primary mass or site of suspected metastatic disease, particularly when removal of the primary tumor is not possible or indicated.

Staging and Management:

Although the classical TNM stage exists for pancreatic cancer, in practical terms physicians approach the disease as a) resectable; b) borderline resectable/locally advanced or c) metastatic disease.

Unfortunately only 15 to 20% of the patients have resectable disease at the time of the diagnosis. In cases where the tumor is localized in the head of the pancreas, eligible patient

will undergo the so-called Whipple procedure that consists in of removal of the distal half of the stomach (antrectomy), the gall bladder and its cystic duct (cholecystectomy), the common bile duct (choledochectomy), the head of the pancreas, duodenum, proximal jejunum and regional lymph nodes. On the other hand, patient with disease localized in the tail of the pancreas will undergo a distal pancreatectomy.

Patients with borderline or locally advanced disease frequently are initially treated with chemotherapy regimens that are used in the metastatic setting for lack of better options. Radiation therapy is often used as well, in the attempt to reduce tumor size facilitating a complete resection of the primary tumor.

For patients with metastatic disease, the traditional treatment is chemotherapy. For many years the only standard of care was the use of a chemotherapy drug called gemcitabine, which was approved in 1997 by the Food and Drug Agency (FDA). This FDA approval was based in the data from a randomized phase III clinical trial showed that gemcitabine-treated patients had increased median overall survival compared to 5-fluorouracil (5-FU) treated patients of 5.65 and 4.41 months respectively ($P = 0.0025$). (9) The survival rate for gemcitabine at one year was 18% compared to 2% for 5-FU. Since then, several other drugs have been tested in the setting of metastatic pancreatic cancer with little success. However, in the last few years, different combinations of chemotherapy have emerged as options for the initial treatment of metastatic pancreatic cancer. The most significant survival benefit has been reported with the FOLFIRINOX (oxaliplatin, irinotecan, leucovorin and 5-fluorouracil) regimen in patients with metastatic disease. This combination when compared with gemcitabine showed a median overall survival of 11.1 versus 6.8 months. (3) However FOLFIRINOX is associated with significant toxicity. In 2013, Von Hoff and colleagues

reported that the combination of gemcitabine and nab-paclitaxel achieved a median overall survival of 8.5 months as compared with 6.7 months in the gemcitabine alone group of patients that were randomized in a phase III trial. (10) The survival rate was 35% in the nab-paclitaxel-gemcitabine group versus 22% in the gemcitabine group at 1 year. With all these options, the decision of using single agent gemcitabine versus folinic acid, fluorouracil, irinotecan, oxaliplatin (FOLFIRINOX) or gemcitabine-nab Paclitaxel routinely dependents on the patient's fitness, performance status, clinician judgment and patient's personal preferences.

In other tumor histologies target therapy had made dramatically progress. Nevertheless, target therapy so far has failed to delivery significant improvement in the war against pancreatic cancer. (11) (12) (13) Even Erlotinib, the FDA-approved target therapy for pancreatic cancer has little impact in patient survival. (14) Clearly, a more detailed understanding of the signaling mechanisms that promote survival, proliferations and invasiveness and the complex feedback mechanisms that mediate dug resistant are key to the development novel and effective targets and strategies.

Precursors lesions and genetic basis

Pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasm (MCN), and intraductal papillary mucinous neoplasm (IPMN) (15) (16) are associated with pancreatic cancer. The most common lesions are the pancreatic intraepithelial neoplasias (PanINs). (16, 17) Progression from these noninvasive ductal lesions to infiltrating adenocarcinoma is associated with the accumulation of genetic alterations (18, 19), including activating mutations in KRAS which appear in more than 90% of PDACs and represent an initiating

event, as well as inactivating mutations in tumor suppressors genes, including p53, p16 and SMAD4 (19-22). It is generally accepted that progression of pancreatic carcinogenesis requires activation of signaling pathways leading to sustained cell proliferation. See more details below.

Molecular characterization of pancreatic cancer and activated pathways

Pancreatic cancer carcinogenesis involves a series of somatic alterations. Four genes are commonly altered in pancreatic cancer. The oncogene KRAs is mutated in more than 90% of the tumors. (23) The suppressors genes TP53, p16/CDKN2A and SDMAD4 are mutated in 90, 75 and 55% of tumors respectively. It is increasingly clear that mutations in *KRAS*, *SMAD4*, *TP53* and *CDKN2A/p16* are "driver" mutations of PDAC, *i.e*., mutations that confer a selective growth advantage to the tumor cell. (24) Additionally, such mutated genes are key players within a complex network of core pathways. (25) (26)

RAS activation and MAPK pathway

The KRAS or *more specifically KRAS2* gene is located on chromosome arm 12p and encodes a membrane-bound guanosine triphosphate (GTP)–binding protein, which is activated by point mutations, most often in codon 12. (27)

RAS activation is the first step in activation of the canonical MAPK cascade (see figure 1). Following RAS activation, RAF (A-RAF, B-RAF, or RAF-1 also known as C-RAF) is recruited to the cell membrane through binding to RAS and activated in a complex process involving phosphorylation and multiple cofactors that is not completely understood. RAF proteins directly activate MEK1 and MEK2 via phosphorylation of serine residues. MEK1

and MEK2 are themselves tyrosine and threonine/serine dual-specificity kinases that subsequently phosphorylate threonine and tyrosine residues in ERK1 and ERK2 resulting in activation. The cellular functions of ERK are diverse and include regulation of cell proliferation, survival, mitosis, and migration. (28). The second best-characterized RAS effector family is phosphoinositide 3-kinases (PI3Ks), which play important roles as mediators of RAS-mediated cell survival and proliferation. (29) (30)

Targeting KRAS directly has proven to be challenging (31) and additional signaling pathways downstream from *KRAS,* including BRAF-MAPK and PI3K-AKT which themselves have activated mutations, became attractive alternatives. In fact, several generations of MEK inhibitors are currently under development and being tested in clinical trials. (32) However, interfering with elements of the MAPK pathway is also associated with several negative feedback loops. (30, 33) For example, MEK inhibition leads to AKT over-activation via PI3K/AKT activation in several tumor types. (34) (35) It is tempting to hypothesize that this failure is due to unleashing negative feedbacks related to MEK inhibition. Indeed, a recent phase 2 clinical trial using Trametimib (the first FDA-approved MEK inhibitor) and gemcitabine in patients with pancreatic cancer showed disappointing results. (35) This trial results reinforce the notion that understanding the effects of interfering with the downstream effectors of KRAS needs to be better understood and study.

<u>Overview of PI3K/AKT/mTOR pathway</u>

Multicellular organisms have developed highly efficient mechanisms of receptor-mediated cell communication to integrate and coordinate the function and proliferation of individual cell types. In this context, the phosphoinositide 3-kinase (PI3K)/Akt/ mammalian

target of rapamycin (mTOR) pathway plays a critical role in regulating multiple normal and abnormal biological processes, including metabolism, migration, survival, autophagy, lysosome biogenesis and growth (36). This pathway is another signaling pathway highly involved in pancreatic cancer. (37, 38)

In response to different stimuli, including ligands of G protein-coupled receptors (GPCRs) and tyrosine kinase receptors (TRKs), PI3K catalyzes the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), a membrane lipid second messenger that coordinates the localization and activation of a variety of downstream effectors the most prominent of which are the isoforms of the Akt family (39). The isoforms of the Akt family (Akt1, Akt2, and Akt3) possess a lipid-binding PH domain and conserved residues (Thr-308 and Ser-473 in Akt1, the most commonly expressed isoform in normal cells), which are critical for Akt activation. Specifically, Akt, translocated to the plasma membrane in response to products of PI 3-kinase, is activated by phosphorylation at Thr-308 in the activation loop by PDK1 and by phosphorylation within the carboxy-terminus at Ser-473 by mTORC2 (40). Akt has been shown to phosphorylate multiple substrates, including the product of the tuberous sclerosis complex (TSC) 2 gene, also termed tuberin, at Ser-939 and Thr-1462 (41, 42). TSC2 forms a heterodimer with TSC1 (hamartin) that represses mTOR activity (43, 44). It is important to emphasize that mTOR functions as a catalytic subunit in two structurally distinct multiprotein complexes, mTORC1 and mTORC2 (36, 45). mTORC1, a complex of mTOR, the substrate binding subunit Raptor, GβL, and PRAS40, senses nutrients and growth factors. The TSC1/TSC2 heterodimer represses mTOR activity by acting as the GTPase-activator protein (GAP) for Rheb (Ras homolog enriched in brain). Rheb is a potent activator of mTORC1 signaling in its GTP-bound state (46, 47). Phosphorylation of TSC2 by Akt

suppresses its GTPase activity towards Rheb, leading to mTORC1 activation (48) (see figure 1). More recently, a novel mechanism of mTORC1 activation involving the ERK/p90RSK pathway but separate from Akt has been elucidated (49, 50). Specifically, ERK and p90RSK phosphorylate TSC2/tuberin at Ser-664 and Ser-1798, i.e. sites different from those targeted by Akt (Ser-939 and Thr-1462). The phosphorylation of TSC2/tuberin by ERK and p90RSK leads to the dissociation and inactivation of the TSC1/TSC2 complex and to the activation of mTORC1 (49, 50). Furthermore, ERK directly phosphorylates Raptor leading to mTORC1 activation (51). The Rag GTPases activate mTORC1 in response to amino acids, by promoting mTORC1 translocation to lysosomal membrane that contains Rheb-GTP (45). Ras-like (Ral) small GTPases, in their GTP-bound state, also promote mTORC1 activation through a pathway parallel to Rheb (52). Phosphatase and tensin homologue (PTEN) opposes PI3K by degrading PIP_3 to PIP_2 thereby inactivating Akt and mTOR signaling (53).

The second mTOR complex (mTORC2) is assembled by binding of mTOR/GβL to rapamycin-insensitive companion of mTOR (rictor) and mammalian SAPK interacting protein 1 (SIN1). (54, 55) These two unique components SIN1 and rictor most likely carry the regulatory functions of this kinase complex. SIN1 contains N-terminal Conserved Region In the Middle (CRIM) domain, the Raf-like Ras binding domain (RBD) and a C-terminal PH domain. (56) CRIM domain is a highly conserved region in the SIN1 family. SIN1 RBD domain suggests that Ras is a potential regulator of mTORC2. SIN1 PH domain implies the mTORC2 localization at the plasma membrane. However, the functional roles of these domains are yet to be characterized.

Rictor is a more conserved and larger protein than SIN1. The human rictor protein contains 1,708 amino acids and its sequence analysis did not reveal any homology to known

functional domains or proteins. (54) In spite of this, rictor is a phospho-protein and the functional characterization of this post-translational modification might provide insights in regulation of mTORC2. In recent reports, some phosphorylation sites of rictor were identified, and the Thr-1135 phosphorylation is the growth factor dependent. The main downstream effectors of mTORC2 are AKT (57) and AGC kinases members including SGK1 (serum and glucocorticoid induced protein kinase 1) (58) and PKCα (protein kinase Cα) (59, 60)

PI3K/AKT/mTOR pathway in pancreatic cancer

The PI3K/AKT/mTOR pathway plays a pivotal role in the pancreas, mediating acinar-to-ductal metaplasia and PDAC formation (61, 62) and is active in premalignant pancreatic lesions and pancreatic cancer tissues. (62-64) Additionally, the PI3K/mTOR pathway, like the mitogen-activated protein kinase (MAPK) pathway, functions downstream of Ras (30) and plays a key role in insulin/IGF receptor signaling.

Pancreatic ductal adenocarcinoma (PDAC) cells express insulin and IGF-1 receptors and over-express IRS-1 and IRS-2 (65-67). Differently from normal tissue, PDAC display activated (phosphorylated) IGF-1R (68). Gene variations in the IGF-1 signaling system have been associated to worse survival in patients with PDAC (69). Inactivation of p53, as seen during the progression of 50-70% of PDAC, up-regulates the insulin/IGF-1/mTORC1 pathway (70). Crosstalk between insulin/IGF-1 receptors and G protein-coupled receptor (GPCR) signaling systems potently stimulate mTORC1, DNA synthesis and cell proliferation in a panel of PDAC cells (63, 64, 71, 72). Therefore, blocking mTORC1 signaling has emerged as an attractive therapeutic target in PDAC.

Feedback loops between MAPK and PI3K/AKT/mTOR pathways and strategies to block mTOR signaling

In addition to growth-promoting signaling, mTORC1/S6K also mediates negative feedback loops that restrain signaling through insulin/IGF receptor and other tyrosine kinase receptors via phosphorylation and transcriptional repression of IRS-1 (73-78) and phosphorylation of Grb10 (79). Suppression of mTORC1 activity by allosteric mTORC1 inhibitors such as rapamycin prevents inhibitory IRS-1 phosphorylation and degradation, thereby augmenting PI3K/Akt activation in several cancer cell types (78, 80-82). These studies imply that the potential anti-cancer activity of rapamycin or analogs can be counterbalanced by release of feedback inhibition of PI3K/Akt activation (73, 78, 80-82).

In an effort to target the mTOR pathway more effectively, a second generation of inhibitors that act at the catalytic active site (active-site mTOR inhibitors) have been developed, including PP242 (83), Torin (84) and KU63794 (85). These compounds inhibit 4E-BP-1 phosphorylation at rapamycin-resistant sites (e.g. $Thr^{37/46}$) and block Akt phosphorylation at Ser^{473} through inhibition of mTORC2. As drug development evolves, several pharmaceutical companies are investing in the so-called PI3K/ mTOR dual inhibitors (86) in the attempt to make target therapy more effective. Many of these inhibitors are already being tested in clinical trials alone or in combinations (87), however, far less is known about the effects of such drugs in pancreatic cancer and even less in regards to their mechanistic effects in PDAC cells.

Metformin, the most widely used drug in the treatment of type 2 diabetes mellitus (T2DM), has emerged as a potential novel agent in cancer therapeutics. It is known that mTORC1 is also negatively regulated by metformin. At the cellular level, metformin

indirectly stimulates AMP–activated protein kinase (AMPK) activation (88), AMPK inhibits mTORC1 activation through stimulation of TSC2 function (89-91), leading to accumulation of Rheb-GDP (the inactive form) and by direct phosphorylation of Raptor, which disrupts its association with mTOR, leading to dissociation of the mTORC1 complex (92). Finally, Insulin/IGF-1-induced mTORC1 activation is attenuated by AMPK by direct phosphorylation of IRS-1 on Ser^{794}, a site that interferes with PI3K activation (77, 93). Interestingly, recent epidemiological studies linked administration of metformin to reduced incidence, recurrence and mortality of a variety of cancers in T2DM patients (72, 94-103), including PDAC (101, 103).

The isoquinoline alkaloid berberine, a phytochemical extracted from a variety of medicinal plants, including plants of the *Berberis* species induces multiple biological effects, including anti-obesity, anti-diabetic, anti-cancer and calorie-restriction effects (104-107). Although the exact mechanisms by which berberine could have anti-cancer effects remain incompletely understood, it is possible that is linked to AMPK activity and AMPK-mediated inhibition of mTORC1. (108)

Emerging evidence shows that pancreatic cancer is a very heterogeneous and complex disease, divided into sub-types that tend to respond differently to interventions.(109) (110) The effects of blocking the mTOR pathway in pancreatic cancer cells using different class of compounds/strategies are not fully understood. This thesis dissertation will characterize the effects of blocking mTOR using different compounds.

In chapter 2, we studied the effects of rapamycin, active-site mTOR inhibitors and metformin in pancreatic adenocarcinoma cell lines. We show that active-site inhibitors of mTOR cause a marked increase in ERK activation whereas rapamycin did not have any

stimulatory effect on ERK activation. The results imply that first and second generation of mTOR inhibitors promote over-activation of different pro-oncogenic pathways in PDAC cells, suggesting that suppression of feed-back loops should be a major consideration in the use of these inhibitors for PDAC therapy. In contrast, metformin abolished mTORC1 activation without over-stimulating Akt phosphorylation on Ser^{473} and prevented mitogen-stimulated ERK activation in PDAC cells.

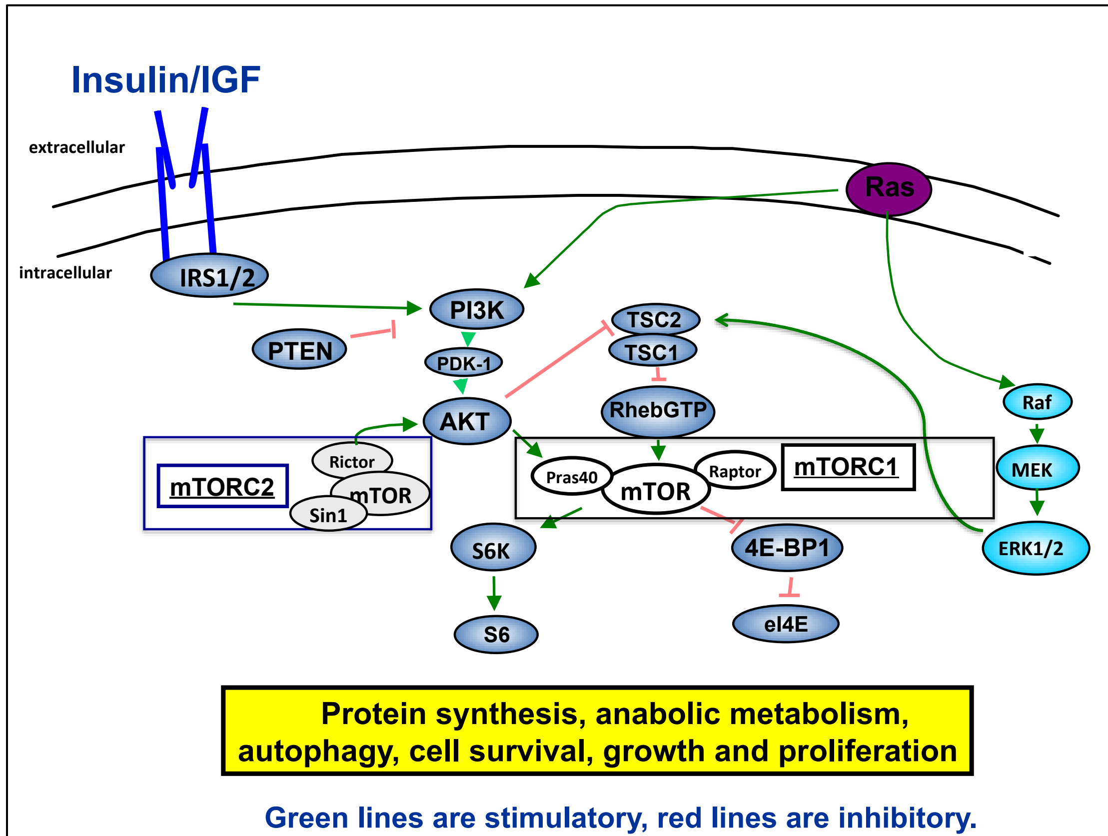
In chapter 3, we show that dual PI3K/mTOR inhibitors, including NVP-BEZ235, suppress a novel negative feedback loop mediated by mTORC2 thereby leading to enhancement of the MEK/ERK pathway activity in pancreatic cancer cells. We also show that MEK inhibitors (U126 or PD0325901) prevented ERK over-activation induced by dual PI3K/mTOR inhibition. The combination of NVP-BEZ235 and PD0325901 caused a more pronounced inhibition of cell growth than that produced by each inhibitor individually.

In chapter 4, we show that berberine inhibits DNA synthesis, cell cycle progression and proliferation in PANC-1 and MiaPaca-2 pancreatic cancer cells. It also inhibits the growth of PDAC tumor xenografts *in vivo* as effectively as metformin. Furthermore, berberine dose-dependently inhibited mTORC1 (phosphorylation of S6K at Thr^{389} and S6 at $Ser^{240/244}$) and ERK activation in PDAC cells stimulated by insulin and neurotensin or fetal bovine serum. Knockdown of α_1 and α_2 catalytic subunit expression of AMPK reversed the inhibitory effect produced by treatment with low concentrations of berberine on mTORC1, ERK and DNA synthesis in PDAC cells. However, at higher concentrations, berberine inhibited mitogenic signaling (mTORC1 and ERK) and DNA synthesis through an AMPK-independent mechanism.

Finally in chapter 5, we review negative feedback mechanisms that restrain signaling

via upstream elements of the PI3K/Akt/mTOR pathway as well as mechanisms leading to the compensatory activation of other pro-oncogenic pathways, including MEK/ERK. The studies discussed in this chapter underscore the importance of unintended pathway activation in the development of drug resistance to clinically relevant inhibitors of mTOR, Akt, PI3K or PI3K/mTOR.

Figure 1. PI3K/AKT/mTOR and MAPK pathways



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Chapter II.

Different patterns of Akt and ERK feedback activation in response to rapamycin, active-site mTOR inhibitors and metformin in pancreatic cancer cells

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Different Patterns of Akt and ERK Feedback Activation in Response to Rapamycin, Active-Site mTOR Inhibitors and Metformin in Pancreatic Cancer Cells

Heloisa P. Soares[1,2], Yang Ni[1,3], Krisztina Kisfalvi[1], James Sinnett-Smith[1], Enrique Rozengurt[1]*

1 Division of Digestive Diseases, Department of Medicine; CURE: Digestive Diseases Research Center David Geffen School of Medicine and Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California, United States of America, 2 Division of Hematology-Oncology, Department of Medicine, University of California at Los Angeles, Los Angeles, California, United States of America, 3 Department of Thoracic Surgery, Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, China

Abstract

The mTOR pathway is aberrantly stimulated in many cancer cells, including pancreatic ductal adenocarcinoma (PDAC), and thus it is a potential target for therapy. However, the mTORC1/S6K axis also mediates negative feedback loops that attenuate signaling via insulin/IGF receptor and other tyrosine kinase receptors. Suppression of these feed-back loops unleashes over-activation of upstream pathways that potentially counterbalance the antiproliferative effects of mTOR inhibitors. Here, we demonstrate that treatment of PANC-1 or MiaPaCa-2 pancreatic cancer cells with either rapamycin or active-site mTOR inhibitors suppressed S6K and S6 phosphorylation induced by insulin and the GPCR agonist neurotensin. Rapamycin caused a striking increase in Akt phosphorylation at Ser^{473} while the active-site inhibitors of mTOR (KU63794 and PP242) completely abrogated Akt phosphorylation at this site. Conversely, active-site inhibitors of mTOR cause a marked increase in ERK activation whereas rapamycin did not have any stimulatory effect on ERK activation. The results imply that first and second generation of mTOR inhibitors promote over-activation of different pro-oncogenic pathways in PDAC cells, suggesting that suppression of feed-back loops should be a major consideration in the use of these inhibitors for PDAC therapy. In contrast, metformin abolished mTORC1 activation without over-stimulating Akt phosphorylation on Ser^{473} and prevented mitogen-stimulated ERK activation in PDAC cells. Metformin induced a more pronounced inhibition of proliferation than either KU63794 or rapamycin while, the active-site mTOR inhibitor was more effective than rapamycin. Thus, the effects of metformin on Akt and ERK activation are strikingly different from allosteric or active-site mTOR inhibitors in PDAC cells, though all these agents potently inhibited the mTORC1/S6K axis.

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* E-mail: erozengurt@mednet.ucla.edu

These authors contributed equally to this work.

Introduction

The mammalian target of rapamycin (mTOR) is a highly evolutionarily conserved protein kinase that plays a key role in the integration of growth factor, nutrient and energy status of the cells [1]. mTOR functions as a catalytic subunit in two distinct multiprotein complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1, characterized by the regulatory subunit Raptor, controls at least two regulators of protein synthesis, the 40S ribosomal protein subunit S6 kinase (S6K) and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1, referred as 4E-BP1 [1,2]. The heterodimer of the tumor suppressor TSC2 (tuberin) and TSC1 (hamartin) represses mTORC1 signaling by acting as the GTPase-activator protein for the small G protein Rheb (Ras homolog enriched in brain), a potent activator of mTORC1 signaling in its GTP-bound state [3,4]. Phosphorylation of TSC2 by Akt and/or ERK/p90RSK suppresses its GTPase activating activity towards Rheb, leading to mTORC1 activation [5]. mTORC1 is acutely and allosterically inhibited by rapamycin through binding to FKBP12. mTORC2, characterized by Rictor, is not inhibited by short-term treatment with this agent and phosphorylates several AGC protein kinases, including Akt at Ser^{473} [6,7]. The mTORC1 pathway plays a key role in insulin/IGF receptor signaling [8,9] and is aberrantly activated in many cancers, including pancreatic ductal adenocarcinoma (PDAC), one of the most lethal human diseases. Accordingly, PDAC cells express insulin and IGF-1 receptors and over-express IRS-1 and IRS-2 [10 12] and PDAC (but not normal) tissue display activated (phosphorylated) IGF-1R [13]. Gene variations in the IGF-1 signaling system have been associated to worse survival in patients with PDAC [14]. Inactivation of p53, as seen during the progression of 50 70% of PDAC, up-regulates the insulin/IGF-1/mTORC1 pathway [15]. Crosstalk between insulin/IGF-1 receptors and G protein-coupled receptor (GPCR) signaling systems potently stimulate mTORC1, DNA synthesis and cell proliferation in a panel of PDAC cells [16 20]. mTORC1

signaling plays a pivotal role in the proliferation and survival of PDAC cells [21] and is activated in pancreatic cancer tissues [20,22 24]. Consequently, mTORC1 has emerged as an attractive therapeutic target in PDAC and other common malignancies.

In addition to growth-promoting signaling, mTORC1/S6K also mediates negative feedback loops that restrain signaling through insulin/IGF receptor and other tyrosine kinase receptors via phosphorylation and transcriptional repression of IRS-1 [25 30] and phosphorylation of Grb10 [31,32]. Consequently, suppression of mTORC1 activity by rapamycin prevents inhibitory IRS-1 phosphorylations and degradation, thereby augmenting PI3K/Akt activation in several cancer cell types [30,33 35]. These studies imply that the potential anti-cancer activity of rapamycin (or analogs) can be counterbalanced by release of feedback inhibition of PI3K/Akt activation [25,30,33 35]. Furthermore, rapamycin incompletely inhibits 4E-BP-1 phosphorylation [36 40]. Accordingly, the clinical antitumor activity of rapamycin and its analogs (rapalogs) has been rather limited in many types of cancer [41,42], including PDAC [43,44]. In an effort to target the mTOR pathway more effectively, novel inhibitors of mTOR that act at the catalytic active site (active-site mTOR inhibitors) have been identified, including PP242 [37], Torin [45], KU63794 [38] and its analogue AZD8055 [46]. These compounds inhibit 4E-BP-1 phosphorylation at rapamycin-resistant sites (e.g. $Thr^{37/46}$) and block Akt phosphorylation at Ser^{473} through inhibition of mTORC2. However, active-site mTOR inhibitors also eliminate feedback loops that restrain PI3K activation [25] and consequently, their therapeutic effectiveness can also be diminished by activation of upstream pathways that oppose their anti-proliferative effects.

mTORC1 is also negatively regulated by metformin, the most widely used drug in the treatment of type 2 diabetes mellitus (T2DM). Metformin is emerging as a potential novel agent in cancer chemoprevention. Recent epidemiological studies linked administration of metformin to reduced incidence, recurrence and mortality of a variety of cancers in T2DM patients [20,47 56], including PDAC [54,56]. At the cellular level, metformin indirectly stimulates AMP activated protein kinase (AMPK) activation [57], though other mechanisms of action have been proposed at very high concentrations of this biguanide. AMPK inhibits mTORC1 activation through stimulation of TSC2 function [58 60], leading to accumulation of Rheb-GDP (the inactive form) and by direct phosphorylation of Raptor, which disrupts its association with mTOR, leading to dissociation of the mTORC1 complex [61]. The precise consequence of suppression of negative feedback loops mediated by the mTORC1/S6K axis in response to metformin remains poorly defined and, in particular, it is not known whether rapamycin, active-site mTOR inhibitors and metformin lead to over-activation of similar upstream pathways in PDAC cells.

Here, we demonstrate that treatment of PANC-1 or MiaPaCa-2 pancreatic cancer cells with either rapamycin or active-site mTOR inhibitors suppressed S6K and S6 phosphorylation induced by insulin, a combination of insulin and the GPCR agonist neurotensin or serum. Rapamycin caused a striking augmentation of Akt phosphorylation at Ser^{473} while the active-site mTOR inhibitors KU63794 and PP242 completely abrogated Akt phosphorylation at this site. A salient feature of the results presented here is that active-site inhibitors of mTOR, in contrast to rapamycin, cause a marked increase in ERK activation in PDAC cells. The results imply that first and second generation mTOR inhibitors promote over-activation of different pro-oncogenic pathways in PDAC cells, namely Akt and ERK. Metformin also abolished mTORC1 activation but without over-stimulating Akt phosphorylation on Ser^{473}. Furthermore, metformin prevented ERK activation in response to cross-talking agonists in PDAC cells. Our results demonstrate that the effects of metformin on Akt and ERK activation are strikingly different from those elicited by allosteric or active-site mTOR inhibitors, though all these agents potently inhibited the mTORC1/S6K axis.

Materials and Methods

Cell culture

The human pancreatic cancer cell lines PANC-1 and MiaPaCa-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cell lines harbor activating mutations in the *KRAS* oncogene. Cells were grown in Dulbecco's modified Eagle Medium (DMEM) with 2 mM glutamine, 1 mM Na-pyruvate, 100 units/mL penicillin, and 100 µg/mL streptomycin and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 10% CO_2.

Western blot analysis

Confluent cultures of PANC-1 or MIA PaCa-2 cells grown on 3 cm dishes were washed and then incubated for 24 hr with DMEM containing 5 mM glucose and 1% FBS. The cells were washed twice with DMEM containing 5 mM glucose and incubated in serum-free medium for 4 h and then treated as described in individual experiments. The cultures were then directly lysed in 2 × SDS-PAGE sample buffer [200 mM Tris-HCl (pH 6.8), 2 mM EDTA, 0.1 M Na_3VO_4, 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol], followed by SDS-PAGE on 10% gels and transfer to Immobilon-P membranes (Millipore, Billerica, MA). Western blots were then performed on membranes incubated overnight with the specified antibodies in phosphate-buffered saline (PBS) containing 0.1% Tween-20. The immunoreactive bands were detected with ECL (enhanced chemiluminescence) reagents (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). The antibodies used detected the phosphorylated state of S6K at Thr^{389}, S6 at $Ser^{235/236}$, 4E-BP1 at $Thr^{37/46}$ and Thr^{70}, Akt at Ser^{473} and Thr^{308} and ERK1/2 at Thr^{202} and Tyr^{204} or the total levels of these proteins.

Anchorage-dependent cell proliferation. PANC-1 cells (10^5) were plated on 35 mm tissue culture dishes in DMEM containing 10% FBS. After 24 h of incubation at 37°C, groups of cultures were incubated with neurotensin and insulin with or without metformin in DMEM containing 0.25% FBS or Rapamycin, KU63794 or Metformin in DMEM containing 2.5% FBS. The cultures were then incubated for 4 d, and the total cell count was determined from a minimum of six wells 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.

Materials

DMEM was obtained from Invitrogen (Carlsbad, CA). Neurotensin and insulin were obtained from Sigma Chemical (St. Louis, MO). Rapamycin, KU63794 and PP242 were from R&D Systems, Inc. Minneapolis. All antibodies were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase conjugated anti-rabbit IgG and anti-mouse IgG were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). All other reagents were of the highest grade available.

Results

Stimulation of p70S6K and S6 phosphorylation in response to insulin and neurotensin in PDAC cells is completely abolished by rapamycin or KU63794

Initially, we determined the influence of rapamycin and KU63794 on mTORC1-mediated phosphorylation of S6K in PDAC cells. Rapamycin is an allosteric inhibitor of mTORC1 that acts via FKBP-12 whereas KU63794 is a highly specific ATP-competitive inhibitor of mTOR that inhibits both mTORC1 and mTORC2. Cultures of PANC-1 (**Fig. 1**) or MiaPaCa-2 (**Fig. 2**) cells were incubated for 2 h in the absence or presence of rapamycin (at 10 or 100 nM) or KU63794 (at 1 or 5 μM). Then, the cultures were stimulated with a combination of insulin (10 ng/ml) and the GPCR agonist neurotensin (5 nM) for 2 h to elicit positive crosstalk [18,20]. Phosphorylation of S6K on Thr^{389}, a direct target of mTORC1, and phosphorylation of S6 ($Ser^{235/236}$), a substrate of S6K, was monitored using specific antibodies that detect the phosphorylated state of those residues. Stimulation of either PANC-1 or MiaPaCa-2 cells with insulin and neurotensin induced robust phosphorylation of S6K on Thr^{389} and S6 (**Figs. 1** and **2**). Exposure to either rapamycin or KU63794 completely prevented the increase in the phosphorylation of these proteins in response to stimulation by insulin and neurotensin in either PANC-1 (**Fig. 1**) or MiaPaCa-2 cells (**Fig. 2**). We verified that the total levels of S6K and S6 did not change in response to the treatments. The results indicate that allosteric or active-site inhibitors of mTOR potently blocked the mTORC1/S6K axis at the concentrations used in PDAC cells.

Differential regulation of 4EBP1 phosphorylation sites in response to mitogenic stimulation, rapamycin and KU63794 in PDAC cells

The phosphorylation of 4EBP1 was also monitored by using site-specific 4E-BP1 antibodies that detect p-$Thr^{37/46}$ or p-Thr^{70} in lysayes of PANC-1 (**Fig. 1**) or MiaPaCa-2 (**Fig. 2**) cells. These cells displayed a high basal level of 4E-BP1 phosphorylation at $Thr^{37/46}$ that was not further increased by stimulation with insulin and neurotensin. However, cell stimulation reduced the mobility of 4E-BP1 in SDS/PAGE, a response suggestive of increased phosphorylation at other sites. Indeed, treatment of PANC-1 or MiaPaCa-2 cells with neurotensin and insulin markedly stimulated 4E-BP1phosphorylation on Thr^{70}. The constituve phosphorylation of 4E-BP1 on $Thr^{37/46}$ was abolished by treatment with KU63794 but was not affected by rapamycin at either 10 or 100 nM, in agreement with reports that rapamycin and its analogs do not inhibit 4E-BP1 phosphorylation at these sites in other cell types. In contrast, the signal responsive phosphorylation of 4E-BP1 on Thr^{70} was prevented by treatment with either KU63794 or rapamycin at 100 nM. We verified that the total levels of 4E-BP1 did not change in response to the treatments.These results revealed an unappreciated regulation of 4E-BP1 phosphorylation on different residues in response to external signals and demonstrate that rapamycin inhibits inducible but not constitutive 4E-BP1 phosphorylation in PDAC cells whereas active-site mTOR inhibitors suppress phosphorylation of 4E-BP1 at all sites.

Rapamycin and KU63794 induce over-stimulation of different upstream pathways in PDAC cells stimulated with insulin and neurotensin or insulin alone

In order to determine whether allosteric and active-site mTOR inhibitors eliminate feedback loops that restrain the activity of upstream signaling pathways in PDAC cells, we examined the effect of these inhibitors on the phosphorylation of Akt in response to mitogenic signaling in PANC-1 and Mia PaCa-2 cells. Stimulation of these cells with insulin and neurotensin induced a marked increase in Akt phosphorylation on Ser^{473} (**Figs. 1** and **Fig. 2**). Treatment with either 10 nM or 100 nM rapamycin promoted over-stimulation of Akt phosphorylation on Ser^{473}, consistent with suppression of mTORC1/S6K axis feedback loops. In contrast, prior exposure to the active-site mTOR inhibitor KU63794, which inhibits both mTORC1 and mTORC2, blocked Akt phosphorylation on Ser^{473} in PANC-1 (**Fig. 1**) and MiaPaCa-2 cells (**Fig. 2**), in line with the notion that mTORC2 is the major protein kinase that phosphorylates Akt on Ser^{473} in PDAC cells. KU63794 did not prevent Akt phosphorylation at Thr^{308}.

The ERK/RSK pathway, which plays a pivotal role in PDAC cell proliferation also leads to mTORC1 activation [5,62]. In breast and bladder cancer cells, inhibition of the mTORC1/S6K axis by rapamycin induced feedback activation of ERK [63]. Consequently, we examined the effects of rapamycin and KU63794 on ERK activation in PDAC cells. In agreement with previous studies [16,64,65], stimulation of either PANC-1 or MiaPaCa-2 cells with insulin and neurotensin markedly activated ERK (ERK phosphorylated on Thr^{202} and Tyr^{204}), as illustrated in **Figs. 1** and **2**. In contrast to the results obtained in other cell types [63], treatment with either 10 or 100 nM rapamycin for 2 h did not alter the basal or the stimulated level of ERK phosphorylation in PANC-1 and MiaPaCa-2 cells. Similar results were obtained when these PDAC cells were treated with rapamycin for 4 or 24 h (results not shown). In contrast, exposure to KU63794 (1 5 μM) increased the basal level of ERK phosphorylation and strikingly enhanced the stimulation of ERK phosphorylation induced by insulin and neurotensin in either PANC-1 or MiaPaCa-2 cells. Quantification of the results with ERK is illustrated in the lower panels of **Figs. 1** and **2** (bars). These results demonstrate that rapamycin, an allosteric inhibitor of mTORC1, and KU63794, an active-site inhibitor of mTOR, lead to over-activation of different upstream pro-oncogenic pathways in PDAC cells.

Stimulation of PANC-1 cells or MiaPaCa-2 with insulin alone induced robust increase in PI3K/Akt/mTORC1 but does not induce significant increase in ERK phosphorylated on Thr^{202} and Tyr^{204} (**Fig. 3**). Consequently, we determined whether the differential effects of rapamycin and KU63794 depicted in PDAC stimulated with the combination of insulin and neurotensin (Figs. 1 and 2) can also be produced when PANC-1 and MiaPaCa-2 cells are challenged with insulin alone. Cultures of these cells were incubated for 2 h in the absence or presence of rapamycin (10 100 nM) or KU63794 (1 5 μM) and then stimulated with insulin (10 ng/ml). We monitored phosphorylation of S6K on Thr^{389}, S6 on $Ser^{235/236}$, Akt on Ser^{473} and Thr^{308} and ERK on Thr^{202} and Tyr^{204}. Prior exposure to either rapamycin or KU63794 abolished the increase in the phosphorylation of S6K and S6 in response to insulin in either PANC-1 or MiaPaCa-2 cells (**Fig. 3**). Exposure to rapamycin over-activated whereas treatment with KU63794 abolished Akt phosphorylation on Ser^{473} in the insulin-stimulated PDAC cells. Rapamycin did not produce any detectable effect on ERK activation in un-stimulated or insulin-treated cells. A salient feature of the results shown in **Fig. 3** is that exposure to KU63794 induced a marked increase in the phosphorylation of ERK on Thr^{202} and Tyr^{204}. These results corroborated that the allosteric inhibitor of mTORC1 and the active-site site inhibitor of mTOR promote over-activation of different upstream pathways in PDAC cells challenged with insulin or insulin and neurotensin, a

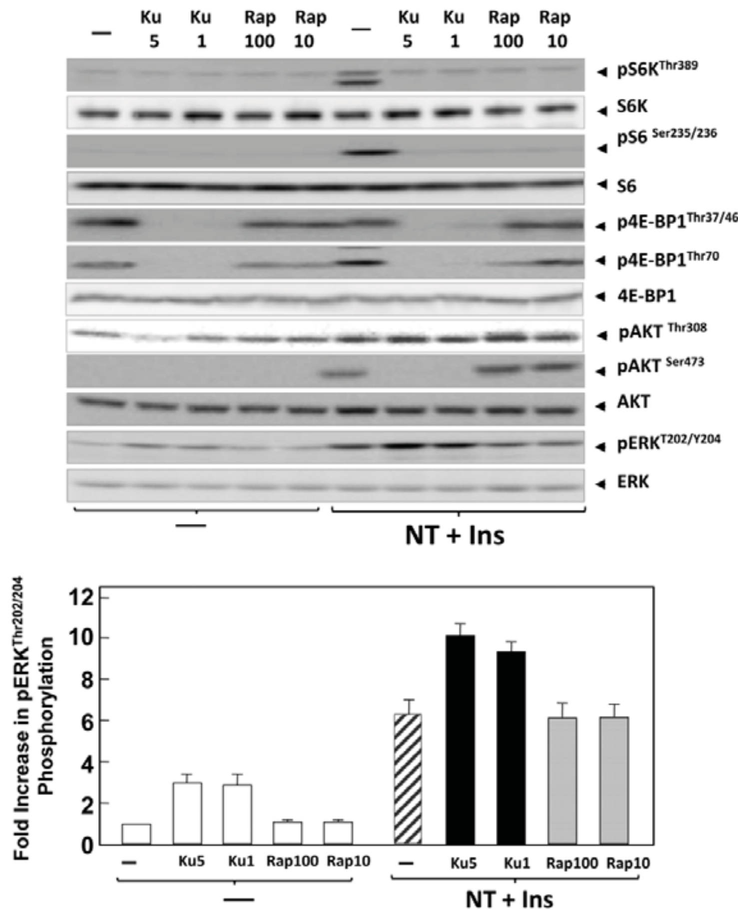

Figure 1. Differential feedback activation of Akt and ERK phosphorylation by rapamycin and KU63794 in PANC-1 cells. Cultures of PANC-1 cells were incubated in the absence (−) or in the presence of KU63794 (Ku) at 1 μM or 5 μM or rapamycin (Rap) at 10 or 100 nM for 2 h in DMEM containing 5 mM glucose, as indicated. Then, the cells were stimulated for 2 h with 5 nM neurotensin (NT) and 10 ng/ml insulin (Ins) and lysed with 2×SDS–PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of S6K at Thr^{389}, S6 at $Ser^{235/236}$, 4E-BP1 at $Thr^{37/46}$ and Thr^{70}, Akt at Ser^{473} and Thr^{308} and ERK at Thr^{202} and Tyr^{204}. Immunoblotting with antibodies that recognize total S6K, S6, 4E-BP1, Akt and ERK was used to verify that the cell treatments did not change the total level of these proteins and confirm equal gel loading. Fold increase in ERK phosphorylation was quantified using Multi Gauge V3.0 and plotted as bars. Similar results were obtained in 3 independent experiments.
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combination that elicits crosstalk between insulin/IGF and GPCR signaling systems.

PP242, like KU63794, enhances ERK activation in PANC-1 cells stimulated with insulin and neurotensin

Subsequently, we determined whether the striking over-activation of ERK by the active-site mTOR inhibitor KU63794 could be also produced by a structurally unrelated active-site mTOR inhibitor. Cultures of PANC-1 were incubated for 2 h in the absence or presence of PP242 (1 5 μM), a recently identified active-site mTOR inhibitor [37], and stimulated for 2 h with insulin and neurotensin. We monitored phosphorylation of S6K on Thr^{389}, S6 on $Ser^{235/236}$, 4EBP1 on $Thr^{37/46}$, Akt on Ser^{473} and ERK on Thr^{202} and Tyr^{204}. As shown in **Fig. 4 A**, prior exposure to PP242 abolished the phosphorylation of S6K, S6, 4EBP1 and Akt in PANC-1 cells. The key feature of the results is that PP242, like KU63794, induced a marked increase in the phosphorylation of ERK on Thr^{202} and Tyr^{204} (**Fig. 4A** and quantification in **Fig. 4B**). Because PP242 is a less selective mTOR inhibitor [66], we determined whether the concentrations of PP242 that promoted ERK activation coincide with those that inhibit mTORC1 activity. As shown in **Fig. 4C**, PP242 enhanced ERK activation and inhibited S6 phosphorylation at almost identical concentrations.

We verified that the active-site mTOR inhibitors KU63794 and PP242, at concentrations that markedly enhanced ERK activation and inhibited Akt phosphorylation on Ser^{473} did not prevent Akt phosphorylation at Thr^{308} in PDAC cells (**Fig. 4D**). In fact, the specific mTOR inhibitor KU63794 slightly enhanced Akt phosphorylation at Thr^{308}, consistent with suppression of feedback loops that restrain PI3K activity (**Fig. 4D**). PP242 was less

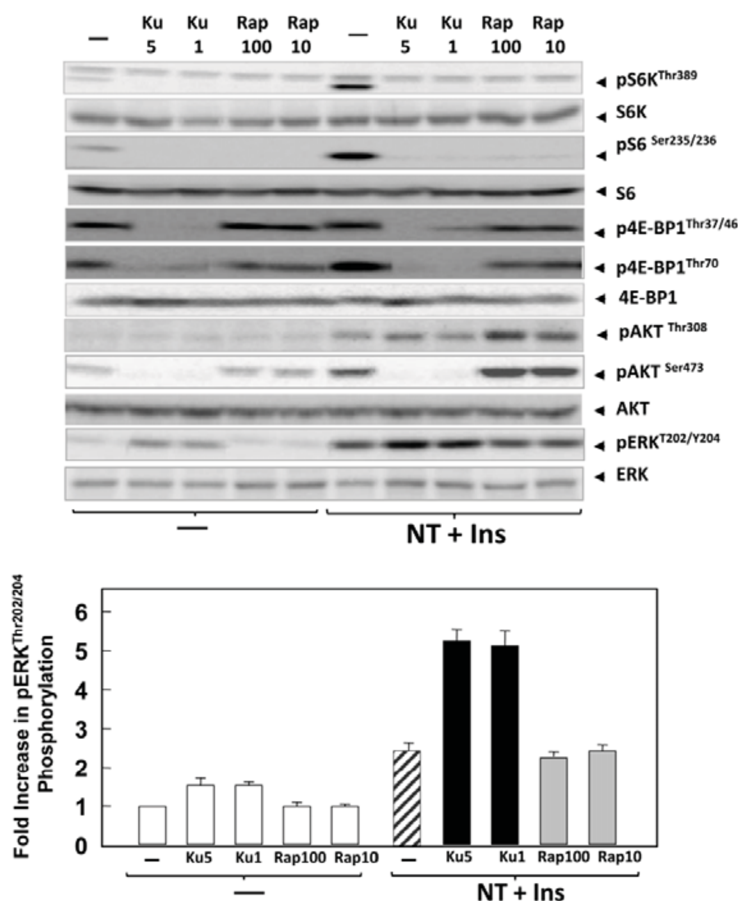

Figure 2. Differential feedback activation of Akt and ERK phosphorylation by rapamycin and KU63794 in MiaPaCa-2 cells. Cultures of MiaPaCa-2 cells were incubated in the absence (−) or in the presence of KU63794 (Ku) at 1 μM or 5 μM or rapamycin (Rap) at 10 or 100 nM for 2 h in DMEM containing 5 mM glucose, as indicated. Then, the cells were stimulated for 2 h with 5 nM neurotensin (NT) and 10 ng/ml insulin (Ins) and lysed with 2×SDS–PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of S6K at Thr^{389} (pS6K), S6 at $Ser^{235/236}$ (pS6), 4E-BP1 at $Thr^{37/46}$ and Thr^{70}, Akt at Ser^{473} and ERK at Thr^{202} and Tyr^{204}. Immunoblotting with antibodies that recognize total S6K, S6, 4E-BP1, Akt and ERK was used to verify that the cell treatments did not change the total level of these proteins and confirm equal gel loading. Fold increase in ERK phosphorylation was quantified using Multi Gauge V3.0 and plotted as bars. Similar results were obtained in 3 independent experiments.
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effective than KU63794 in enhancing Akt phosphorylation at Thr^{308}, most likely reflecting off-target inhibition of PI3K [66]. Thus, the specific active-site site mTOR inhibitors KU63794 and PP242 suppressed Akt phosphorylation on Ser^{473}, did not decrease Akt phosphorylation on Thr^{308} and stimulated over-activation of ERK phosphorylation at Thr^{202} and Tyr^{204} in PDAC cells.

The mechanism by which active-site inhibitors enhance ERK activation is not well understood. Our results do not support the existence in PDAC cells of a putative mTORC1/S6K/PI3K/ERK feedback loop, proposed in other cell types [63], since potent inhibition of the mTORC1/S6K axis by either rapamycin or everolimus did not produce overstimulation of ERK in PDAC cells. To substantiate this conclusion, PANC-1 cells were treated with KU63794 or PP242 and stimulated with insulin and neurotensin in the absence or presence of A66 [67], a selective inhibitor of the 110α catalytic subunit of PI3K. As shown in **Fig. 4D**, exposure to A66 did not prevent enhancement of ERK activation in response to exposure to either KU63794 or PP242. We corroborated that A66, at the concentration used, potently inhibited PI3K within PANC-1 cells since it prevented insulin-induced Akt phosphorylation at Thr^{308}, the key residue in the Akt activation loop phosphorylated by PI3K-dependent PDK1.

In order to obtain further insight of the mechanism by which treatment with KU63794 induces over-activation of ERK we also determined the effect of this active-site mTOR inhibitor on the activation of MEK, the upstream kinase that phosphorylates ERK. MEK activation was scored by assessing the phosphorylation of Ser^{217} and Ser^{221} in its activation loop. As shown in **Fig. S1**, treatment of PANC-1 or MiaPaCa-2 cells with KU63794 markedly enhanced MEK phosphorylation induced by insulin and neurotensin. Collectively, the results demonstrate that active-

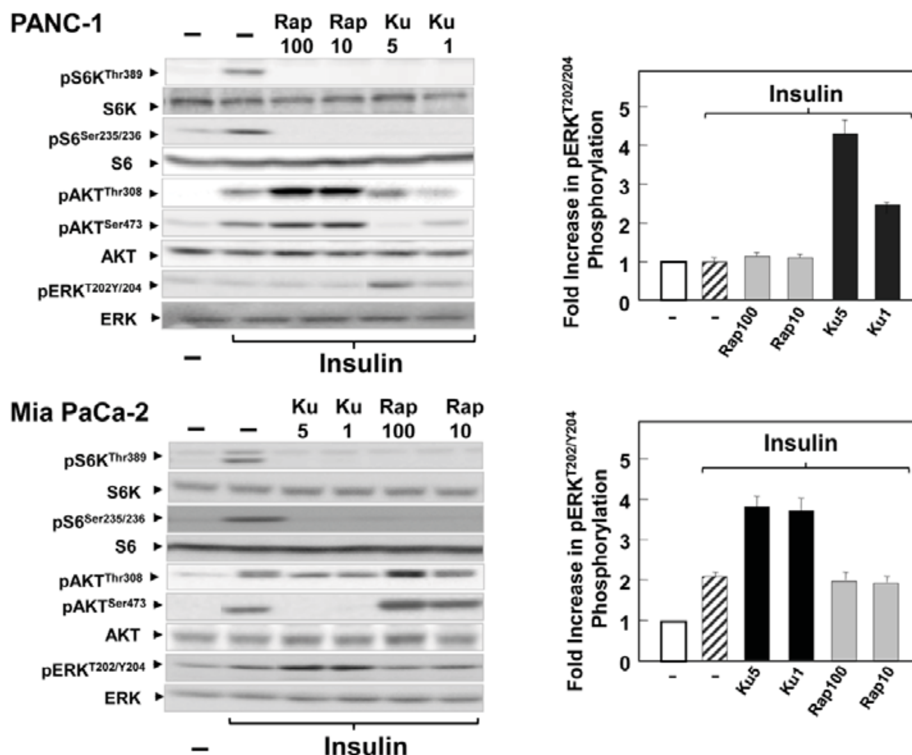

Figure 3. Differential feedback activation of Akt and ERK phosphorylation by rapamycin and KU63794 in insulin-stimulated MiaPaCa-2 and PANC-1 cells. The cultures of PANC-1 (upper panels) and MiaPaCa-2 (lower panels) were incubated in the absence (−) or in the presence of KU63794 (Ku) at 1 μM or 5 μM or rapamycin (Rap) at 10 or 100 nM for 2 h in DMEM containing 5 mM glucose, as indicated. Then, the cells were stimulated for 2 h with 10 ng/ml insulin and lysed with 2×SDS–PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of S6K at Thr^{389}, S6 at $Ser^{235/236}$, Akt at Ser^{473} and Thr^{308} and ERK at Thr^{202} and Tyr^{204}. Immunoblotting with total S6K, S6, Akt and ERK was used to verify equal gel loading. Fold increase in ERK phosphorylation was quantified using Multi Gauge V3.0 and plotted as bars. Similar results were obtained in 3 independent experiments.
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site mTOR inhibitors led to MEK/ERK hyper-activation through a PI3K/S6K-independent feedback loop in PDAC cells.

Differential patterns of Akt and ERK activation in response to rapamycin, everolimus, KU63794 and PP242 in PANC-1 cells stimulated with serum

The preceding results were obtained with PDAC cells stimulated with defined mitogens that act through specific receptors. To extend further these findings we also tested whether differential patterns of Akt and ERK activation are produced when the cells are stimulated with fetal bovine serum. Cultures of PANC-1 cells were incubated for 2 h in the absence or presence of rapamycin (100 nM), everolimus (100 nM), KU63794 (1 μM) or PP242 (1 μM) and stimulated with medium containing fetal bovine serum. We monitored phosphorylation of S6 on $Ser^{235/236}$, Akt on Ser^{473} and ERK on Thr^{202} and Tyr^{204}. Prior exposure to rapamycin, everolimus, KU63794 or PP242 abolished the increase in the phosphorylation of S6 in response to serum (**Fig. 5**). Exposure to rapamycin or everolimus over-activated whereas treatment with KU63794 or PP242 abolished Akt phosphorylation on Ser^{473} in serum-stimulated PDAC cells. Rapamycin or everolimus did not produce any detectable effect on ERK activation whereas exposure to KU63794 or PP242 induced a marked increase in the phosphorylation of ERK on Thr^{202} and Tyr^{204} in serum-treated cells (**Fig. 5**). These results corroborated that allosteric and active-site site inhibitors of mTOR promote over-activation of different upstream pathways in PDAC cells under a variety of experimental conditions, including cells challenged with insulin, insulin and the GPCR agonist neurotensin or with fresh fetal bovine serum.

Metformin, in contrast to allosteric and active-site mTOR inhibitors, inhibits ERK activation and does not induce over-stimulation of Akt in PDAC cells

Like rapamycin and active-site mTOR inhibitors, metformin also inhibits stimulation of the mTORC1/S6K axis but its effects on feedback loops regulating Akt and ERK activation have not been examined in PDAC cells. Recently, we demonstrated that the sensitivity of PDAC cells to the inhibitory effects of metformin are markedly enhanced by culturing PDAC cells in medium containing physiological (5 mM) rather than supra-physiological (25 mM) concentrations of glucose [68]. In order to determine the effect of metformin on Akt and ERK signaling in PDAC cells, PANC-1 and MiaPaCa-2 cells grown in medium containing 5 mM glucose were treated with or without metformin (1 mM) and then stimulated with insulin and the GPCR agonist

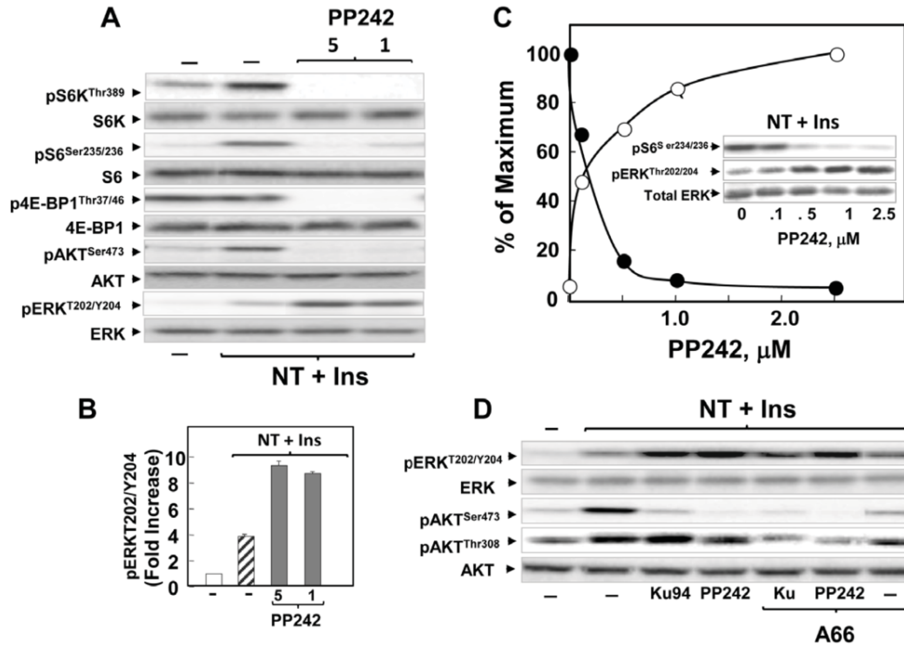

Figure 4. Feedback activation of ERK phosphorylation by PP242: role of PI3K. ***A,*** Cultures of PANC-1 cells were incubated in the absence (−) or in the presence of PP242 at 1 μM or 5 μM for 2 h in DMEM containing 5 mM glucose, as indicated. Then, the cells were stimulated for 2 h with 5 nM neurotensin (NT) and 10 ng/ml insulin (Ins) and lysed with 2×SDS–PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of S6K at Thr^{389}, S6 at $Ser^{235/236}$, 4E-BP1 at $Thr^{37/46}$, Akt at Ser^{473} and ERK at Thr^{202} and Tyr^{204}. Immunoblotting with antibodies that recognize total S6K, S6, 4E-BP1, Akt and ERK was used to verify that the cell treatments did not change the total level of these proteins and confirm equal gel loading. Similar results were obtained in 3 independent experiments. ***B,*** The bars represent the increase in ERK phosphorylation induced by insulin (Ins) and neurotensin (NT) in cells without or with prior exposure to PP242. Quantification was performed using Multi Gauge V3.0 ***C,*** Cultures of PANC-1 cells were incubated as in panel **A** but in the presence of increasing concentrations of PP242. The samples were analyzed to detect the phosphorylated state of S6 at $Ser^{235/236}$ and ERK at Thr^{202} and Tyr^{204}. Immunoblotting with total ERK and S6 (not shown) was used to verify equal gel loading. Quantification was performed using Multi Gauge V3.0. ***D,*** Cultures of PANC-1 cells were incubated in the absence (−) or in the presence of either KU63794 (Ku) or PP242 at 5 μM for 2 h. Then, the cells were stimulated for 2 h with 5 nM neurotensin (NT) and 10 ng/ml insulin (Ins) and lysed with 2×SDS–PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of ERK at Thr^{202} and Tyr^{204}, Akt at Ser^{473} and Thr^{308}. Immunoblotting with total Akt and ERK was used to verify equal gel loading.

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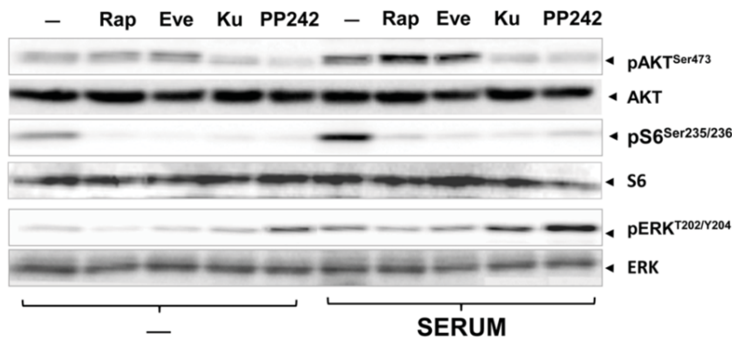

Figure 5. Differential feedback activation of Akt and ERK by rapamycin, everolimus, KU63794 and PP242 in serum-stimulated PANC-1 cells. The cultures were incubated in the absence (−) or in the presence of 5 μM KU63794 (Ku), 5 μM PP242, 100 nM rapamycin (Rap) or 100 nM everolimus for 2 h in DMEM containing 5 mM glucose, as indicated. Then, the cells were stimulated for 2 h with fetal bovine at a final dilution of 2% (SERUM) and lysed with 2×SDS–PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of Akt at Ser^{473}, S6 at $Ser^{235/236}$, and ERK at Thr^{202} and Tyr^{204}. Immunoblotting with total Akt, S6 and ERK was used to verify equal gel loading. Similar results were obtained in 3 independent experiments.

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neurotensin. mTORC1 activity was determined by phosphorylation of S6K at Thr^{389} and phosphorylation of S6 (Ser$^{235/236}$) and ERK activation by detecting ERK phosphorylated on Thr^{202} and Tyr^{204}. Metformin, like rapamycin, virtually abolished mTORC1 activation induced by insulin and neurotensin in PANC-1 and MiaPaCa-2 cells (pS6K, pS6 in **Fig. 6, A** and **B**) without changing the total levels of either S6K or S6. However, metformin did not over-stimulated Akt phosphorylation on Ser^{473} in the PDAC cells (p-Akt^{473} in **Fig. 6, A** and **B**), a result strikingly different from that obtained with rapamycin and everolimus. The salient feature of the results in **Fig. 6 A** and **B** is that metformin, in sharp contrast to the effects of active-site mTOR inhibitors, prevented ERK activation in PANC-1 and MiaPaCa-2 cells in multiple independent experiments (depicted by the bars) but did not alter the level of total ERK. We verified that under these experimental conditions, metformin markedly induced AMPK activation, as shown by the phosphorylation of acetyl-CoA carboxylase (ACC) at Ser^{79}, a residue directly phosphorylated by AMPK and used as a biomarker of its activity within intact cells.

We next determined whether metformin inhibits ERK activation at concentrations (0.05 0.1 mM) that are close to the therapeutic range. As shown in **Fig. 6 C**, metformin dose-dependently inhibited phosphorylation S6K at Thr^{389} and ERK activation at concentrations as low as 0.05 0.1 mM. Metformin, at these concentrations, also induced AMPK activation, as shown by ACC phosphorylation at Ser^{79}. Quantification of the immunoblotting results is illustrated in **Fig. 6 D**. Our results demonstrate that the effects of metformin on Akt and ERK activation are strikingly different from those elicited by allosteric or active-site mTOR inhibitors, though all these agents potently inhibited the mTORC1/S6K axis.

Effects of metformin, KU63974 and rapamycin on the proliferation of PANC-1 cells

The differential effects of metformin, KU63794 and rapamycin on the activation of PI3K/Akt and MEK/ERK in PDAC cells, prompted us to determine the effects of these agents on the proliferation of these cells. Initially, we assessed the effect of increasing concentrations of metformin on the increase in the number of PANC-1 cells induced by stimulation with neurotensin and insulin in the presence of 0.25% serum for 4 days (**Fig. 7, A**). Metformin prevented the increase in the number of PANC-1 cells in a dose-dependent manner. A marked inhibitory effect was induced by metformin at a concentration as low as 0.1 mM and complete suppression of cell proliferation was achieved by metformin at 1 mM. The concentrations of metformin that inhibited PANC-1 cell proliferation coincided with the concentration of metfornin that prevented mTORC1 and ERK signaling in these cells.

Next, we examined the effect of 1 mM metformin, 5 µM KU63794 and 100 nM rapamycin on the proliferation of PANC-1 incubated in medium containing serum. Each agent was tested at a concentration that produced maximal inhibition of the mTORC1/S6K axis in PDAC cells. As seen in **Fig. 7 B**, the agents inhibited PANC-1 cell proliferation but with important differences in their efficacy. Metformin induced a more pronounced inhibition of proliferation than either KU63794 or rapamycin while, the active-site mTOR inhibitor was more effective than rapamycin (all these differences were statistically significative). The results suggest that the effects of inhibiting mTORC1/S6K by the allosteric or active-site inhibitors is compensated by over-activation of Akt (rapamycin) or ERK (KU63794). The comparatively stronger inhibition of PDAC cell proliferation by metformin could be attributed, at least in part, to inhibition of ERK signaling.

Discussion

Aberrant stimulation of the mTOR pathway in many cancer cells, including PDAC, is eliciting intense interest for targeting this pathway [1]. However, it is increasingly appreciated that the mTORC1/S6K axis also mediates negative feedback loops that attenuate signaling via insulin/IGF receptor and other tyrosine kinase receptors. Suppression of these feed-back loops unleashes over-activation of upstream pathways that potentially counterbalance the anti-proliferative effects of mTOR inhibitors. Consequently, the identification of negative feedback loops by either allosteric or active-site mTOR inhibitors has emerged as an area of major interest in cancer therapy. Because the operation of these complex feedback loops is cell-context specific, we examined the patterns of Akt and ERK feedback activation in response to mTORC1 inhibition by rapamycin, active-site mTOR inhibitors and metformin in human PDAC cells. PDAC is one of the most lethal human diseases, with overall 5-year survival rate of only 3 5% and a median survival period of 4 6 months. The incidence of this disease in the US has increased to more than 44,000 new cases in 2011 and is now the fourth leading cause of cancer mortality in both men and women [69]. As the current therapies offer very limited survival benefits, novel molecular therapeutic targets and strategies are urgently needed to treat this aggressive disease.

Our results demonstrate that treatment of PDAC cells with allosteric mTORC1 inhibitors (rapamycin, everolimus) augmented Akt phosphorylation at Ser^{473} while the active-site inhibitors of mTOR (KU63794 and PP242) completely abrogated Akt phosphorylation at this site consistent with the notion that mTORC2 is the major kinase that phosphorylates Akt at Ser^{473}. A salient feature of our results is that active-site inhibitors of mTOR promoted a marked increase in ERK activation in PDAC cells stimulated with insulin, insulin and neurotensin or serum. These results indicate that first and second generations of mTOR inhibitors promote over-activation of different upstream pro-oncogenic pathways in PDAC cells.

While augmentation of Akt phosphorylation at Ser^{473} by rapalogs is well known in other cell types [30,33 35], the enhancing effect of active-site mTOR inhibitors on ERK has been much less explored. In order to understand the mechanism by which active-site mTOR inhibitors promote ERK activation in PDAC cells, we determined here the role of a feedback loop involving mTORC1/S6K/PI3K/ERK, proposed to mediate ERK activation in other cell types [63]. Several lines of evidence dissociated this feedback loop from the enhancement of ERK activation induced by active-site mTOR inhibitors in PDAC cells. Firstly, neither rapamycin nor everolimus, at concentrations that completely blocked the mTORC1/S6K axis, produced any detectable enhancement of ERK activation in PDAC cells under a variety of experimental conditions. Secondly, KU63794 induced ERK hyper-activation even in PDAC cells treated with A66, a potent and selective inhibitor of the 110α catalytic subunit of PI3K [67]. These results, indicating that active-site inhibitors enhance ERK through a PI3K-independent pathway, are in agreement with a recent report using PP242 in multiple myeloma cells [70]. However, PP242 inhibits a number of protein kinases *in vitro*, including MEK, whereas KU63794 did not inhibit any protein kinase other than mTOR [66]. The possibility that PP242 could induce ERK via off-target effects is an important consideration. Our results demonstrate, for the first time, that the highly selective inhibitor of mTOR KU63794 enhances MEK/ERK activation

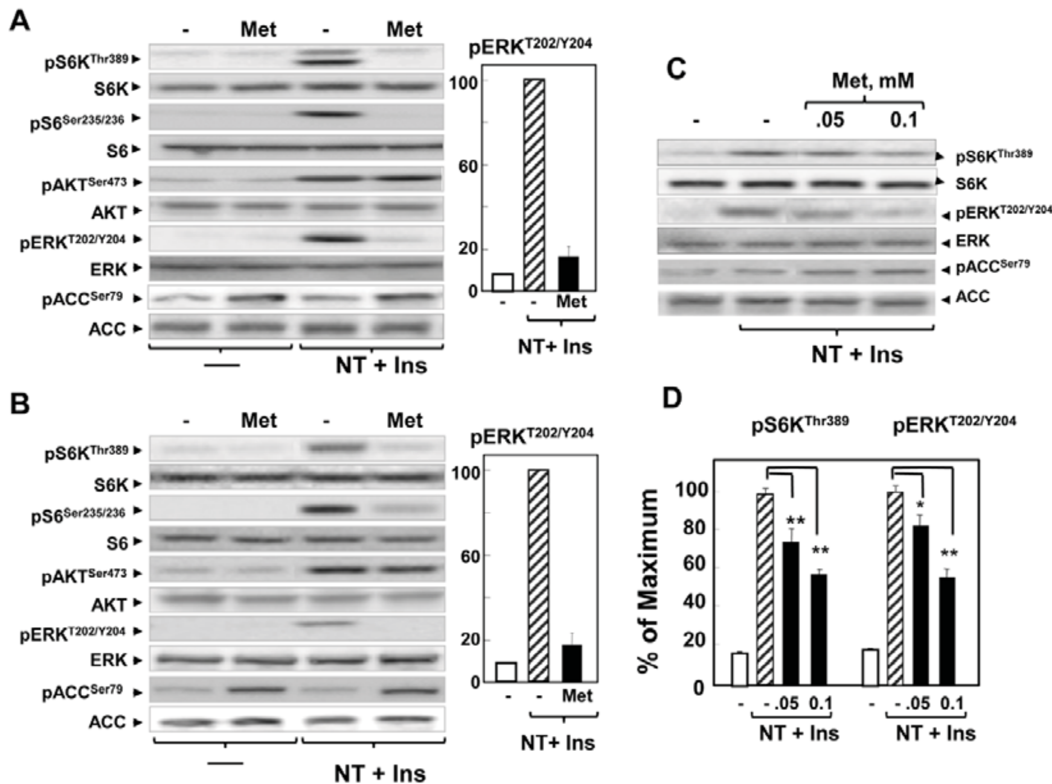

Figure 6. Metformin inhibits mTORC1 and ERK signaling without over-activating Akt in PDAC cells incubated in medium containing a physiological glucose concentration. **A**) Cultures of MiaPaca-2 (**A**) and PANC-1 (**B**) cells were incubated in the absence (−) or in the presence of 1 mM metformin (Met) for 16 h in DMEM containing 5 mM glucose, as indicated. Then, the cells were stimulated for 2 h with 5 nM neurotensin and 10 ng/ml insulin (NT+Ins) and lysed with 2×SDS–PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of S6K at Thr^{389}, S6 at $Ser^{235/236}$, ACC at Ser^{79}, Akt at Ser^{473} and ERK at Thr^{202} and Tyr^{204}. Immunoblotting with antibodies that recognize total S6K, S6, Akt, ERK and ACC was used to verify that the cell treatments did not change the total level of these proteins and confirm equal gel loading. Similar results were obtained in 3 independent experiments. The bars in panels *A* and *B* represent the % of the maximal ERK phosphorylation (mean ±SEM) induced by insulin (Ins) and neurotensin (NT) in cells without or with prior treatment with 1 mM metformin. The results of ERK phosphorylation were obtained in multiple independent experiments (N = 12 for PANC-1 and N = 8 for MiaPaca-2) Quantification was performed using Multi Gauge V3.0 **C**). Mia PaCa-2 cells were incubated with DMEM containing 5 mM glucose either in absence or presence of 0.05 mM or 0.1 mM metformin for 16 h. Then, the cells were treated with NT+Ins, as above, and lysates analyzed by immunoblotting. Similar results were obtained in 6 independent experiments. **D**) The experiment presented in panel C was representative of 6 independent experiments. Quantification of these experiments was performed using Multi Gauge V3.0. Results are expressed as the percentage of maximum mean ±SEM, n = 6. P values were determined using the t-test (Sigma Plot 12.) *$p<0.05$; **$p<0.001$; n = 6.
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through a PI3K-independent pathway. Given that active-site mTOR inhibitors are increasingly considered for clinical use [1], the findings presented here imply that suppression of feed-back loops by these inhibitors should be a major consideration in the use of these inhibitors for PDAC therapy.

Many epidemiological studies have linked obesity and long-standing type 2 diabetes mellitus (T2DM), with increased risk for developing PDAC and other clinically aggressive cancer [71,72]. Obesity and T2DM are multifaceted but characterized by peripheral insulin resistance and compensatory overproduction of insulin by the β cells of the islet leading to increase circulating levels of insulin and enhanced bioavailability of IGF-1. Further, epidemiological studies are linking administration of metformin, the most widely used drug in the treatment of T2DM, with reduced incidence, recurrence and mortality of a variety of cancers in T2DM patients [20,47 56], including PDAC. Indeed, T2DM patients who had taken metformin had a 62% lower adjusted incidence of PDAC compared with those who had not taken metformin [54], a result recently substantiated in a different patient population [56]. Here we demonstrate that metformin abolishes mTORC1/S6K activation in PDAC cells but in contrast to rapamycin, metformin treatment did not overactivate Akt phosphorylation on Ser^{473}. We verified that, under our conditions, metformin stimulated AMPK in PDAC cells. In this context it is relevant that AMPK not only blocks mTORC1 activation but also mediates IRS-1 phosphorylation at Ser^{794}, an inhibitory site that attenuates PI3K/Akt activation [29,73]. We confirmed that metformin (but not rapamycin) induced IRS-1 phosphorylation at Ser^{794} in PANC-1 cells (our unpublished results). Consequently, it is plausible that metformin, via AMPK-mediated phosphorylation of IRS-1 at Ser^{794}, attenuates PI3K overstimulation caused by interrupting feedback loops mediated by the mTOR/S6K axis

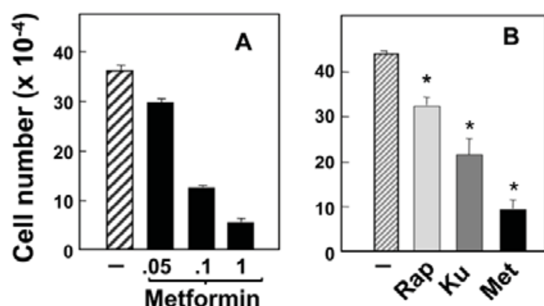

Figure 7. Metformin inhibits PANC-1 cell proliferation more potently than rapamycin or KU63794. ***A,*** Single-cell suspensions of PANC-1 cells were plated at a density of 10^5 cells per dish. After 24 h, the cultures were shifted to media containing 0.25% FBS without (−) or with 10 nM neurotensin and 10 ng/ml insulin (NT+Ins) in the absence (open bars) or presence (closed bars) of increasing concentrations of metformin, as indicated. After 4 days, cell numbers were determined from 6 plates per condition. Results are presented as mean ± SEM. Similar results were obtained in two independent experiments. p values compared to control were all <0.05 n = 6. ***B,*** Single-cell suspensions of PANC-1 cells were plated at a density of 10^5 cells per dish. After 24 h, the cultures were shifted to media containing 2.5% FBS without (−) or with 1 mM metformin, 5 μM KU63794 (Ku) or 100 nM rapamycin (Rap) as indicated. After 4 days, cell numbers were determined from 6–8 plates per condition. Results are presented as mean ± SEM. Similar results were obtained in two independent experiments. * All p values compared to control were <0.05 n = 6. Anova analysis showed that metformin inhibition of cell proliferation was statistically significant ($<p<0.05$) from either rapamycin or KU63794. In turn, KU63794 was statistically different from rapamycin ($p<0.05$).
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and thereby avoids hyper-activation of Akt phosphorylation on Ser^{473}. More importantly, we found that metformin inhibited rather than enhanced ERK activation (like active-site mTOR inhibitors) in PDAC cells. The inhibitory effects of metformin on ERK were elicited at low concentrations when the cells were cultured in medium containing physiological concentrations of glucose [68]. Thus, the effects of metformin on Akt phosphorylation and ERK activation are strikingly different from allosteric or active-site mTOR inhibitors, though all these agents potently inhibited the mTORC1/S6K axis. In line with differential effects on feedback loops, further findings revealed that metformin inhibited cell proliferation of PDAC cells more efficiently than allosteric or active-site mTOR inhibitors in PDAC cells. Although the elucidation of the precise mechanism(s) involved requires further experimental work, it is tempting to speculate that the favorable effects of metformin on preventing over-activation of pro-oncogenic pathways, such as Akt and ERK, may contribute to its antiproliferative effects *in vitro* and ultimately explain its beneficial anticancer effects.

Supporting Information

Figure S1 Treatment with KU63794 causes over-activation of MEK and ERK phosphorylation in PANC-1 and MiaPaCa-2 cells. The cultures of PANC-1 and MiaPaCa-2 were incubated in the absence (−) or in the presence of KU63794 (Ku) at 5 mM for 2 h in DMEM containing 5 mM glucose, as indicated. Then, the cells were stimulated with 10 ng/ml insulin and 5 nM neurotensin (NT+Ins) for 2 h and lysed with 2×SDS PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of MEK at $Ser^{217/221}$, ERK at Thr^{202} and Tyr^{204} and Akt at Ser^{473} Immunoblotting with total MEK, ERK and Akt was used to verify equal gel loading.
(TIF)

Author Contributions

Conceived and designed the experiments: ER HPS YN JSS KK. Performed the experiments: HPS YN JSS KK. Analyzed the data: ER HPS YN JSS KK. Wrote the paper: ER HPS JSS.

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Chapter III.

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

Cancer Biology and Signal Transduction

Molecular Cancer Therapeutics

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

Heloisa P. Soares[1], Ming Ming[2], Michelle Mellon[2], Steven H. Young[2,3], Liang Han[2], James Sinnet-Smith[2,3], and Enrique Rozengurt[2,3,4]

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 $Ser^{240/244}$), mTORC1/4E-BP1 (assayed by 4E-BP1 phosphorylation at $Thr^{37/46}$), and mTORC2-mediated AKT phosphorylation at Ser^{473}, 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 Ser^{473}, 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. *Mol Cancer Ther; 14(4); 1014–23. ©2015 AACR.*

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 Ser^{473}. 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 IGFIR (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/IGFI/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

[1]Division of Hematology-Oncology, David Geffen School of Medicine, Los Angeles, California. [2]Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine, Los Angeles, California. [3]CURE: Digestive Diseases Research Center, University of California at Los Angeles, Los Angeles, California. [4]Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California.

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Current address for Liang Han: Department of Hepatobiliary Surgery, First Affiliated Hospital of Medical College, Xi' an Jiaotong University, Xi'an, China.

Corresponding Author: Enrique Rozengurt, Department of Medicine, David Geffen School of Medicine at UCLA, 900 Veteran Avenue, Warren Hall Room 11-124, Los Angeles, CA 90095-1786. Phone: 310-794-6610; Fax: 310-267-1953; E-mail: erozengurt@mednet.ucla.edu

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receptor (GPCR) signaling systems potently stimulates mTORC1, DNA synthesis, and cell proliferation in a panel of PDAC cells (16, 17). Consequently, mTORC1, downstream of PI3K/AKT, has emerged as an attractive therapeutic target in PDAC and other common malignancies.

In addition to growth-promoting signaling, the mTORC1/S6K axis also mediates negative feedback loops that restrict signaling via insulin/IGF receptor and other tyrosine kinase receptors (18). Indeed, suppression of mTORC1/S6K feedback loops unleashes overactivation of PI3K/AKT (7) that potentially counterbalances the antiproliferative effects of mTOR inhibitors in many cancer cell types (19–22), including PDAC cells (23). In an effort to prevent PI3K/AKT overactivation in response to allosteric mTORC1 inhibition, dual PI3K and mTOR kinase inhibitors (PI3K/TOR-KIs), including BEZ235 (24, 25), PKI-587 (26, 27), and GDC-0980 (28) have been developed. Although these inhibitors are well suited to prevent activation of PI3K/AKT caused by suppression of mTORC1/S6K, much less is known about negative feedback loops impinging on other pro-oncogenic pathways (e.g., MEK/ERK) and/or concerning mTORC2 instead mTORC1. Here, we show that clinically relevant PI3K/TOR-KIs, including BEZ235, PKI-587, and GDC-0980, induce MEK/ERK pathway overactivation in human PDAC cells. On the basis of our results, we propose that mTORC2 mediates a feedback loop that curtails the activity of the MEK/ERK pathway in PDAC cells.

Materials and Methods

Cell culture

The human pancreatic cancer cell lines PANC-1, MiaPaCa-2, AsPC-1 and BxPC-3 were obtained from the ATCC. PANC-1 and MiaPaCa-2 were chosen because they harbor mutations typical of human pancreatic cancer (29), including activating mutations in *KRAS*, *TP53* (encoding the p53 protein), and *CDKN2A* (also known as p16 or p16^{INK4a}). These cell lines, authenticated by ATCC by short-tandem repeat analysis, were used within 15 passages and cultured for less than 6 months after recovery from frozen stocks (no authentication was done by the authors). Cells were obtained from ATCC at the following dates: MiaPaca-2 (June 2012, August 2013 and October 2014); PANC-1 (January 2012 and October 2014); BxPC-3 (June 2013); and AcPC-1 (December 2009). Cells were grown in DMEM with 2 mmol/L glutamine, 1 mmol/L Na-pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin and 10% FBS at 37°C in a humidified atmosphere containing 10% CO_2.

Western blot analysis

Confluent cultures of PANC-1 or MiaPaCa-2 cells, grown on 35-mm tissue culture dishes, were washed and then incubated for 24 hours in DMEM containing 5 mmol/L glucose and 1% FBS. The cells were washed twice with DMEM containing 5 mmol/L glucose and incubated in serum-free medium for 4 hours and then treated as described in individual experiments. The cultures were then directly lysed in 2× SDS-PAGE sample buffer [200 mmol/L Tris-HCl (pH 6.8), 2 mmol/L EDTA, 0.1 mol/L Na_3VO_4, 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol], followed by SDS-PAGE on 10% gels and transfer to Immobilon-P membranes (Millipore). Western blot analyses were then performed on membranes incubated overnight with the specified antibodies in PBS containing 0.1% Tween-20. The immunoreactive bands were detected with ECL (enhanced chemiluminescence) reagents (GE Healthcare Bio-Sciences Corp). In most experiments, the antibodies used detected the phosphorylated state of S6 at Ser $^{240/244}$, S6K at Thr^{389}, 4E-BP1 at Thr$^{37/46}$, AKT at Ser^{473} and at Thr^{308}, MEK at Ser$^{217/221}$ and ERK at Thr^{202} and Tyr^{204} or the total levels of these proteins.

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 (Achroplan 40/.75W Carl Zeiss, Inc.). Images were captured as uncompressed 24-bit TIFF 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 10^5 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 nmol/L of BEZ235, 5 nmol/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 Thr^{389}, a site directly phosphorylated by mTORC1 and S6 at $Ser^{240/244}$, 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 $Thr^{37/46}$ (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 Ser^{473}, a site directly phosphorylated by mTORC2 and at Thr^{308}, a site phosphorylated by PDK1 in response to PI3K activation. AKT phosphorylation on both Ser^{473} and Thr^{308} 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

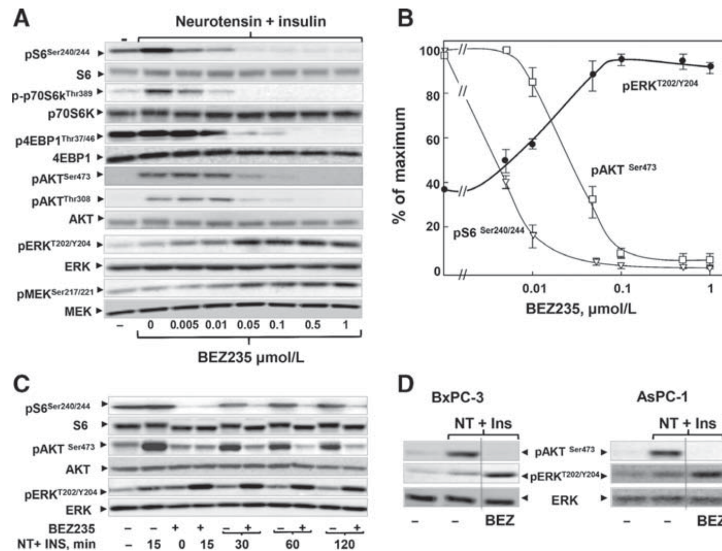

Figure 1.

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 Thr^{202} and Tyr^{204}, S6 at Ser $^{240/244}$ and AKT at Ser^{473} 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/TOR-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 nmol/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 overactivated ERK phosphorylation on Thr^{202} and Tyr^{204} 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 by 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 $Ser^{240/244}$ and AKT on Ser^{473}, 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 PD0325901. 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/TOR-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 $Ser^{240/244}$ and AKT on Ser^{473} 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

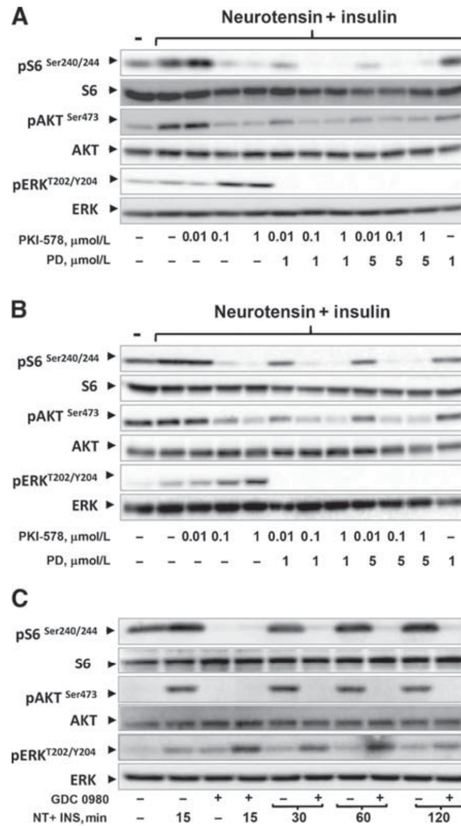

Figure 3.
MEK inhibition blunts the enhancement of ERK activity induced by PKI-587 or GDC-0980. A and B, cultures of MiaPaCa-2 cells (A) and PANC-1 (B) cells were incubated for 2 hours in the absence or presence of increasing doses of PKI-587 with or without PD0325901 (PD) at 1 or 5 μmol/L. Then, the cells were stimulated for 90 minutes with 5 nmol/L neurotensin and 10 ng/mL insulin and lysed with SDS-PAGE sample buffer. All samples were analyzed by SDS-PAGE and immunoblotting using the antibodies that detect phosphorylated or total proteins, as described in each panel. C, cultures of MiaPaCa-2 cells were incubated in the absence or presence of 1 μmol/L of GDC-0980 for 2 hours, and then the cells were stimulated for 15, 30, 60, or 120 minutes with 5 nmol/L neurotensin (NT) and 10 ng/mL insulin (ins) and lysed with SDS-PAGE sample buffer. The extracts were then analyzed as in A and B.

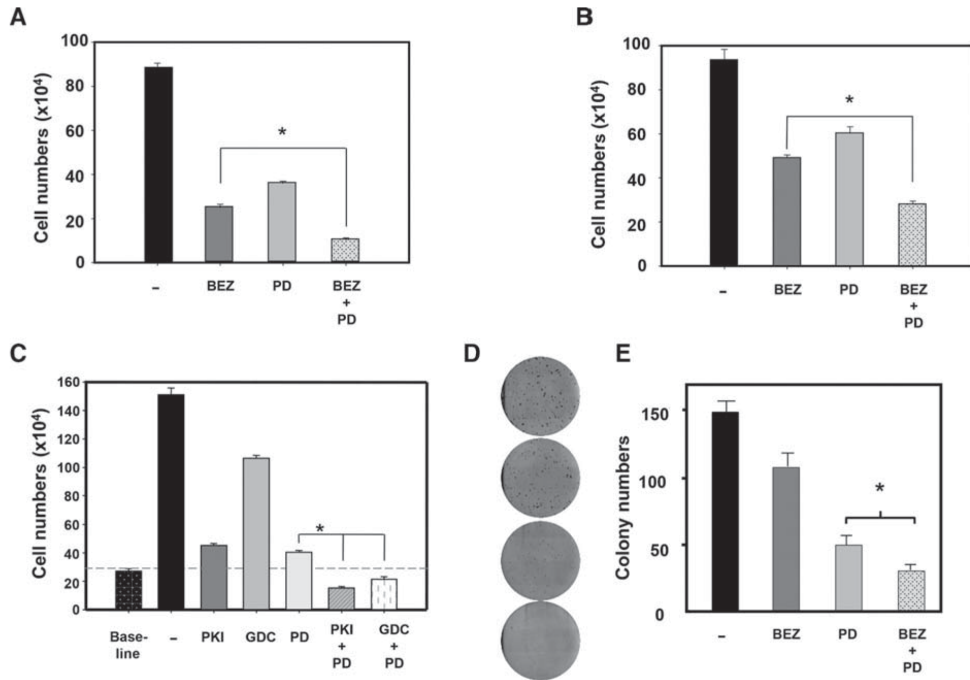

Figure 4.
Dual PI3K/mTOR kinase inhibitors and PD0325901 inhibit the proliferation of PDAC cells. A, single-cell suspensions of MiaPaCa-2 cells were plated at a density of 10^5 cells per dish. After 24 hours, the cultures were shifted to media containing FBS with 100n BEZ235 (BEZ), 100 nmol/L PD0325901 (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 10^5 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 10^5 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.

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 PI3K-generated accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) 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 PIP_3 (36, 37). In unstimulated cells, the PIP_3 sensor did not display any detectable accumulation at the plasma membrane (Fig. 5A and Supplementary Fig. S5). 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 PIP_3 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.

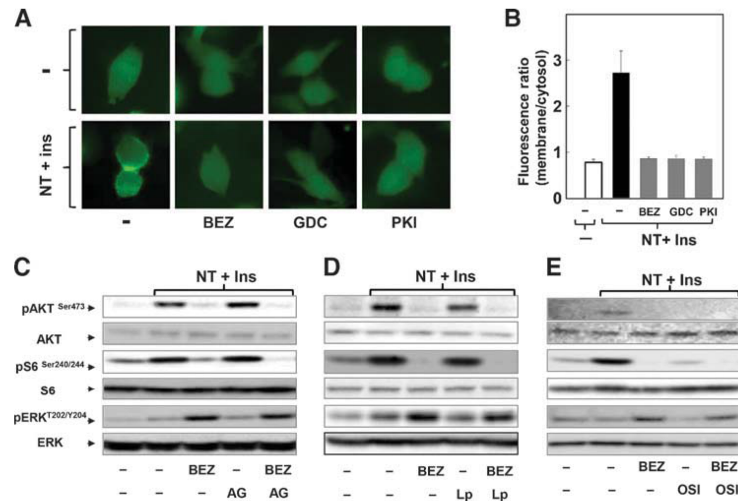

Figure 5.

BEZ235 enhances ERK pathway activation through a pathway that does not require PI3K, EGFR, HER2, insulin receptor, or IGFIR. A, MiaPaCa-2 cells were transiently transfected with a plasmid encoding a fusion protein between GFP and the PH domain of AKT (AKT-PH-GFP). After 24 hours, the cultures were incubated in DMEM without or with BEZ235 (BEZ), PKI-587 (PKI), or GDC-0980 (GDC) each at 1 μmol/L for 1 hour before stimulation with 5 nmol/L neurotensin and 10 ng/mL insulin. The intracellular distribution of AKT-PH-GFP was monitored under a fluorescence microscope. The selected cells displayed in the figures are representative of 90% of the population of GFP-positive cells. B, graphic represents quantification from A (ratio of membrane/cytoplasm fluorescence). C–E, cultures of MiaPaCa-2 cells were incubated for 2 hours in the absence or presence of 1 μmol/L BEZ235 (BEZ) with or without 1 μmol/L of AG-1478 (AG; C), 1 μmol/L of lapatinib (Lp; D), or 1 μmol/L of OSI-906 (OSI; E). Then, the cells were stimulated with 5 nmol/L neurotensin and 10 ng/mL insulin and lysed with SDS-PAGE sample buffer. All samples were analyzed by SDS-PAGE and immunoblotting with the antibodies that detect phosphorylated or total S6, AKT, and ERK proteins, as indicated in each panel.

BEZ235 enhances ERK activation independently of EGFR, HER2, insulin receptor, and IGFI receptor

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/mTOR 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 Ser^{473}, 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 but did not alter the expression of S6, AKT, or ERK (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 Ser^{473}, a function mediated by mTORC2. Surprisingly, 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 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 Ser^{259} (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

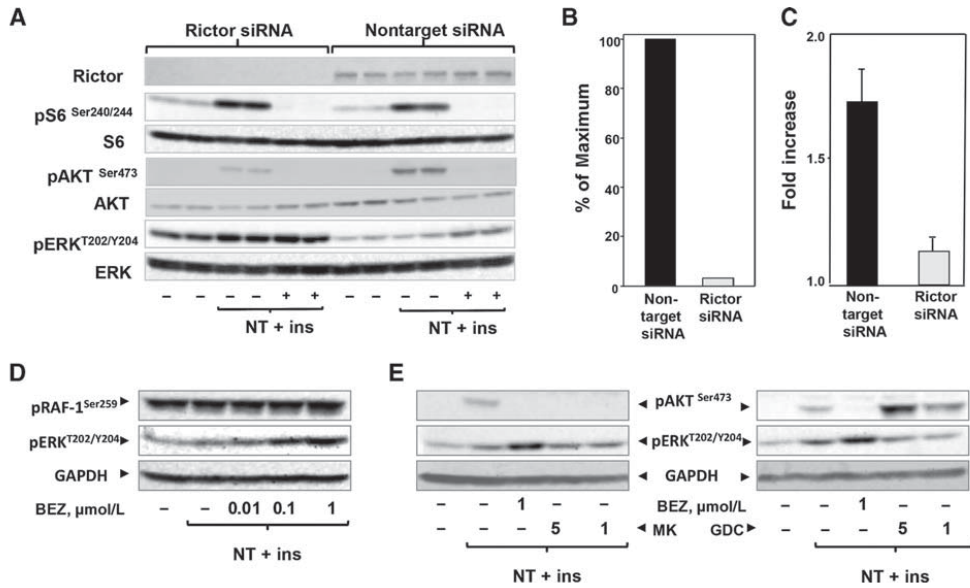

Figure 6.
BEZ235 enhances ERK activation through a RICTOR (mTORC2)-dependent but AKT-independent pathway. A, MiaPaCa-2 cells were transfected with siRNA targeting RICTOR or nontargeted siRNA. After 3 days, cells were incubated without (−) or with 1 µmol/L BEZ235 (+) for 2 hours. Then, the cells were stimulated for 60 minutes with 5 nmol/L neurotensin and 10 ng/mL insulin and lysed with SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. B, representation of quantification of levels of RICTOR protein after transfection with nontargeted siRNA or siRNA targeting RICTOR. C, fold increase of phosphorylated ERK. Quantification of phosphorylated ERK at Thr^{202} and Tyr^{204} from three independent experiments was performed using Multi Gauge V3.0. D, MiaPaCa-2 cells were incubated for 2 hours in the absence or presence of increasing doses of BEZ235 (BEZ) and then stimulated for 90 minutes with 5 nmol/L neurotensin (NT) and 10 ng/mL insulin (ins) and lysed with SDS-PAGE sample buffer. All samples were analyzed by SDS-PAGE and immunoblotting using the antibodies that detect phosphorylated RAF-1 (at Ser^{259}) and ERK or total GAPDH as a loading control. E, MiaPaCa-2 cells were incubated for 2 hours in the absence or presence of 1 µmol/L BEZ235 (BEZ) and either MK2206 (MK) at 1 or 5 µmol/L or GDC-0068 (GDC) at 1 or 5 µmol/L, as indicated. The cultures were then stimulated for 60 minutes with 5 nmol/L neurotensin (NT) and 10 ng/mL insulin and lysed with SDS-PAGE sample buffer. All samples were analyzed by SDS-PAGE and immunoblotting using the antibodies that detect phosphorylated AKT and ERK or total GAPDH as a loading control. Note that MK2206 (allosteric inhibitor) inhibited AKT phosphorylation, whereas GDC0068 (active-site inhibitor) increased AKT phosphorylation, presumably by stabilizing a conformation that prevents AKT dephosphorylation.

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 PIP_3 (36, 37). 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 PIP_3 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/IGFI receptor and HER3, thereby leading to enhancement of ERK (38, 39, 42–45). This gene expression loop should be distinguished from the effects induced by dual PI3K/mTOR inhibitors in this study since the enhancement of MEK/ERK activation occurred rapidly (within minutes) and was not prevented by inhibitors of insulin/IGFI receptor (OSI-906) or EGFR and HER2 (lapatinib). Our results with PDAC cells with *KRAS* mutations also contrast with a recent study demonstrating that acute inhibition of PI3K transiently inhibits ERK in breast cancer cells harboring HER-2 amplification or lacking PTEN but expressing wild-type *RAS* (46). We conclude that the impact of suppressing feedback loops mediated by the PI3K/AKT/mTOR pathway depends on cell context and leads to different MEK/ERK outcomes in cancer cells harboring mutations in different pro-oncogenic pathways.

Although many studies demonstrated negative feedback regulation by mTORC1 (18), a similar role for mTORC2 is only emerging. For example, a recent study revealed that mTORC2 can also regulate the cellular level of IRS-1 and concluded that mTORC1 and mTORC2 cooperate in promoting IRS-1 degradation (47). We noted that the doses of BEZ235 that enhanced MEK/ERK activation coincided with those that inhibited mTORC2-mediated AKT phosphorylation on Ser^{473} raising the possibility that BEZ235 suppresses a negative feedback loop operated through mTORC2. To test this possibility, we used RNAi to silence RICTOR, an essential and specific component of mTORC2. Knockdown of RICTOR increased baseline levels of ERK phosphorylation and treatment with BEZ235 failed to produce a significant further enhancement of ERK activation. On the basis of our results, 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. 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 PD0325901 suppressed enhanced ERK activation induced by PI3K/TOR-KIs and enhanced the growth-inhibitory 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
Development of methodology: H.P. Soares, L. Han
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.P. Soares, M. Ming, S.H. Young
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.P. Soares, S.H. Young, L. Han, E. Rozengurt
Writing, review, and/or revision of the manuscript: H.P. Soares, S.H. Young, J. Sinnet-Smith, E. Rozengurt
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Mellon, J. Sinnet-Smith
Study supervision: H.P. Soares

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Chapter IV.

Dose-Dependent AMPK-Dependent and Independent Mechanisms of Berberine and Metformin Inhibition of mTORC1, ERK, DNA Synthesis and Proliferation in Pancreatic Cancer Cells

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RESEARCH ARTICLE

Dose-Dependent AMPK-Dependent and Independent Mechanisms of Berberine and Metformin Inhibition of mTORC1, ERK, DNA Synthesis and Proliferation in Pancreatic Cancer Cells

Ming Ming[1], James Sinnett-Smith[1,4], Jia Wang[1], Heloisa P. Soares[2], Steven H. Young[1,4], Guido Eibl[3,4], Enrique Rozengurt[1,4,5]*

1. Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California, United States of America, 2. Division of Hematology-Oncology, Department of Medicine David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California, United States of America, 3. Department of Surgery, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California, United States of America, 4. CURE: Digestive Diseases Research Center, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California, United States of America, 5. Molecular Biology Institute, University of California Los Angeles, Los Angeles, California, United States of America

*erozengurt@mednet.ucla.edu

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Abstract

Natural products represent a rich reservoir of potential small chemical molecules exhibiting anti-proliferative and chemopreventive properties. Here, we show that treatment of pancreatic ductal adenocarcinoma (PDAC) cells (PANC-1, MiaPaCa-2) with the isoquinoline alkaloid berberine (0.3–6 μM) inhibited DNA synthesis and proliferation of these cells and delay the progression of their cell cycle in G1. Berberine treatment also reduced (by 70%) the growth of MiaPaCa-2 cell growth when implanted into the flanks of nu/nu mice. Mechanistic studies revealed that berberine decreased mitochondrial membrane potential and intracellular ATP levels and induced potent AMPK activation, as shown by phosphorylation of AMPK α subunit at Thr-172 and acetyl-CoA carboxylase (ACC) at Ser^{79}. Furthermore, berberine dose-dependently inhibited mTORC1 (phosphorylation of S6K at Thr^{389} and S6 at $Ser^{240/244}$) and ERK activation in PDAC cells stimulated by insulin and neurotensin or fetal bovine serum. Knockdown of α_1 and α_2 catalytic subunit expression of AMPK reversed the inhibitory effect produced by treatment with low concentrations of berberine on mTORC1, ERK and DNA synthesis in PDAC cells. However, at higher concentrations, berberine inhibited mitogenic signaling (mTORC1 and ERK) and DNA synthesis through an AMPK-independent

mechanism. Similar results were obtained with metformin used at doses that induced either modest or pronounced reductions in intracellular ATP levels, which were virtually identical to the decreases in ATP levels obtained in response to berberine. We propose that berberine and metformin inhibit mitogenic signaling in PDAC cells through dose-dependent AMPK-dependent and independent pathways.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease, with overall 5-year survival rate of only 6% [1]. The incidence of this disease in the US is estimated to increase to more than 44,000 new cases in 2014 and is now the fourth leading cause of cancer mortality in both men and women [2]. Total deaths due to PDAC are projected to increase dramatically to become the second leading cause of cancer-related deaths before 2030 [1] As the current therapies offer very limited survival benefits, novel strategies to treat and prevent this aggressive disease are urgently required [3].

G protein-coupled receptors (GPCRs) and their cognate agonists are increasingly implicated as autocrine/paracrine growth factors for multiple solid tumors, including small cell lung cancer, colon, prostate, breast and pancreas [4–8]. We showed that pancreatic cancer cell lines express multiple GPCRs [9] and a variety of GPCR agonists, including neurotensin, angiotensin II and bradykinin, stimulated DNA synthesis in pancreatic cancer cell lines, including PANC-1 and MiaPaca-2 [9–12]. Furthermore, a broad-spectrum GPCR antagonist [13, 14], inhibited the growth of pancreatic cancer cells either *in vitro* or xenografted into nu/nu mice [15]. Other studies demonstrated increased expression of GPCRs in pancreatic cancer tissues [16–19]. Subsequently, we identified positive crosstalk between insulin/IGFI receptors and GPCR signaling systems in pancreatic cancer cells, leading to mTORC1 signaling and ERK activation, and synergistic stimulation of DNA synthesis and cell proliferation [20–22]. These findings assume an added importance in view of the large number of epidemiological studies linking long standing type-2 diabetes mellitus (T2DM), obesity and metabolic syndrome, characterized by peripheral insulin resistance and compensatory overproduction of insulin, with increased risk for developing pancreatic cancer [23–32].

The biguanide metformin (1,1-dimethylbiguanide hydrochloride) derived from galegine, a phytochemical from *Galega officinalis*, is the most widely prescribed drug for treatment of T2DM, *worldwide* [33, 34]. Systemically, metformin lowers blood glucose levels through reduced hepatic gluconeogenesis, increases glucose uptake in skeletal muscles and adipose tissue [34, 35] and reduces the circulating levels of insulin and IGF-1 [36, 37]. At the cellular level, metformin indirectly stimulates AMP–activated protein kinase (AMPK) activation [38] via inhibition

of mitochondrial function, though other mechanisms of metformin action have been also suggested at high doses [39]. Major downstream targets of AMPK include TSC2 and Raptor [40–43]. The AMPK-mediated phosphorylation of these targets inhibits mTOR complex 1 (mTORC1) activity in a variety of cell types, including PDAC cells [44, 45] and disrupts positive crosstalk between insulin/IGFI receptors and GPCR signaling systems [21, 46]. Interestingly, a number of observational studies suggest that metformin reduces incidence and improved prognosis of a variety of cancers in patients with T2DM [47, 48], though this this conclusion is under scrutiny [49]. In the setting of PDAC, diabetic patients who had received metformin appear to have lower adjusted incidence and better survival compared with those who had not taken metformin or used other anti-diabetic agents [47, 50–52]. We hypothesized that structurally unrelated natural or synthetic compounds that interfere with mitochondrial-mediated ATP synthesis and target mTORC1 and ERK pathways, could provide novel anti-PDAC agents.

Natural products represent a rich reservoir of potential small chemical molecules exhibiting diverse pharmacological properties. The isoquinoline alkaloid berberine [53–55], a phytochemical extracted from a variety of medicinal plants, including plants of the *Berberis* species induces multiple biological effects, including anti-obesity, anti-diabetic, anti-cancer and calorie-restriction effects [55–62]. The cellular mechanism(s) involved, however, remains incompletely understood. Berberine has been reported to inhibit mitochondrial function and induce AMPK activation [63] but other mechanisms of action of this alkaloid have been proposed when added at high concentrations [64, 65]. Despite its potential clinical implications, there is no understanding of the precise mechanism(s) by which berberine inhibits the proliferation of cancer cells and it is not known whether this agent has any direct effect on signaling and proliferation of PDAC cells harboring *KRAS* mutations, characteristic of >90% of ductal pancreatic carcinomas.

In this study, we show that berberine inhibits DNA synthesis, cell cycle progression and proliferation in PANC-1 and MiaPaca-2 pancreatic cancer cells. Furthermore, berberine administration inhibits the growth of PDAC tumor xenografts *in vivo* as effectively as metformin. In mechanistic studies, we demonstrate that berberine, like metformin decreases mitochondrial membrane potential and ATP levels and concomitantly induces AMPK activation. Based on results using siRNA-mediated knockdown of AMPK, we propose that the inhibitory effects of berberine and metformin are mediated through AMPK-dependent and AMPK-independent pathways depending on the dose of each agent. This conclusion provides a plausible explanation for apparently contradictory reports on the role of AMPK in the mechanism of action of berberine and metformin in other model systems.

Materials and Methods

Chemicals and Reagents

Dulbecco's modified Eagle Medium (DMEM) was obtained from Invitrogen (Carlsbad, CA). Neurotensin, insulin, berberine and metformin were obtained from Sigma Chemical (St. Louis, MO). All antibodies were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). All other reagents were of the highest grade available.

Cells and Culture Conditions

The human pancreatic cancer cell lines PANC-1 and MiaPaCa-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cell lines were chosen because they harbor mutations typical of human pancreatic cancer [66], including activating mutations in KRAS, TP53 (encoding the p53 protein) and CDKN2A (also known as p16 or p16INK4a). Indeed, there is an excellent correlation between point mutation frequencies in PDAC cell lines and primary tumors [67]. Cells were grown in DMEM supplemented with 2 mM glutamine, 1 mM Na-pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 10% CO2. In the experiments, the glucose concentration in DMEM was adjusted to 5 mM, a physiological level in human serum.

[^{3}H]-Thymidine Incorporation into DNA

PANC-1 and MiaPaCa-2 cells (1×10^5) were plated and grown in 3.5 cm tissue culture plates for 5 days in DMEM supplemented with 10% FBS. The cells were washed twice and incubated for 24 h with DMEM containing 5 mM glucose and 1% FBS. To start the experiment, fresh medium containing the specified concentration of agonist and/or inhibitor was added after washing twice with DMEM (4 cultures used for each condition), and then the cells were incubated for 17 h and then pulse labeled for 6 h with [^{3}H]-thymidine (0.25 μCi/ml). The cells were fixed with 5% trichloroacetic acid and washed twice with ethanol. Acid-insoluble pools were dissolved in 0.1 N NaOH with 1% SDS and the radioactivity incorporated was counted in a liquid scintillation counter.

Mitochondrial Membrane Potential

The cell-permeable JC-1 dye (Invitrogen), which exhibits potential-dependent accumulation in the mitochondria, was used as an indicator of mitochondrial membrane potential. Following treatment without or with berberine or metformin, JC-1 was added to cultures of PANC-1 or MIA PaCa-2 cells at 1 μg/ml for 30 min. Then, the media was exchanged for Hanks Buffered Saline Solution containing 5 mM glucose and cells were immediately imaged using rhodamine and fluorescein optics. Images were stored from several visual fields.

The histogram analysis feature of Photoshop (Adobe) was used to measure the average red and average green fluorescence intensity from about 50 cells in a visual field. At least 5 independent fields were measured in each condition. The results are expressed as an average ratio of red/green florescent intensity in a single visual field (mean $\pm$ SEM). The ratio of red/green fluorescence intensity indicates mitochondrial membrane potential with a decreased ratio indicating a loss of potential.

ATP Determination

PANC-1 and MiaPaCa-2 cells (5×10^4) were plated and grown in 24 well culture plates for 5 days in DMEM and 10% FBS. The cells were then incubated for 24 h with DMEM containing 5 mM glucose and 1% FBS. The cells were washed twice with DMEM containing 5 mM glucose and incubated in serum-free medium for 17 h in the absence or presence of berberine or metformin. ATP levels were determined using the firefly luciferase/D-luciferin ATP determination Kit according to the manufacturer's protocol (Life Technologies Grand Island, NY).

Knockdown of AMPK levels via siRNA transfection

Silencer Select siRNAs were purchased from Life Technologies (Grand Island, NY) and designed to target either human $AMPK_{\alpha 1}$ or human $AMPK_{\alpha 2}$. Cells were transfected using the reverse transfection method. Either Silencer Select non-targeting negative control or a mixture of 10 nM $AMPK_{\alpha 1}$ and 10 nM $AMPK_{\alpha 2}$ siRNA (AMPKα1, α2 siRNA) were mixed with Lipofectamine RNAi MAX (Life Technologies Grand Island, NY) according to the manufacturer's protocol and added to 24 well plates. PANC-1 cells were then plated on top of the siRNA/ Lipofectamine RNAiMAX complex at a density of 10^5 cells/well.in DMEM containing 5 mM glucose and 10% FBS. Three days after transfection the cells were incubated for 24 h with DMEM containing 5 mM glucose and 1% FBS. The cells were washed twice with DMEM containing 5 mM glucose and incubated in serum-free medium for 17 h in the absence or presence of berberine or metformin and then treated as described in the corresponding figure legends.

Flow cytometric/cell cycle analysis

The proportion of cells in the G_0/G_1, S, G_2, and M phases of the cell cycle was determined by flow cytometric analysis. PANC-1 cells (2×10^4 cells) were seeded in DMEM containing 2.5% FBS. After 24 h the cultures were treated without or with berberine in medium containing 2.5% FBS for 3 days. Cells were then harvested by trypsinization, centrifuged at 1,000 *g* for 5 min and resuspended in a final concentration of 10^6 cells/ml in hypotonic propidium iodide (PI) solution containing 0.1% Sodium citrate, 0.3% Trixon-X 100, 0.01% PI and 0.002% Ribonuclease A. Cells were incubated in 4℃ for 30 min and analyzed on a FACScan (Becton-Dickinson, Franklin Lakes, NJ) using the software CELLQuest. One hundred thousand cells were collected for each sample. Excitation occurred

at 488 nm and data was collected using the FL2 channel and analyzed using FCS ExpressV3.

Western Blot Analysis

Confluent cultures of PANC-1 or Mia PaCa-2 cells grown on 3.5 cm dishes were washed and then incubated for 24 h with DMEM containing 5 mM glucose and 1% FBS. The cells were then washed twice with DMEM containing 5 mM glucose and incubated in serum-free medium in the absence or presence of berberine, metformin or A-769662, as described in the individual experiments. The cultures were then directly lysed in 2 × SDS-PAGE sample buffer [200 mM Tris-HCl (pH 6.8), 2 mM EDTA, 0.1 M Na_3VO_4, 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol], followed by SDS-PAGE on 10% gels and transfer to Immobilon-P membranes (Millipore, Billerica, MA). Western blots were then performed on membranes incubated overnight with the specified antibodies in phosphate-buffered saline (PBS) containing 0.1% Tween-20. The immunoreactive bands were detected with ECL (enhanced chemiluminescence) reagents (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). The antibodies used detected the phosphorylated state of S6K at Thr^{389}, S6 at $Ser^{240/244}$ and ERK1/2 at Thr^{202} and Tyr^{204}, AMPKα at Thr^{172}, ACC at Ser^{79} ^{and} Raptor at Ser^{792}. In addition, the total level of these proteins was also evaluated. Routinely, the membranes used with the phospho-specific antibodies were stripped and re-blotted with the antibodies that detect the total level of the corresponding protein. Occasionally, stripping and re-blotting of the same membrane was not satisfactory for obtaining both loading and expression controls. In these cases, we used a separate blot for assessing total protein expression and immunoblotted the original membrane for other proteins (e.g. actin, S6K, S6, ERK) that migrate at a different position in the original gel for verifying equal loading.

Cell Proliferation

Cultures of PANC-1 and MiaPaCa-2 cells, 3–5 days after passage, were washed and suspended in DMEM containing 5 mM glucose. Cells were then disaggregated by two passes through a 19-guage needle into an essentially single-cell suspension as judged by microscopy. Cell number was determined using a Coulter Counter, and 2×10^4 cells were seeded in 35 mm tissue culture plates in DMEM containing 5 mM glucose and 10% FBS. After 24 h of incubation, the medium was removed and the cultures shifted to DMEM containing 5 mM glucose without or with 3% FBS. The cultures were then incubated in a humidified atmosphere containing 10% CO_2 at 37°C for 4 days and the total cell count was determined from a minimum of four dishes per condition using a Coulter counter, after cell clumps were disaggregated by passing the cell suspension ten times through a 19- and subsequently a 21-gauge needle.

Mice xenografts

Early-passage MiaPaCa-2 cells were harvested, and 2×10^6 cells were implanted into the right flanks of male *nu/nu* mice. The male *nu/nu* mice were maintained in specific pathogen-free facility at University of California at Los Angeles (UCLA). The UCLA Chancellor's Animal Research Committee approved all the animal experiments. The animals were randomized into control and treated groups (10 mice per group) and were given punched ear tags to allow identification. Treatment was initiated when the tumors reached a mean diameter of 2 mm, and the 1st day of treatment in both cases was designated as day 0. For injection into animals, metformin (250 mg/Kg), berberine (5 mg/kg) or vehicle (control) was given intraperitoneally once daily intraperitoneally (50 µL/mouse). Tumor volume (V) was measured by external caliper every 4 days and it was calculated as V=0.52 (length $\times$ width^2). At the end of the experiment, the tumors were dissected weighted and measured. The volume of the excised tumors was calculated as V=0.52 (length $\times$ width $\times$ depth).

Statistical analysis

Values are means $\pm$ SE. Differences between groups were analyzed with the unpaired Student's *t*-test.

Results

Berberine inhibits DNA synthesis, cell cycle progression and cell proliferation in PDAC cells

Initially, we determined the effect of the isoquinoline alkaloid berberine on DNA synthesis in PANC-1 and MiaPaCa-2 cells, which have been used extensively as models of PDAC cells. Cultures of these cells grown in medium containing 10% fetal bovine serum were washed and transferred to serum-free medium for 24 h. Then, the cells were switched to medium containing a physiological concentration of glucose (5 mM), increasing doses of berberine and a combination of insulin (10 ng/ml) and the GPCR agonist neurotensin (5 nM) to elicit potent mitogenic crosstalk signaling [20, 21, 46]. Treatment with berberine inhibited the stimulation of DNA synthesis in a dose-dependent manner in both PANC-1 and MiaPaCa-2 cells. At a concentration of 3 µM, berberine inhibited DNA synthesis by 82% in MiaPaCa-2 cells and by 76% in PANC-1 cells. The incorporation of [^{3}H]-thymidine was blocked by 97% in MiaPaCa-2 cells and by 94% in PANC-1 cells in response to 6 µM berberine (Fig. 1 **A**).

The assays of [^{3}H]-thymidine incorporation were complemented by flow cytometric analysis to determine the proportion of PDAC cells in the various phases of the cell cycle. As shown in Fig. 1B, exposure of PANC-1 cells to berberine (1.5–3 µM) induced a marked increase in the proportion of cells in G_1 (from 61$\pm$0.2% in cells with FBS to 79$\pm$2% in cells with FBS and berberine) and a corresponding decrease in the proportion of cells that were in S and G_2/M phase

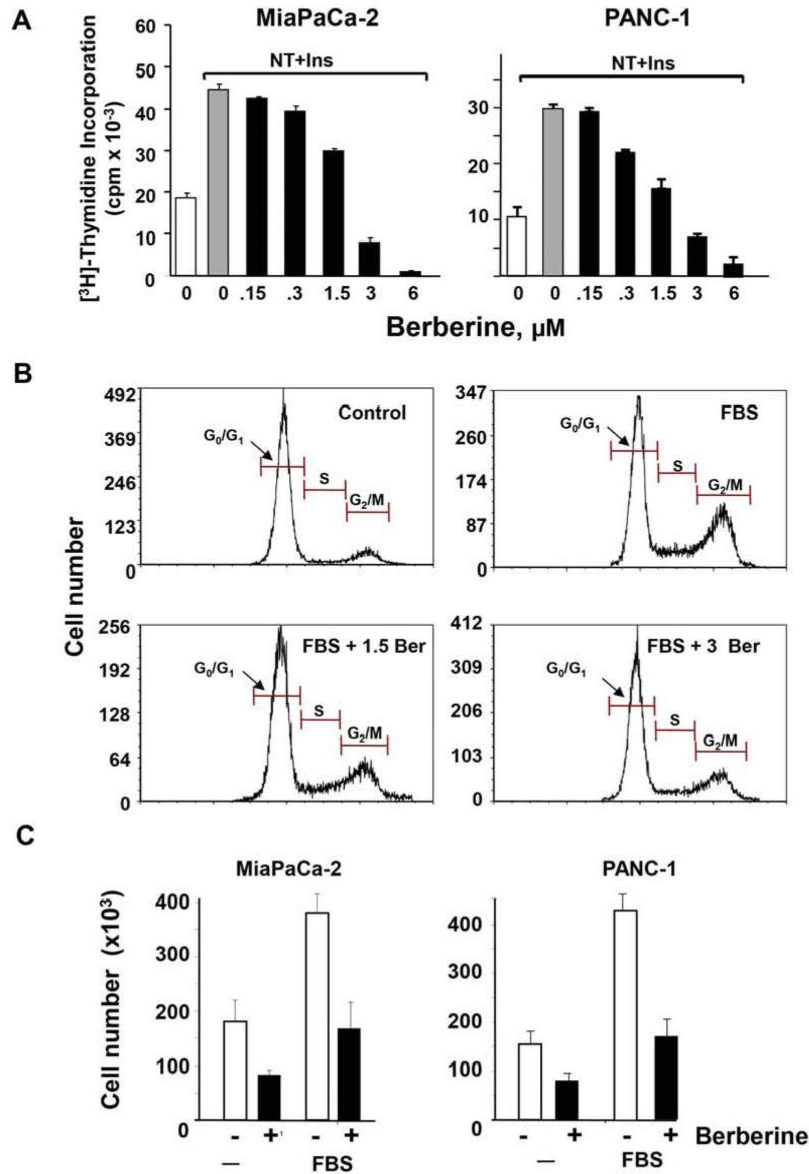

Fig. 1. Berberine inhibits DNA synthesis, cell cycle progression and proliferation in PANC-1 and Mia PaCa-2 cells. A, Mia PaCa-2 or PANC-1 cells were incubated without (*open bars*) or with 5 nM neurotensin and 10 ng/ml insulin (*closed bars*) in the presence of increasing concentration of berberine for 17 h at 37˚C prior to the addition of [^{3}H]-thymidine for 6 h. The radioactivity incorporated into acid-insoluble pools was measured in a scintillation counter, as described in "Materials and Methods. The values shown are the mean ± SEM obtained in 3 independent experiments; **B**, PANC-1 cells were treated without (control) or with berberine at 1.5 μM or 3 μM in medium containing 2.5% FBS for 3 days (indicated by cont., FBS, FBS +1.5 Ber and FBS +3 Ber). Cell cycle

was analyzed by PI-staining and flow cytometry. Similar results were obtained in 3 independent experiments. **C**, Single-cell suspension of Mia PaCa-2 or PANC-1 cells were plated on tissue culture dishes at a density of 2×10^4 cells per dish. After 24 h of incubation the medium was removed and the cultures shifted to medium without or with 3% FBS in the absence (open bars) or presence (closed bars) of 3 μM berberine. The cultures were incubated for 4 days as described in "Materials and Methods". Cell count was determined from 4 to 6 replicate plates per condition using a Coulter Counter. Results are presented as mean ± SEM of 3 biological replicates.

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of the cell cycle (from 36 ± 0.1% to 19 ± 0.7%). These results indicate that berberine delays the progression of the PDAC cell cycle at G_1.

We next examined the effect of berberine on the proliferation of pancreatic cancer cells. Single cell suspensions of either PANC-1 or MiaPaCa-2 cells were plated and incubated in media supplemented without or with 3% FBS in the absence or presence of 3 μM berberine. Treatment with berberine markedly inhibited proliferation in both PDAC cells (Fig. 1C) without affecting cell viability at the doses tested (results not shown). Taken together, the results in Fig. 1 demonstrate that berberine inhibits DNA synthesis, cell cycle progression and proliferation in pancreatic cancer cells.

Berberine and metformin inhibit the growth of a PDAC xenograft in nude mice

Given our results showing inhibitory effects of berberine on PDAC cell proliferation *in vitro*, we subsequently determined whether this compound inhibits pancreatic cancer growth *in vivo* using MiaPaca-2 tumor xenografts in nude mice. The xenografts were derived by implantation of 2×10^6 cells into the right flanks of male *nu/nu* mice. The animals were randomized into control and berberine-treated groups (10 mice per group). Berberine was given once daily intraperitoneally at 5 mg/kg for the duration of the experiment. As shown in Fig. 2, administration of berberine decreased the growth of MiaPaca-2 cells xenografted in nude mice by 70%. The tumor volumes at the end of the experiment (day 29) were 781 mm^3 in the control and 240 mm^3 in the berberine treated group ($p < 0.001$). The dose of berberine used (5 mg/kg) is 12-fold lower than the LD_{50}, based on preliminary range-finding studies and previous work published by others [53, 68, 69]. Berberine was well tolerated and did not significantly affect the weight of the mice during the treatment (Fig. 2, **B**). The inhibitory effect of berberine was comparable to that induced by metformin given at 250 mg/Kg (Fig. 2), a dose that induces maximal inhibitory effect on PDAC tumor growth [70]. Indeed, the curves corresponding to the growth of MiaPaca-2 xenografts in mice treated with berberine or metformin were virtually super-imposable during the first 24 days (Fig. 2, **A**). At day 29, metformin was slightly more effective than berberine as assessed by tumor volume ($p < 0.05$) but the effects did not reach statistical significance ($p > 0.07$) when scored by tumor weight (Fig. 2, **B**). These results indicate that berberine inhibits the growth of human pancreatic cancer cells xenografted into nude mice with efficacy comparable to that achieved by a maximal dose of metformin.

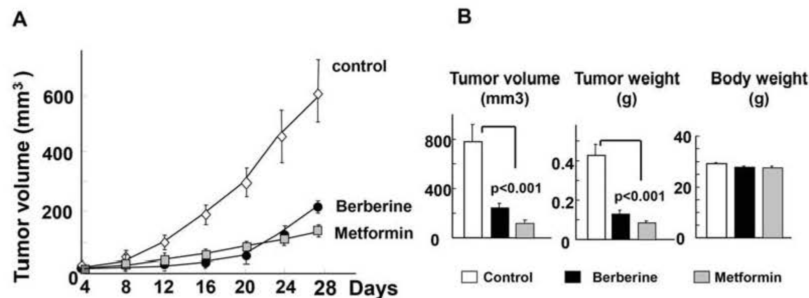

Fig. 2. Berberine inhibits the growth of MiaPaCa-2 tumor xenografts as effectively as metformin. Xenografts of MiaPaca-2 were generated by implantation of 2×10^6 cells into the right flanks of male *nu/nu* mice. When the tumors reached a mean diameter of 2 mm the animals were randomized into control and treated groups (10 mice per group). Berberine was given once daily intraperitoneally at 5 mg/kg for the duration of the experiment. For comparison, metformin was given intraperitoneally to another group of mice at 250 mg/kg. The 1st day of treatment was designated as day 0. Control animals received an equivalent volume of saline. **A**, Tumor volumes were measured every 4 days as described in "Materials and Methods". At the end of the experiment (day 29, the tumors were removed, weighted and measured and tumor volumes estimated as V 0.52 (length $\times$ width $\times$ depth). The results are shown in panel **B** (mean $\pm$ SEM). Treatment of mice with berberine significantly reduced the volume and weight of the tumors as compared with the tumors from the control group ($p<0.001$), as indicated. A similar inhibition of tumor growth was obtained by administration of metformin ($p<0.001$). The curves corresponding to the growth of MiaPaca-2 xenografts in mice treated with berberine or metformin were superimposable during the first 24 days. At day 29, metformin was slightly more effective than berberine as assessed by tumor volume ($p<0.05$) but the difference of the effects between these drugs did not reach statistical significance ($p>0.07$) when scored by tumor weight (**Fig. 2, B**). At the concentrations used, berberine and metformin were well tolerated with no apparent toxicity based on body weight changes.

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Berberine induces mitochondrial membrane depolarization, reduces the levels of ATP and stimulates AMPK in pancreatic cancer cells

Having established that berberine inhibits PDAC cell proliferation *in vitro* and *in vivo*, we next explored the mechanisms involved. In other cell types, berberine is thought to inhibit complex I of the mitochondrial respiratory chain [71, 72], resulting in reduced ATP synthesis and concomitant increase in cellular AMP and ADP which are potent allosteric activators of AMPK [73–75]. Because it is not known whether berberine has any effect on mitochondrial function in pancreatic cancer cells, we initially examined whether this phytochemical interferes with mitochondrial membrane potential in these cells. Cultures of MiaPaCa-2 and PANC-1 cells were incubated in the absence or presence of 3 μM berberine or 1 mM metformin, included for comparison. Then, mitochondrial membrane potential, a key component driving ATP synthesis, was assessed using the mitochondrial-specific fluorescent probe JC-1 [76]. As shown in Fig. 3 **A**, berberine caused a significant fall in mitochondrial membrane potential in MiaPaCa-2 and PANC-1 cells. The effect was comparable to that induced by metformin (Fig. 3 **A**). These results indicate that berberine, like metformin, targets mitochondrial function in PDAC cells. Accordingly, exposure to increasing concentrations of berberine or metformin produced a marked dose-dependent decrease in the intracellular ATP levels in both PANC-1 and MiaPaCa- 2 cells (Fig. 3 **B**).

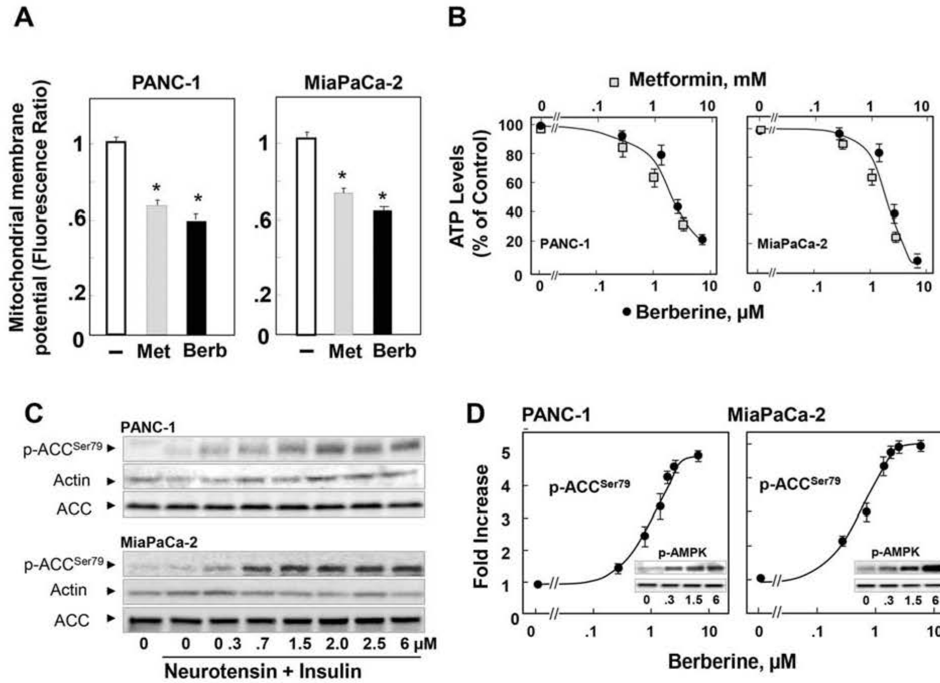

Fig. 3. Berberine and metformin induce mitochondrial membrane depolarization, reduce ATP levels and activate AMPK in PDAC cells. A, Cultures of PANC-1 and MiaPaca-2 cells were incubated in the absence or in the presence of 3 μM berberine (Berb) or 1 mM metformin (Met) for 17 h in DMEM containing 5 mM glucose. The change in mitochondrial membrane potential was measured using the mitochondrial membrane potential indicator JC-1. The results are expressed as an average ratio of red/green florescent intensity in a single visual field (mean ± SEM). At least 5 fields were studied in each condition. P values were determined using the t-test (SigmaPlot 12.); $*p<0.002$. **B,** Cultures of PANC-1 and MiaPaca-2 cells were incubated in the absence or in the presence of berberine or metformin at the indicated concentrations for 17 h in DMEM containing 5 mM glucose and 2.5% FBS. **C,** Cultures of PANC-1 (upper panels) and MiaPaCa-2 (lower panels) were incubated in the absence or in the presence of berberine at the indicated doses for 17 h. Then, the cells were stimulated for 1 h with 5 nM neurotensin and 10 ng/ml insulin and lysed with 2X SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of Acetyl-CoA Carboxylase (ACC) at Ser^{79}. Western blotting for actin was used to verify equal loading in the same membrane and a separate gel confirmed that expression of total ACC protein was not changed by any of the treatments. **D,** Quantification was performed using Multi Gauge V3.0. The values represent the mean ± SEM; n 3, fold increase in ACC phosphorylation at Ser^{79}. Inset, phosphorylated state of AMPK at Thr^{172} at the indicated concentrations of berberine (μM).

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We next determined whether berberine stimulates AMPK activity within intact MiaPaCa-2 and PANC-1 cells. Lysates of these cells were analyzed by immunoblotting using antibodies that detect the phosphorylated state of acetyl-CoA carboxylase (ACC) at Ser^{79}, a residue directly phosphorylated by AMPK and used as a biomarker of AMPK activity within intact cells [75]. Treatment with berberine induced a marked increase in the phosphorylation of ACC at Ser^{79} in a dose-dependent manner in both PANC-1 and MiaPaCa-2 cells (Fig. 3, **C**; quantification in Fig. 3 **D**). Maximal effect was elicited at doses >2 μM in both PDAC cells (Fig. 3 **D**). Furthermore, treatment of MiaPaCa-2 or PANC-1 cells with berberine induced a striking increase in the phosphorylation of AMPK at

Thr^{172}, the residue in the kinase domain of the catalytic subunit (α) of AMPK critical for activation (Inserts in Fig. 3 **D**). Collectively, the results demonstrate that berberine decreases mitochondrial membrane potential and lowers intracellular levels of ATP thereby stimulating AMPK activity in PDAC cells cultured in medium containing a physiological concentration of glucose (5 mM).

Berberine inhibits mTORC1 and ERK activation in PDAC cells

The activation of the PI3K/Akt/mTORC1 and MEK/ERK pathways plays a pivotal role in stimulating DNA synthesis, cell cycle progression and proliferation of PDAC cells and are negatively regulated by AMPK [21]. Consequently, we determined whether berberine inhibits mTORC1 and ERK activation in PDAC cells. Cultures of MiaPaCa-2 cells were treated with increasing doses of berberine and then stimulated with a combination of insulin and neurotensin to elicit positive crosstalk (Fig. 4). Lysates of these cells were analyzed by immunoblotting using antibodies that detect the phosphorylated state of S6K at Thr^{389}, a residue directly phosphorylated by mTORC1, using Western blot analysis with antibodies that specifically detect the phosphorylated state of this residue. To corroborate that phosphorylation of S6K at Thr^{389} reflects its activation within PDAC cells, we examined the phosphorylation of the 40S ribosomal protein subunit S6, a downstream target of S6K [77]. As shown in Fig. 4 **A**, stimulation with insulin and neurotensin induced a marked increase in mTORC1 activity, as scored by phosphorylation of S6K and S6 protein (pS6K and pS6). Treatment with berberine prevented mTORC1 activation in a dose-dependent manner (Fig. 4A; quantification in Fig. 4 **B**).

Given the pivotal importance of the RAS/MEK/ERK pathway in PDAC development and maintenance, we also analyzed the effect of increasing concentrations of berberine on ERK activation by detecting ERK phosphorylated on Thr^{202} and Tyr^{204}. The results in Fig. 4 **A, B** demonstrated that berberine prevented ERK activation in MiaPaCa-2 cells, in a dose-dependent manner. The doses of berberine that inhibited ERK activation were similar to those that blunted mTORC1 activation and produced AMPK activation.

Similar to the results obtained with MiaPaCa-2 cells, berberine inhibited mTORC1 and ERK activation by insulin and neurotensin in a dose-dependent manner in PANC-1 cells (Fig. 4 **C**; quantification in Fig. 4 **D**). We also found that berberine inhibited mTORC1 and ERK signaling in PANC-1 cells stimulated with FBS instead of insulin and neurotensin (S1 Figure). The results presented so far demonstrate that berberine inhibited mTORC1, ERK and cell proliferation in PDAC cells at doses that reduced the intracellular ATP levels and induced robust AMPK activation.

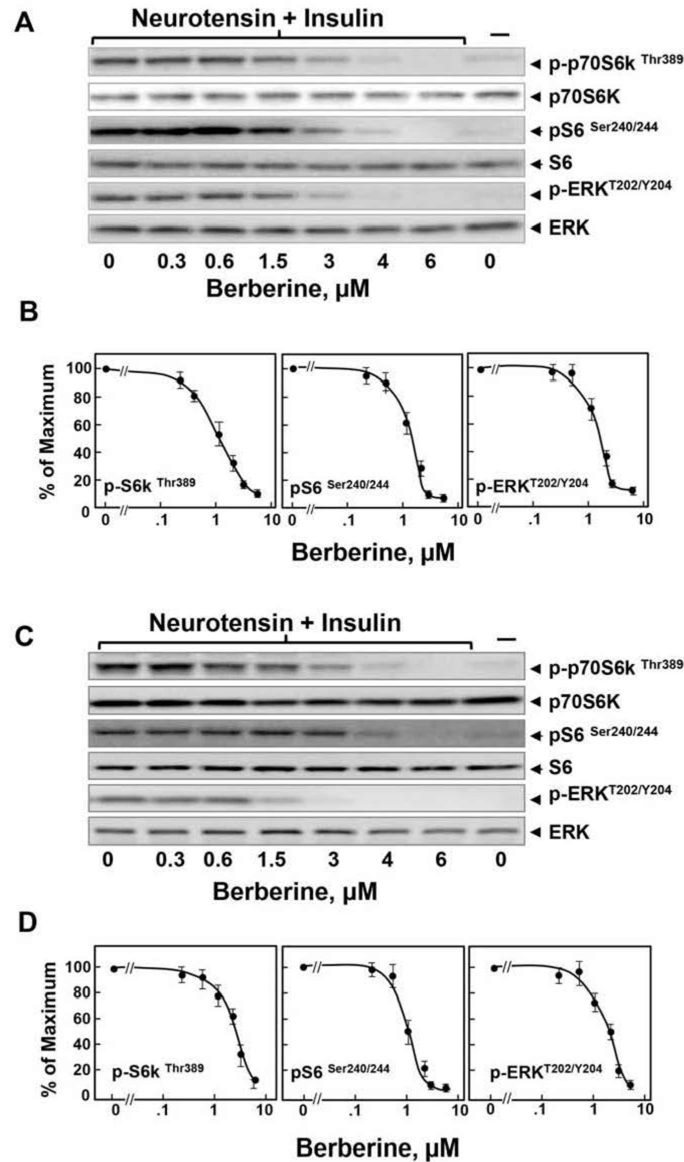

Fig. 4. Berberine inhibits mTORC1 signaling and ERK activation in PDAC cells. Cultures of MiaPaCa-2 (Panels **A** and **B**) or PANC-1 cells (panels **C** and **D**) were incubated in the absence or in the presence of increasing concentrations of berberine. Then, the cells were stimulated for 1 h with 5 nM neurotensin and 10 ng/ml insulin and lysed with 2X SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of S6K at Thr^{389}, S6 at $Ser^{240/244}$, and ERK at Thr^{202} and Tyr^{204}. Immunoblotting with total S6K, S6 and ERK was used to verify equal gel loading.

The quantification of the immune signals was performed using Multi Gauge V3.0. The results are presented in the plots shown in panels **B** and **D**. The values represent the mean $\pm$ SEM (n 3) of S6K, S6 and ERK phosphorylation expressed as a percentage of the maximal response obtained in 3 independent experiments.

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Knockdown of the α subunits of AMPK reverses inhibition of mTORC1, ERK and DNA synthesis induced by low doses of berberine or metformin: evidence for AMPK-dependent and AMPK-independent mechanisms

In order to determine the role of AMPK in mediating berberine-induced inhibition of PDAC cell signaling and proliferation, we used short interfering RNA (siRNA), which effectively (>90%) knockdown the protein expression of both α_1 and α_2 catalytic subunits of AMPK, as compared with cells transfected with non-targeting siRNA (Fig. 5, **A**). Accordingly, berberine-induced increases in the phosphorylation of ACC at Ser^{79} and Raptor at Ser^{792}, were blunted in the cells treated with siRNA targeting the α_1 and α_2 catalytic subunits of AMPK (Fig. 5, **A**; quantification in Fig. 5, **B**). The salient feature in Fig. 5, **A** is that knockdown of AMPK prevented the inhibitory effect produced by treatment with low doses of berberine (<3 μM) on the stimulation of mTORC1 (scored by phosphorylation of S6K at Thr^{389} and of its substrate S6) and ERK activation in PDAC cells. In contrast, knockdown of AMPK expression prevented only partially the inhibitory effect berberine added at 6 μM. It was conceivable that the inhibitory effect of berberine at the high concentration was due to incomplete elimination of AMPK after silencing. However, the dose-response relationships presented in Figs. 2 and 5 argue against this possibility. Specifically, ACC phosphorylation at Ser^{79} reached a plateau in cells challenged with berberine at doses 2–6 μM (Fig. 2) or 3–6 μM (Fig. 5B). The faint ACC phosphorylation remaining after knockdown of AMPK followed a similar dose-response relationship, i.e. phosphorylation was increased only slightly and to same level by either 3 μM or 6 μM berberine in AMPK-depleted cells (Fig. 5). Furthermore, the marked increase in Raptor phosphorylation at Ser^{792} in response to berberine at 6 μM was also blocked by knockdown of AMPK expression (Fig. 5A; quantification in Fig. 5, **B**). Thus, knockdown of AMPK prevented berberine-induced phosphorylation of the AMPK substrates ACC at Ser^{79} and Raptor at Ser^{792} when added at either at 3 μM or 6 μM. Consequently, berberine hampered mTORC1 and ERK activation through AMPK signaling at low doses but at higher concentrations, berberine inhibited mitogenic signaling, at least in part, through an AMPK-independent pathway in PDAC cells.

In accord with this conclusion, knockdown of α_1 and α_2 catalytic subunit expression of AMPK substantially prevented the inhibitory effect produced by low doses of berberine on the stimulation of DNA synthesis in PDAC cells (Fig. 5, **C**). In contrast, at higher concentrations, berberine inhibited DNA synthesis through an AMPK-independent mechanism. These results reinforce the notion that berberine inhibits mitogenic signaling in PDAC cells through distinct AMPK-dependent and independent mechanisms in a dose-dependent manner.

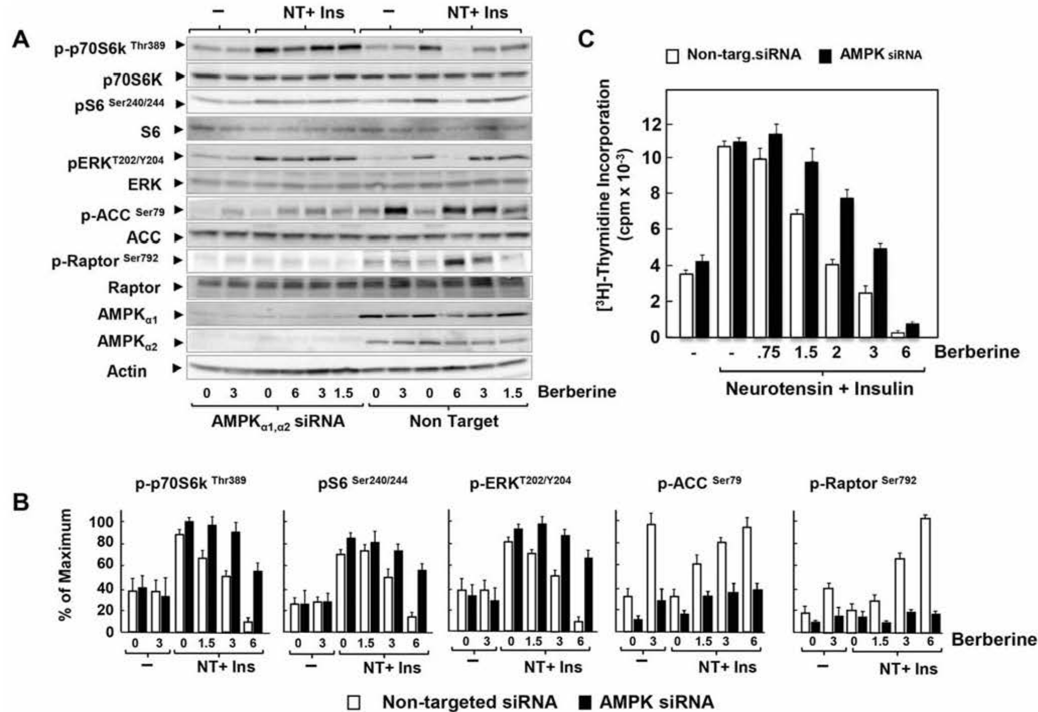

Fig. 5. Knockdown of the α subunits of AMPK reverses inhibition of mTORC1, ERK and DNA synthesis induced by low but not high doses of berberine. **A,** PANC-1 cells were transfected with either non-targeting negative control (Non Target.) or 10 nM AMPKα1 and 10 nM AMPKα2 siRNA (AMPKα1, α2 siRNA) in DMEM containing 5 mM glucose and 10% FBS. After 3 days the cells were incubated either in the absence or presence of berberine for 17 h in serum free DMEM containing 5 mM glucose. Then, the cells were stimulated for 1 h with 5 nM neurotensin and 10 ng/ml insulin and lysed with 2X SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with the following phospho antibodies: S6K at Thr^{389}, S6 at $Ser^{240/244}$, and ERK at Thr^{202} and Tyr^{204}, ACC at Ser^{79} and Raptor at Ser^{792}. Shown here is a representative autoluminogram; similar results were obtained in 4 independent experiments. **B,** Quantification was performed using Multi Gauge V3.0. Results are expressed as the percentage of the maximum (mean ±SEM: n 4). **C,** PANC-1 cells were transfected with either non-targeting negative control (open bars) or 10 nM AMPKα1 and 10 nM AMPKα2 siRNA (black bars) in DMEM containing 5 mM glucose and 10% FBS. After 3 days the cells were incubated for 6 h in serum-free medium containing 5 mM glucose. Then, 5 nM neurotensin and 10 ng/ml insulin and the indicated concentration of berberine were added for 17 h at 37°C prior to the addition of $[^3H]$-thymidine for 6 h. The radioactivity incorporated into acid-insoluble pools was measured in a scintillation counter, as described in "Materials and Methods". Results are expressed as the percentage of maximum mean ± SEM obtained in 4 independent experiments (3 replicate cultures per point in each experiment).

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Previously, we demonstrated that metformin, at low concentrations, inhibited DNA synthesis through an AMPK-dependent mechanism in PANC-1 cells incubated in medium containing physiological concentrations of glucose [44]. The results obtained here with berberine prompted us to examine further the notion that inhibitors of mitochondrial function impede mitogenic signaling through AMPK-dependent and independent mechanisms in a dose-dependent manner. Knockdown of α_1 and α_2 catalytic subunit expression of AMPK prevented the increase in the phosphorylation of ACC at Ser^{79} and Raptor at Ser^{792} in cells treated with metformin at either 1 mM or 3 mM (Fig. 6 **A**;

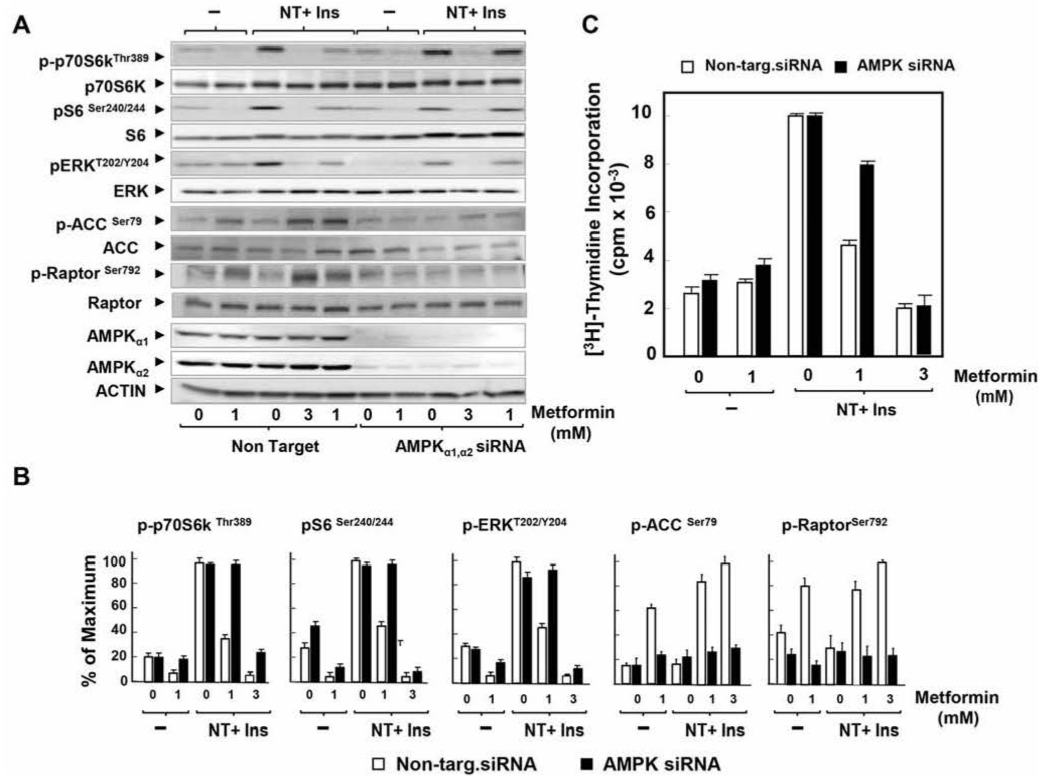

Fig. 6. Knockdown of the α subunits of AMPK reverses inhibition of mTORC1, ERK and DNA synthesis induced by low doses of metformin. A, PANC-1 cells were transfected with either non-targeting negative control (Non Target.) or 10 nM AMPKα1 and 10 nM AMPKα2 siRNA (AMPKα1, α2 siRNA) in DMEM containing 5 mM glucose and 10% FBS. After 3 days the cells were incubated either in the absence or presence of 1 mM or 3 mM metformin (as indicated) for 17 h in serum free DMEM containing 5 mM glucose. The samples were analyzed by SDS-PAGE and immunoblotting with the following phospho antibodies: S6K at Thr^{389}, S6 at Ser$^{240/244}$, ERK at Thr^{202} and Tyr^{204}, ACC at Ser^{79} and Raptor at Ser^{792} Shown here is a representative autoluminogram; similar results were obtained in 3 independent experiments. **B,** Quantification was performed using Multi Gauge V3.0. Results are expressed as the percentage of maximum (mean ±SEM; n 3). **C,** PANC-1 cells were transfected with either non-targeting negative control (open bars) or 10 nM AMPKα1 and 10 nM AMPKα2 siRNA (black bars) in DMEM containing 3 mM glucose and 10% FBS. After 3 days the cells were incubated for 6 h in serum-free medium containing 5 mM glucose. Then, 5 nM neurotensin and 10 ng/ml insulin and metformin at either 1 mM or 3 mM were added for 17 h at 37°C prior to the addition of [^{3}H]-thymidine for 6 h. The radioactivity incorporated into acid-insoluble pools was measured in a scintillation counter, as described in "Materials and Methods". Results are expressed as the percentage of maximum mean ±SEM obtained in 3 independent experiments (3 replicate cultures per point in each experiment).

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quantification in Fig. 6, **B**). Interestingly, siRNA-mediated depletion of AMPK reversed the inhibitory effect produced by 1mM metformin on mTORC1 (scored by phosphorylation of S6K at Thr^{389} and of its substrate S6) and ERK activation in PDAC cells (Fig. 6 **A**; quantification in Fig. 6, **B**). In contrast, knockdown of AMPK did not prevent the inhibitory effect of metformin on mTORC1 and ERK when added at 3 mM (Fig. 6 **A**; quantification in Fig. 6, **B**). Furthermore, knockdown of α_1 and α_2 catalytic subunit expression of AMPK substantially

reversed the inhibitory effect produced by 1 mM metformin on the stimulation of DNA synthesis in PDAC cells but did not prevent inhibition of DNA synthesis produced by 3 mM metformin. Collectively, these results support the notion that structurally unrelated inhibitors of mitochondrial function, including berberine and metformin, inhibit mitogenic signaling in PDAC cells through AMPK-dependent and independent mechanisms in a dose-dependent manner.

To substantiate the operation of AMPK-mediated inhibition of mitogenic signaling in pancreatic cancer cells, we determined whether treatment with A-769662, a direct AMPK agonist [78, 79], inhibits mTORC1 and DNA synthesis in PANC-1 cells. As expected for a compound that acts directly on AMPK rather than through inhibition of mitochondrial function, A-769662 neither reduced mitochondrial membrane potential nor decreased ATP levels in PANC-1 cells but induced robust phosphorylation of ACC at Ser^{79} and Raptor at Ser^{792} (S2 Figure). At the concentrations used, A-769662 inhibited mTORC1-mediated phosphorylation of S6K at Thr^{389} and of S6 at $Ser^{240/244}$ and DNA synthesis in PANC-1 cells (S2 Figure). The results corroborate that AMPK activation inhibits mitogenic signaling in pancreatic cancer cells.

Discussion

The studies presented here were designed to explore the hypothesis that structurally unrelated natural or synthetic compounds that interfere with mitochondrial-mediated ATP synthesis and target mTORC1 and ERK pathways, provide novel anti-PDAC agents. Our results demonstrate that treatment of pancreatic cancer PANC-1 and MiaPaCa-2 cells with berberine potently inhibited DNA synthesis, cell cycle progression and proliferation in a dose-dependent manner. We noticed that most previous studies examining effects of berberine *in vitro* were carried out with cancer cells cultured in medium supplemented with supra-physiological concentrations of glucose (e.g. 25 mM, as in DMEM) and used berberine at doses as high as 50 μM [61, 80, 81]. When PDAC cells were cultured in medium containing a physiological concentration of glucose, as in this study, berberine induced growth-suppressive effects at a dose as low as 1.5–3 μM. In view of the inhibitory effects of berberine on the proliferative responses of PDAC cells *in vitro*, we examined whether this compound inhibits pancreatic cancer growth using the MiaPaca-2 tumor xenograft model in nude mice. Our results show that administration of berberine markedly inhibited the growth of human pancreatic cancer cells xenografted into nude mice, as effectively as metformin. Given that berberine inhibited PDAC cell proliferation both *in vitro* and *in vivo*, it was important to elucidate its mechanism of inhibitory action in these cells.

Berberine has been proposed to inhibit complex I of the mitochondrial respiratory chain, reduce ATP synthesis and thereby activate AMPK, a highly conserved sensor of cellular energy being activated when ATP concentrations decrease and 5′-AMP concentrations increase [73]. Accordingly, we demonstrate

here that exposure of PDAC cells to berberine decreased mitochondrial membrane potential and induced a marked, dose-dependent decline in the intracellular levels of ATP. Concomitantly, berberine produced a pronounced dose-dependent stimulation of AMPK, as judged by the increase in the phosphorylation of ACC at Ser^{79}, a reliable biomarker of AMPK activity within intact cells [75] and phosphorylation Raptor at Ser^{792}. AMPK has been proposed to inhibit mTORC1 activation by phosphorylation of TSC2 [40–42], Raptor [82] and IRS-1 [83, 84]. Accordingly, we demonstrate here that berberine inhibited mTORC1 activity in PDAC cells, as shown by monitoring the phosphorylated state of S6K at Thr^{389}, a residue directly phosphorylated by mTORC1 and the phosphorylation of S6, a downstream target of S6K [77]. Furthermore, berberine also inhibited ERK activation in PDAC cells. The inhibitory effects of berberine on mTORC1 and ERK were elicited at doses that hampered mitochondrial function, reduced intracellular levels of ATP and activated AMPK within intact PDAC cells.

Although the preceding results are consistent with the notion that AMPK mediates some of the inhibitory effects of berberine on mitogenic signaling in PDAC cell, the precise role of AMPK in the proliferation and survival of cancer cells has become controversial [85]. Specifically, it remains unclear whether AMPK suppresses cancer cell proliferation (tumor suppressive function) or alternatively enhances cancer cell survival under conditions of metabolic stress (tumor promoter function). A tumor suppressive activity of AMPK is strongly implicated in Myc-induced lymphomagenesis [86], aerobic glycolysis [86] and in the mechanism underlying the gain of oncogenic function of certain p53 protein mutants [87]. Conversely, several recent reports have also shown that AMPK promotes tumorigenesis via protecting cancer cell viability under energy stress conditions [88] and enhances metabolic transformation [89]. These contrasting views prompted us to determine the role of AMPK in human pancreatic cancer cells. Specifically, we examined whether knockdown of the protein expression of both α_1 and α_2 catalytic subunits of AMPK in these cells opposes or facilitates the inhibitory effects induced by berberine and metformin, two agents that induce metabolic stress via inhibition of mitochondrial function and interference with ATP synthesis.

Our results led us to propose a novel mechanism of action for these agents that is sharply dependent on the dose used. We found that knockdown of the α subunits of AMPK reversed the inhibition of mTORC1 and ERK induced by low doses of berberine. Consequently, we propose that berberine inhibits mitogenic signaling through an AMPK-dependent pathway when used at low concentrations and in PDAC cells cultured in physiological concentration of ambient glucose. However, at higher concentrations, berberine inhibited mitogenic signaling (mTORC1 and ERK) and DNA synthesis through an AMPK-independent mechanism. Importantly, AMPK knockdown prevented the increase in the phosphorylation of ACC at Ser^{79} and Raptor at Ser^{792} induced by berberine at either low or high doses.

A number of studies, using high concentrations of metformin (e.g. 10 mM, as in [39, 90]) indicated that the inhibitory effect of metformin on mTORC1 is not

dependent on AMPK but the significance of results obtained with metformin at such high doses has been questioned. The results presented here with berberine prompted us to examine the generality of the notion that inhibitors of mitochondrial function, including metformin, hinder mitogenic signaling through AMPK-dependent and independent mechanisms in a dose-dependent manner. We found that metformin inhibited mitogenic signaling (mTORC1, ERK and DNA synthesis) through an AMPK-dependent pathway when used at 1 mM and in PDAC cells cultured in physiological concentration of ambient glucose (5 mM). In this context, it will be of interest to examine whether berberine and metformin display synergistic effects with other molecules that act directly on AMPK, including A-769662 [91, 92]. However, at higher concentrations, metformin inhibited mitogenic signaling and DNA synthesis through an AMPK-independent mechanism. In support of this conclusion, AMPK knockdown prevented the increase in the phosphorylation of ACC at Ser^{79} and Raptor at Ser^{792} induced by metformin at either low or high doses. Remarkably, these results were obtained with metformin used at doses that induced either modest or pronounced declines in intracellular ATP levels, which were virtually identical to the decreases in ATP levels obtained in response to berberine (Fig. 3 B). It is plausible that the decline in ATP levels produced by higher doses of either berberine or metformin interferes with ATP-consuming processes required for anabolic metabolism and cell proliferation in an AMPK-independent manner. We therefore propose that berberine and metformin inhibit mitogenic signaling in PDAC cells through an AMPK-dependent pathway at low concentrations but act via AMPK-independent pathways in the same cells when added at higher doses. This conclusion provides a plausible explanation for apparently contradictory reports on the role of AMPK in the mechanism of action of berberine and metformin in other model systems and emphasizes the need of using detailed dose-response studies to define the anticancer mechanisms of action of agents that produce metabolic stress via inhibition of mitochondrial function.

In conclusion, our results raise the attractive possibility that treatment with berberine, a widely used agent used in traditional medicine, directly inhibits pancreatic cancer cell proliferation. Our mechanistic studies with berberine and metformin provide evidence in favor of a dose-dependent tumor suppressive role of AMPK in PDAC cells and offer the bases for novel therapeutic strategies for the treatment of pancreatic cancer, a devastating disease with limited survival option.

Supporting Information

S1 Figure. Berberine inhibits mTORC1 signaling and ERK activation in PDAC cells stimulated with fetal bovine serum (FBS). Cultures of PANC-1 cells were incubated in the absence or in the presence of increasing concentrations of berberine. Then, the cells were stimulated for 1 h with 2.5% FBS and lysed with 2X SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of S6K at

Thr^{389}, S6 at Ser$^{240/244}$, and ERK at Thr^{202} and Tyr^{204}. Immunoblotting with total S6K, S6 and ERK was used to verify equal gel loading.
doi:10.1371/journal.pone.0114573.s001 (PDF)

S2 Figure. A769662 inhibits mTORC1 signaling and DNA synthesis in PANC-1 cells. A) Cells were incubated without or with 50 mM A769662 or 1 mM metformin and stimulated with 5 nM neurotensin (NT) and 10 ng/ml insulin (Ins). Lysates were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of ACC at Ser^{79}, Raptor at Ser^{792}, S6K at Thr^{389} and S6 at Ser$^{240/244}$. Irrelevant lanes in the original autoradiograph were removed and relevant ones yuxtaposed (indicated by the vertical line). **B)** A769662 (50 mM) does affect mitochondrial membrane potential (fluorescence ratio) measured with JC-1 or reduces ATP levels. **C** Dose-dependent inhibition of [^{3}H]-thymidine incorporation into DNA by increasing concentrations of A769662 in PANC-1 cells stimulated with neurotensin and insulin. **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.
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Author Contributions

Conceived and designed the experiments: ER JSS MM GE. Performed the experiments: MM JSS JW SHY HPS. Analyzed the data: ER JSS GE SHY. Wrote the paper: ER JSS MM HPS.

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Chapter V.

Suppression of feedback loops mediated by PI3K/mTOR induces multiple overactivation of compensatory pathways: an unintended consequence leading to drug resistance

Molecular Cancer Therapeutics

Review

Suppression of Feedback Loops Mediated by PI3K/mTOR Induces Multiple Overactivation of Compensatory Pathways: An Unintended Consequence Leading to Drug Resistance

Enrique Rozengurt[1,2,3], Heloisa P. Soares[4], and James Sinnet-Smith[1,2]

Abstract

The development of drug resistance by cancer cells is recognized as a major cause for drug failure and disease progression. The PI3K/AKT/mTOR pathway is aberrantly stimulated in many cancer cells and thus it has emerged as a target for therapy. However, mTORC1 and S6K also mediate potent negative feedback loops that attenuate signaling via insulin/insulin growth factor receptor and other tyrosine kinase receptors. Suppression of these feedback loops causes overactivation of upstream pathways, including PI3K, AKT, and ERK that potentially oppose the antiproliferative effects of mTOR inhibitors and lead to drug resistance. A corollary of this concept is that release of negative feedback loops and consequent compensatory overactivation of promitogenic pathways in response to signal inhibitors can circumvent the mitogenic block imposed by targeting only one pathway. Consequently, the elucidation of the negative feedback loops that regulate the outputs of signaling networks has emerged as an area of fundamental importance for the rational design of effective anticancer combinations of inhibitors. Here, we review pathways that undergo compensatory overactivation in response to inhibitors that suppress feedback inhibition of upstream signaling and underscore the importance of unintended pathway activation in the development of drug resistance to clinically relevant inhibitors of mTOR, AKT, PI3K, or PI3K/mTOR. *Mol Cancer Ther; 13(11); 2477–88. ©2014 AACR.*

Introduction

Multicellular organisms have developed highly efficient mechanisms of receptor-mediated cell communication to integrate and coordinate the function and proliferation of individual cell types. In this context, the PI3K/AKT/mTOR pathway plays a critical role in regulating multiple normal and abnormal biologic processes, including metabolism, migration, survival, autophagy, lysosome biogenesis, and growth (1). In response to different stimuli, including ligands of G protein–coupled receptors (GPCR) and tyrosine kinase receptors (TRK), PI3K catalyzes the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), a membrane lipid second messenger that coordinates the localization and activation of a variety of downstream effectors, the most prominent of which are the isoforms of the AKT family (2). The AKTs possess a PH domain and conserved residues (Thr^{308} and Ser^{473} in Akt1, the most commonly expressed isoform in normal cells) which are critical for AKT activation. Specifically, AKT translocated to the plasma membrane in response to products of PI3K is activated by phosphorylation at Thr^{308} in the kinase activation loop and at Ser^{473} in the hydrophobic motif (1). The components of the PI3K pathway and the role of this pathway in disease have been reviewed (1, 3).

mTOR functions as a catalytic subunit in two structurally distinct multiprotein complexes, mTORC1 and mTORC2 (1, 4). mTORC1, a complex of mTOR, the substrate-binding subunit RAPTOR, GβL, and PRAS40, senses nutrients and growth factors. mTORC1 phosphorylates and controls at least two regulators of protein synthesis, the 40S ribosomal protein subunit S6 kinase (S6K) and the inhibitor of protein synthesis 4E-binding protein 1, referred as 4EBP1, which promote translation of cell growth proteins, including c-MYC and cyclin D. mTORC1 also plays a critical role in the regulation of cellular metabolism (5). The heterodimer of the tumor suppressor tuberous sclerosis complex 2 (TSC2; tuberin) and TSC1 (hamartin) represses mTORC1 signaling by acting as the GTPase-activator protein for the small G protein RHEB (RAS homolog enriched in brain), a potent activator of mTORC1 signaling in its GTP-bound state. Phosphorylation of TSC2

[1]Division of Digestive Diseases and Department of Medicine, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California. [2]CURE: Digestive Diseases Research Center, University of California at Los Angeles, Los Angeles, California. [3]Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California. [4]Division of Hematology-Oncology and Department of Medicine, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California.

Corresponding Author: Enrique Rozengurt, David Geffen School of Medicine at UCLA, 900 Veteran Avenue, Warren Hall Room 11-124, Los Angeles, CA 90095-1786. Phone: 310-794-6610; Fax: 1-310-267-1953; E-mail: erozengurt@mednet.ucla.edu

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by AKT and/or ERK/p90RSK uncouples TSC1/TSC2 from RHEB, leading to RHEB-GTP accumulation and mTORC1 activation. The RAG GTPases activate mTORC1 in response to amino acids by promoting mTORC1 translocation to lysosomal membrane that contains RHEB-GTP (4). RAS-like (RAL) small GTPases, in their GTP-bound state, also promote mTORC1 activation through a pathway parallel to RHEB (6). Phosphatase and tensin homolog (PTEN) opposes PI3K by dephosphorylating PIP_3 to PIP_2 thereby inactivating AKT and mTOR signaling (7).

The PI3K/AKT/mTORC1 module is aberrantly activated in many human cancers and plays a pivotal role in insulin/insulin growth factor (IGF) receptor signaling. Inactivation of p53, as seen during the progression of approximately 50% of human malignancies, potently upregulates the insulin/IGFI/mTORC1 pathway (8). Consequently, mTORC1 and the upstream components of the cascade have emerged as attractive therapeutic targets in a variety of common malignancies (9).

Mounting evidence indicates that the mTORC1/S6K axis not only promotes growth-promoting signaling but also mediates potent negative feedback loops that restrain upstream signaling through insulin/IGF receptor and other TRKs in both normal and oncogene-transformed cells (Fig. 1). Suppression of these feedback loops by inhibitors of mTORC1/S6K causes compensatory overactivation of

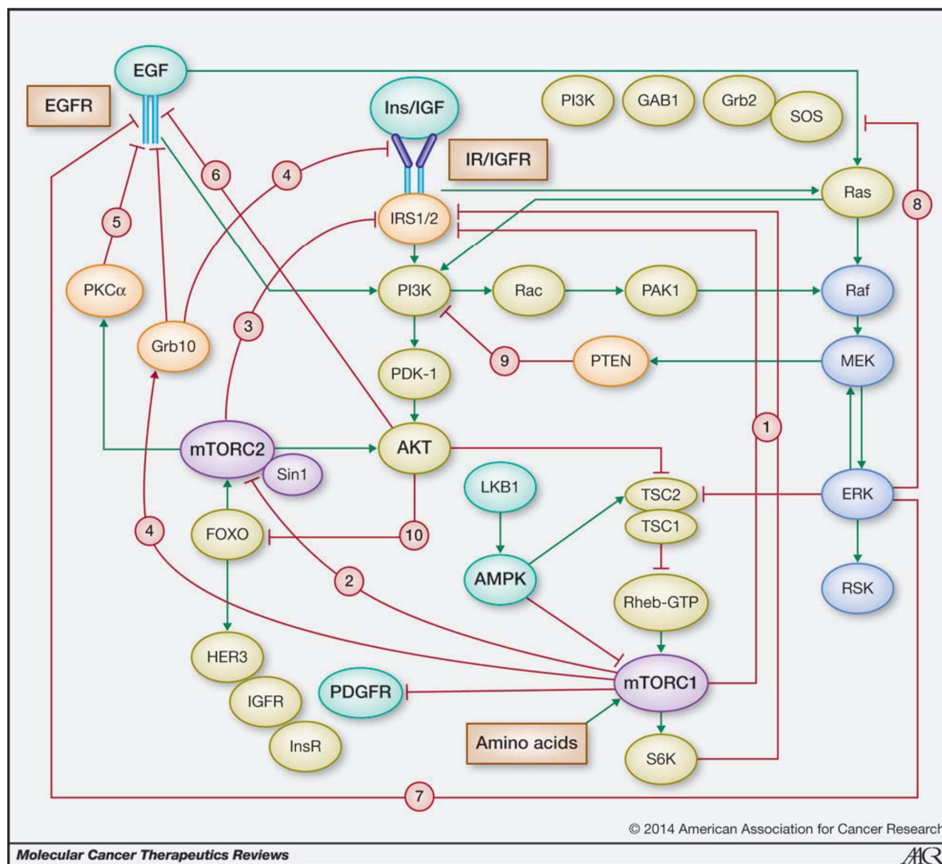

Figure 1. Signaling through the PI3K/mTOR and RAS/RAF/MEK/ERK pathways is controlled by negative feedback loops. Feedback loops emanate from distal elements of the same pathway (intrinsic negative loops) or from other pathways (extrinsic loops) and restrain the activity of upstream signaling nodes thereby fine-tuning the output of the signaling network. These potent negative feedback loops are indicated by the red lines and identified with numbers. Stimulatory connections are in green. See text for detailed description.

upstream signaling nodes, including PI3K, AKT, and ERK that potentially oppose the antiproliferative effects of the inhibitors and lead to drug resistance. To realize the therapeutic potential of targeting mTOR, it is necessary to elucidate the full spectrum of feedback loops that are unleashed by suppression of the PI3K/AKT/mTOR pathway. The detailed understanding of these feedback mechanisms will allow the design of rational combinations of therapeutic agents to overcome drug resistance produced by compensatory activation of upstream pathways and the identification of biomarkers to predict which patient will respond to them. The purpose of this article is to review negative feedback mechanisms that restrain signaling via upstream elements of the PI3K/AKT/mTOR pathway as well as mechanisms leading to the compensatory activation of other pro-oncogenic pathways, including MEK/ERK. The studies discussed here underscore the importance of unintended pathway activation in the development of drug resistance to clinically relevant inhibitors of mTOR, AKT, PI3K, or PI3K/mTOR.

mTORC1 and mTORC2 Mediate Negative Feedback of PI3K/AKT Activation through Inhibition and Degradation of IRS-1

The insulin receptor substrate (IRS) docking proteins, including IRS-1 and IRS-2, play a key role in insulin/IGF signaling through PI3K. These proteins are phosphorylated by these receptors at multiple Tyr residues that play a critical role in downstream signaling, including PI3K activation. The IRS family is also phosphorylated at multiple serine and threonine residues that attenuate signaling and promote degradation. As illustrated in Fig. 1, loop 1, activation of the mTORC1/S6K cascade inhibits IRS-1 function, including PI3K/AKT activation, following its phosphorylation at multiple residues, including $Ser^{636/639}$ by mTORC1 and $Ser^{270/307/636/1001}$ by S6K (10). Accordingly, suppression of mTORC1 activity by rapamycin (sirolimus) and its analogs (rapalogs) prevents inhibitory phosphorylations mediated by mTORC1/S6K (11). Rapalogs, (e.g., RAD001/everolimus) which act as allosteric inhibitors of mTORC1 via FKBP-12 were the first generation of mTOR inhibitors to be tested as anticancer agents.

A prominent consequence of mTORC1/S6K inhibition by rapalogs in cells, preclinical cancer models, and clinical trials has been a striking increase in AKT phosphorylation at Thr^{308} by PDK1 and at Ser^{473} by mTORC2 (11–13). In this context, loss of PTEN expression which can potentiate feedback AKT phosphorylation in response to rapamycin is actually a marker of rapalog resistance rather than a biomarker for the use of mTORC1 inhibitors in human bladder cancer cells (14). mTORC2-mediated phosphorylation of AKT at Ser^{473} in response to rapamycin can be further enhanced by eliminating negative cross-talk from mTORC1/S6K (Fig. 1, loop 2). Specifically, Liu and colleagues reported that phosphorylation of SIN1, a specific component of mTORC2 also known as MAPK-associated protein 1, at Thr^{86} and Thr^{398} suppresses mTORC2 kinase activity by dissociating SIN1 from mTORC2 (15). SIN1 phosphorylation, mediated by S6K in epithelial cells (AKT can also phosphorylate SIN1 in mesenchymal cells), inhibits not only insulin- or IGFI-mediated, but also platelet-derived growth factor (PDGF)- or EGF-induced AKT phosphorylation by mTORC2 (15). Thus, these findings reveal a novel negative feedback loop connecting mTORC1 and mTORC2 and an additional mechanism by which exposure to rapamycin enhances AKT phosphorylation at Ser^{473}.

A recent study by Kim and colleagues (16) revealed that mTORC2 can also regulate the cellular level of IRS-1. These investigators found that despite phosphorylation at the mTORC1-mediated serine sites, inactive IRS-1 accumulated in mTORC2-disrupted cells. Defective IRS-1 degradation was due to diminished expression and phosphorylation of the ubiquitin ligase substrate-targeting subunit, FBW8 (16). mTORC2 stabilizes FBW8 by phosphorylation at Ser^{86}, allowing the insulin-induced translocation of FBW8 to the cytosol where it mediates IRS-1 degradation. Thus, mTORC2 negatively feeds back to IRS-1 via control of FBW8 stability and localization (Fig. 1, loop 3). These findings indicate that mTORC1 and mTORC2 cooperate in promoting IRS-1 degradation and imply that the potential therapeutic benefit of inhibiting mTORC1 with rapamycin is opposed by release of feedback inhibition of PI3K/AKT activation (11, 13), resulting in disease progression. Although rapalogs have been demonstrated to prolong overall survival of patients with metastatic renal cell carcinoma, the clinical antitumor activity of rapamycin analogs in many types of cancer has been rather limited. In some cases, mutated cancer genes can serve as biomarkers of response to targeted agents. So far, the use of PTEN, PI3K mutations, and AKT phosphorylation as biomarkers for predicting rapalog sensitivity has not been successful in clinical settings. In fact, as mentioned above, loss of PTEN expression may be a marker of rapalog resistance, at least in some cancer cells. It is likely that treatment with rapalogs not only interferes with feedback loops that restrain PI3K/AKT activation but also with other signaling pathways that can promote drug resistance, as discussed below.

Rapamycin-Induced ERK Overactivation

In addition to the feedback loop that restrains PI3K/AKT activation, immunohistochemical analysis of biopsies of patients with breast cancer that were treated with the rapalog RAD001 (everolimus) revealed that there was a marked increase in ERK activation, i.e., ERK phosphorylated on the activation loop residues Thr^{202} and Tyr^{204} (17). These results indicated that anticancer therapy with allosteric mTORC1 inhibitors can lead to activation of the ERK pathway, thus adding a new level of complexity to the previously described negative feedback loop involving mTORC1/PI3K/AKT. On the basis of experiments using inhibitors of PI3K (LY294002) and a dominant-negative form of RAS ($RASN^{17}$), Carracedo and colleagues (17) concluded that ERK overactivation in response

to rapamycin depended on the function of PI3K/RAS but the mechanism(s) was not defined. In all the experiments presented, the cells were exposed to the rapamycin for at least 24 hours (17). Thus, it is not clear whether the putative PI3K-dependent pathway is an acute effect of unleashing a rapid feedback loop or a slow feedback loop involving a transcriptional response (see below).

A recent study with breast cancer cells harboring *PI3KCA* mutant or *HER2* amplification but without *RAS* mutations suggest a possible mechanism by which PI3K can lead to ERK pathway activation (18). Specifically, PI3K-mediated PIP_3 accumulation increased the activity of RAC1 (RAC1-GTP) via PIP_3-dependent RAC exchanger 1 (P-Rex1) and of its effector PAK1 leading to phosphorylation of RAF1 at the activating Ser^{338}. These findings imply that robust PI3K-mediated RAC/PAK1 can enhance RAF stimulation and thereby promote MEK/ERK overactivation (Fig. 1). It will be of interest to determine whether rapamycin-induced ERK is correlated to P-Rex1 expression, RAC-GTP, and RAF Ser^{338} phosphorylation. A putative alternative pathway of PIP_3-dependent ERK activation involves the recruitment of the adaptor GRB2-associated binder 1 (GAB1) which in turn recruits GRB2–SOS, leading to RAS/RAF activation (19). In this context, it is also relevant that long-term exposure to rapamycin also initiates transcriptional upregulation of PI3K subunits, e.g., p85α and p110δ (20), potentially reinforcing the PI3K-mediated signaling to RAC/PAK1 and/or GAB1/GRB2/SOS which can lead to MEK/ERK overactivation in response to rapamycin.

Another mTORC1-mediated negative feedback loop restrains the expression of PDGF receptor (PDGFR). Activation of PI3K or AKT or deletion of PTEN in mouse embryonic fibroblasts suppresses PDGFR expression, whereas rapamycin increases PDGFR expression (21). In hepatocellular carcinoma cells, prolonged (>6 hours) treatment with rapamycin induced ERK signaling through increased expression and phosphorylation of PDGFRβ (22). The role of this PDGFRβ-dependent loop leading to ERK signaling in other cancer cells requires further experimental work. The remodeling of the signaling network in response to rapalogs is illustrated in Fig. 2A.

Compensatory Activation of PI3K and ERK Signaling in Response to Active-Site mTOR Inhibitors and Dual PI3K/mTOR Inhibitors

As discussed above, the potential anticancer activity of rapamycin (or analogs) can be counterbalanced by release of feedback inhibition of PI3K/AKT and ERK activation. Furthermore, rapamycin incompletely inhibits 4E-BP1 phosphorylation (23, 24). Specifically, most cells display a high basal level of 4E-BP1 phosphorylation at $Thr^{37/46}$ that is not further increased by growth factor stimulation nor inhibited by rapamycin (25). However, cell stimulation reduced the mobility of 4E-BP1 in SDS/PAGE, a response suggestive of increased phosphorylation at other sites. Indeed, growth factor stimulation of pancreatic cancer cells markedly stimulated 4E-BP1 phosphorylation on Thr^{70}, a response blocked by treatment with rapamycin (25). These results revealed an unappreciated regulation of 4E-BP1 phosphorylation on different residues in response to external signals and demonstrate that rapamycin inhibits inducible but not constitutive 4E-BP1 phosphorylations. More studies are needed to determine whether 4E-BP1 is subject to constitutive and inducible phosphorylations at different sites in different cancer cells.

In an effort to target the mTOR pathway more effectively, novel ATP-competitive inhibitors of mTOR that act at its catalytic active site (active-site mTOR inhibitors) have been identified, including PP242 (26), Torin (27), KU63794 (28), and its analog AZD8055 (29). These compounds inhibit 4E-BP1 phosphorylation at rapamycin-resistant sites (e.g., $Thr^{37/46}$) and block AKT phosphorylation at Ser^{473}, in line with the notion that mTORC2 is the major protein kinase that phosphorylates AKT at this residue. Active-site inhibitors proved more effective inhibitors of cell proliferation than rapamycin in a variety of model systems. However, active-site mTOR inhibitors also eliminate negative feedback loops that restrain PI3K activation and, consequently, their therapeutic effectiveness can also be diminished by activation of upstream pathways that oppose their antiproliferative effects (Fig. 2B). Specifically, active-site mTOR inhibitors enhance PI3K/PDK-dependent AKT phosphorylation at its activation loop (Thr^{308}) and, consequently, these agents do not completely block AKT activity (30).

Surprisingly, short-term exposure of a variety of cell types, including human pancreatic cancer cells (25) and multiple myeloma cells (31), to active-site mTOR inhibitors, such as KU63794 or PP242, induced a striking overactivation of ERK. The mTOR inhibitors also induced MEK overactivation, as scored by assessing the phosphorylation of Ser^{217} and Ser^{221} in the MEK activation loop, and MEK inhibitors abrogated the overactivation of MEK. In contrast, treatment with rapamycin at concentrations that completely prevented the mTORC1/S6K axis, as scored by phosphorylation of S6K on Thr^{389} did not cause any change in ERK phosphorylation in cells harboring *RAS* mutations (25, 31). These results indicated that first and second generations of mTOR inhibitors promote overactivation of different upstream pro-oncogenic pathways in a cell context–dependent manner.

Further evidence supporting that active-site inhibitors enhance ERK overactivation through a PI3K-independent feedback loop was obtained by determining the effect of KU63794 or PP242 on ERK activity in multiple myeloma cells treated with wortmannin (31) or pancreatic cancer cells treated with A66, a selective inhibitor of the p110α catalytic subunit of PI3K (25). Inhibition of PI3K did not prevent enhancement of ERK activation in response to active-site mTOR inhibitors. These results identified a PI3K-independent feedback loop regulating the cross-talk between the mTOR and MEK/ERK pathways which is different from the loop previously identified with

Figure 2. A, compensatory overactivation of signal transduction pathways induced by rapamycin-mediated suppression of negative feedback loops. Rapamycin triggers PI3K activation and AKT phosphorylation at Thr[308] and Ser[473] via suppression of mTORC1/S6K-mediated phosphorylation of IRS-1 and SIN1 (loop 1). mTORC2-mediated phosphorylation of AKT at Ser[473] in response to rapamycin can be further enhanced by eliminating negative cross-talk from S6K (loop 2). Furthermore, mTORC2 negatively feeds back to IRS-1 via control of its stability (loop 3). In some cancer cells, rapalogs also induce MEK/ERK via the PI3K-dependent pathway that could involve RAC/PAK1 and/or PDGFR. B, compensatory overactivation of signal transduction pathways induced by active-site mTOR inhibitors. Active-site mTOR inhibitors that block both mTORC1 and mTORC2 also eliminate feedback loops that restrain PI3K/PDK1 activation. Specifically, these agents disable feedback loops 1, 2, 3, 4, 5, 6, and 10. Active-site mTOR inhibitors enhance AKT phosphorylation at the activation loop (Thr[308]) and consequently these agents do not completely block AKT activity. Acute exposure of a variety of cell types to active-site mTOR inhibitors induced a striking overactivation of the MEK/ERK pathway via a PI3K-independent pathway. Chronic exposure to these agents also disables the negative influence of AKT on FOXO thereby promoting expression of TRKs and adaptors. For additional details, see text. Inhibitory connections are in red. Stimulatory connections are in green. Pathways activated by suppression of negative feedback loops are highlighted in yellow.

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rapamycin (17). The remodeling of the signaling network in response to active-site mTOR inhibitors is illustrated in Fig. 2B.

The fact that PI3K and mTOR have high homology in their kinase domains has made possible the development of dual active-site inhibitors (PI3K/TOR-KIs), including NPV-BEZ235 (32), PKI-587 (33), and GDC-0980 (34). Additional PI3K/TOR-KIs that are being tested in preclinical and clinical trials include XL765, NVP-BKM120, XL147, SF1126, GSK2126458, VS-5584, and PF-04691502. As mentioned above, overactivation of the ERK pathway induced by active-site mTOR inhibitors is mediated through a PI3K-independent pathway (25, 31). Therefore, it could be expected that dual PI3K and mTOR inhibitors also promote ERK overactivation. In line with this prediction, our current studies demonstrate ERK overactivation in pancreatic cancer cells treated with multiple clinically relevant PI3K/TOR-KIs, including NPV-BEZ235, PKI-587, and GDC-0980 (H. P. Soares et al., unpublished). These results with dual PI3K and mTOR catalytic kinase inhibitors provide conclusive evidence identifying a novel PI3K-independent feedback loop that restrains the activity of the MEK/ERK pathway. The remodeling of signaling in response to active-site PI3K/mTOR inhibitors is illustrated in Fig. 3A.

Mechanisms by Which mTOR and PI3K/mTOR Inhibitors Stimulate MEK/ERK Signaling

A plausible mechanism by which active-site mTOR inhibitors or dual PI3K/mTOR inhibitors relieve a negative feedback on receptor tyrosine kinases that leads to RAF/MEK/ERK has been suggested by recent phosphoproteomic studies demonstrating that mTORC1 directly phosphorylates the adaptor protein growth factor receptor–bound protein 10 (GRB10) at multiple sites (35, 36). GRB10 is known to suppress signaling induced by insulin and IGFs (37) and mice lacking GRB10 are larger than normal and exhibit enhanced insulin sensitivity (38). In addition to inhibiting insulin/IGFI receptor tyrosine kinase activity by direct binding, GRB10 also mediates degradation of these receptors through ubiquitination (39). The phosphorylation of GRB10 by mTORC1 enhances its stability and capacity to inhibit insulin/IGF signaling. The sites phosphorylated by mTORC1 were mapped to Ser^{104}, Ser^{150}, Thr^{155}, Ser^{428}, and Ser^{476} (35). While active-site mTOR inhibitors blocked phosphorylation of GRB10 at all five sites, rapamycin only prevented GRB10 phosphorylation at Ser^{476} (35). Therefore, GRB10, like 4E-BP1, is an mTORC1 substrate with both rapamycin-sensitive and -insensitive sites. As the phosphorylation of GRB10 potentiates its inhibitory activity on insulin/IGF receptor signaling, acute suppression of GRB10 phosphorylation at all sites (by direct mTOR inhibitors or dual PI3K/mTOR inhibitors) eliminates its ability to attenuate insulin/IGF signaling (Fig. 1, loop 4) thereby leading to MEK/ERK activation.

Another potential mechanism by which active-site mTOR or dual PI3K/mTOR inhibitors could promote MEK/ERK signaling is via enhanced EGFR activity. The EGFR tyrosine kinase activity and affinity for its ligand is known to be negatively regulated by PKCα via phosphorylation at Thr^{654} (40). Recent studies indicated that mTORC2 mediates PKCα phosphorylation of both the turn and hydrophobic motifs (41, 42). Interestingly, the mTORC2-dependent phosphorylation of PKCα plays an important role in its maturation, stability, and signaling (41, 42). It is plausible, therefore, that suppression of mTORC2-mediated posttranslational processing of PKCα interferes with negative feedback of PKCα on EGFR thereby leading to hyperactivation of EGFR and overactivation of ERK signaling in response to EGFR agonists or GPCR transactivation (43), as illustrated in Fig 1, loop 5.

A recent study examining the role of AKT in EGFR trafficking elucidated a novel negative feedback mechanism (44). Specifically, EGF-induced activation of AKT promotes progression of internalized EGFR through the early endosomes and EGFR degradation. In cells treated with inhibitors of PI3K or AKT, EGFR trafficking was impaired and accumulated in the early endosomes, resulting in increased ERK activation (44). It is conceivable that AKT inhibition interferes with this negative feedback loop (Fig. 1, loop 6) thereby promoting EGFR accumulation and enhanced ERK signaling in cells with a hyperactive EGFR signaling system. Accordingly, it will be of interest to determine whether EGFR inhibitors potentiate the inhibitory effects of AKT inhibitors.

In addition to MEK/ERK, dual PI3K/mTOR inhibitors can also induce compensatory activation of other signaling pathways that mediate resistance to these drugs. In triple-negative breast cancer (TNBC), a clinically aggressive subtype of breast cancer defined by lack of expression of estrogen and progesterone receptors and HER2 amplification, PI3K/mTOR inhibition induced feedback activation of JAK2/STAT5 and secretion of IL8 in cell lines and primary breast tumors (45). In TNBC, inhibition of JAK2 abrogated this feedback loop and combined PI3K/mTOR and JAK2 inhibition synergistically reduced cancer cell number and tumor growth (45).

Mechanisms by Which MEK Inhibitors Stimulate PI3K/AKT Signaling

It is pertinent that cross-talk between PI3K/AKT/mTOR and MEK/ERK pathways also functions in the opposite direction. Specifically, MEK inhibitors have been shown to induce PI3K/AKT activation via EGFR (46), thus revealing a negative feedback loop mediated by ERK phosphorylation of EGFR (e.g., at Thr^{669}) that restrains PI3K/AKT activation and PIP_3 accumulation (47). This negative feedback (Fig. 1, loop 7) could also underlie the different responses of colon and melanoma cancer cells (both with BRAF V600E mutations) to BRAF inhibitors (48, 49). While melanomas are highly sensitive to BRAF inhibitors, colon cancers harboring the identical BRAF V600E mutation are resistant to these agents. Two elegant studies demonstrated that the drug resistance of colon

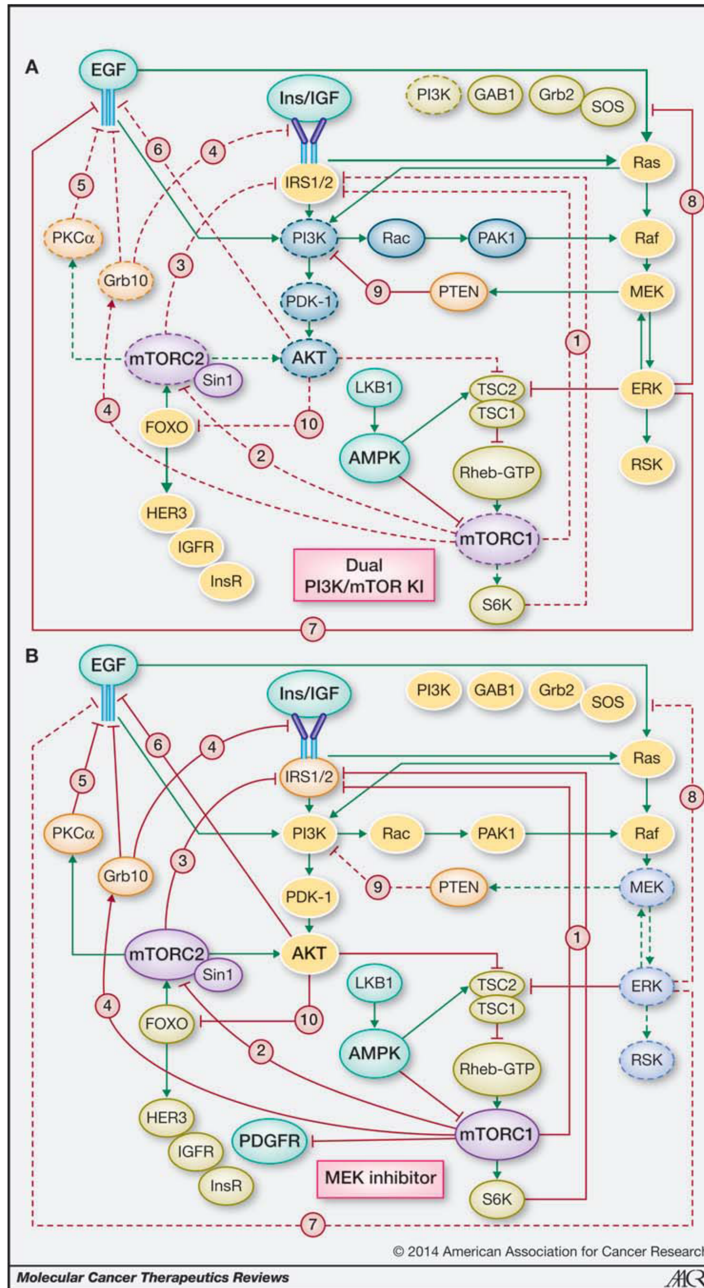

Figure 3. A, compensatory overactivation of signal transduction pathways induced by dual PI3K/mTOR inhibitors. Dual PI3K/mTOR inhibitors disable feedback loops 1, 2, 3, 4, 5, 6, and 10. Acute exposure of a variety of cell types to dual PI3K/mTOR inhibitors induced overactivation of the MEK/ERK pathway via a PI3K-independent pathway, probably involving GRB2/SOS-mediated RAS activation as a result of TRK and/or IRS activation. Chronic exposure to these agents also promotes FOXO-mediated expression of TRKs and adaptors. B, compensatory overactivation of signal transduction pathways induced by MEK inhibitors. These inhibitors disable feedback loops 7, 8, and 9 leading to RAS/RAF and PI3K/AKT overactivation. See text for detailed description. Inhibitory connections are in red. Stimulatory connections are in green. Pathways activated by suppression of negative feedback loops are highlighted in yellow.

cancer cells is due to increased expression and signaling of EGFR in these cells (48, 49). Previously, we mentioned that EGFR signaling is negatively regulated by PKCα (40). Interestingly, PKCα is expressed in melanoma (50) but decreased in most colorectal cancers (51), suggesting another mechanism by which EGFR signaling could be stronger in colon cancers following release of feedback inhibition. Consequently, release of feedback inhibition by BRAF inhibitors induces stronger EGFR activation in colon cells thereby recovering ERK pathway activation via alternative pathways (CRAF instead BRAF) as well as enhancing EGFR-induced PI3K/AKT signaling.

Another feedback loop could also involve the ERK-regulated p90RSK which has been shown to phosphorylate (at Ser^{1101}) and inhibit IRS-1 (52) and ERK-mediated feedback of RAS/RAF activation, for example, via phosphorylation of SOS (Fig. 1, loop 8). More recent work has demonstrated that MEK1 is an essential regulator of the lipid/protein phosphatase PTEN, through which it restrains PIP_3 accumulation and AKT signaling. MEK1 has been shown to be required for PTEN membrane recruitment as part of a ternary complex containing the multidomain adaptor MAGI1 (53). Complex formation depends on phosphorylation of MEK1 at Thr^{292} by activated ERK. Consequently, ERK inhibition by MEK inhibitors prevents PTEN membrane recruitment, increasing PIP_3 accumulation and AKT activation (Fig. 1, loop 9).

Reciprocal feedback loops connecting PI3K/AKT/mTOR and MEK/ERK pathways provide further impetus for developing combination of inhibitors that cotarget both pathways. Indeed, phase I clinical trials cotargeting these pathways with MEK inhibitors plus PI3K/mTOR inhibitors are ongoing (e.g., Clinicaltrial.gov identifiers NCT01347866; NCT01390818). The remodeling of the signaling network in response to MEK inhibitors is illustrated in Fig. 3B.

Chronic Exposure to PI3K/PDK1/AKT Inhibitors Suppresses a Feedback Loop That Mediates Repression of TRK and Survival Protein Expression

In addition to ERK and AKT overactivation in response to acute mTOR pathway inhibition, a number of studies demonstrated that long-term treatment with PI3K, AKT, or PI3K/mTOR inhibitors induces a transcriptional response that also leads to drug resistance (54–59). The forkhead box O (FOXO) transcription factors, which include FOXO1, 3, 4, and 6 in mammals, are major downstream targets of the AKTs. The phosphorylation of FOXO by AKT creates docking sites for 14–3–3 proteins. The binding of 14–3–3 to FOXO promotes its translocation from the nucleus to the cytoplasm. Reciprocally, inhibition of AKT activity releases a feedback loop that promotes nuclear localization of the FOXO transcription factors (Fig. 1, loop 10). Nuclear FOXO family members stimulate transcription of several TRKs, including EGFR, IGFR, and insulin receptor in a spectrum of tumor cells (54). Furthermore, a recent study showed that FOXOs upregulated the expression of RICTOR, thereby enhancing mTORC2 and AKT phosphorylation at Ser^{473} (60), thus creating an amplification loop. At least in some cancer cells, FOXO-mediated transcription cooperates with enhanced cap-independent translation mediated by Pim-1 (58).

Accordingly, recent preclinical and clinical studies revealed that suppression of PI3K, AKT, or PI3K/mTORC1 initiates transcriptional responses that lead to the overexpression of TRKs or adaptor proteins, including HER3, IGFR, FGFR and in some cases, consequent enhancement of ERK. Dimerization of HER3 with EGFR or HER2 then promotes resistance to a number of inhibitors of PI3K signaling (61). Chronic exposure of HER2-positive breast cancer models to NPV-BEZ235 induced activation of HER family of receptors and adaptors leading to ERK overactivation, as shown by increased expression of HER3 and phosphorylation of HER2 and HER3 (56). In these breast cancer cells, ERK overactivation was completely prevented by inhibitors of MEK or HER2, suggesting clear combinatorial strategies to circumvent resistance to PI3K/AKT inhibition. Compensatory activation of HER3 and ERK has been corroborated in clinical samples following treatment with GDC-0068, an inhibitor of AKT catalytic activity (59). In contrast, treatment with a PI3K inhibitor (XL147) promoted expression of several TRKs but did not stimulate ERK overactivation (55). In ovarian cancer cells, NVP-BEZ235 also induces a program leading to expression of receptors and survival proteins, at least in part due to enhanced cap-independent translation, but do not appear to stimulate ERK signaling (57). In conclusion, treatment of a variety of tumor cells with inhibitors that block the PI3K/AKT/mTOR pathway at each level induces a concerted transcriptional response mediated, at least in part, by FOXO family members that oppose the anticancer effects of these agents. This gene expression loop should be distinguished from the rapid MEK/ERK overactivation induced by mTOR and dual PI3K/mTOR inhibitors in other cell types (25, 31). The FOXO-mediated transcriptional response on signaling in response to active-site mTOR inhibitors and dual PI3K/mTOR inhibitors are highlighted in Figs. 2 and 3.

Metformin Inhibits mTORC1 but Does Not Elicit AKT or ERK Overactivation: Role of AMPK

Metformin (1,1-dimethylbiguanide hydrochloride) is the most widely prescribed drug for treatment of type 2 diabetes mellitus (T2DM) worldwide but its mechanism of action remains incompletely understood. At the cellular level, metformin indirectly stimulates AMP-activated protein kinase (AMPK) activation via inhibition of mitochondrial complex I (62), though other cellular mechanisms of action have been proposed, especially at high concentrations (63, 64). AMPK is a conserved regulator of the cellular response to low energy, and it is activated when the ATP concentration decreases and 5′-AMP and ADP concentrations increase. AMPK, a potent inhibitor of

anabolic metabolism, is also implicated in the regulation of epithelial cell polarity (65). The tumor suppressor LKB-1/STK11 (Liver kinase B1/serine–threonine kinase; ref. 11) is the major kinase phosphorylating the AMPK activation loop at Thr^{172}.

Recent epidemiologic studies are linking administration of metformin with reduced incidence, recurrence, and mortality of a variety of cancers in patients with T2DM (66). Although epidemiologic associations do not establish causation, they provide an important line of evidence that supports the need for mechanistic studies. The protective effects of metformin in human cancers could be mediated by direct suppression of mitogenic signaling through AMPK-dependent and/or AMPK-independent pathways. It is well established that metformin inhibits mTORC1 activation in a variety of cancer cell types (67). At low concentrations of metformin, the inhibitory effect on mTORC1 is prominent in cells incubated in medium containing physiologic concentrations of glucose (68). Studies *in vitro* demonstrated that AMPK inhibits mTORC1 activation at several levels: (i) AMPK stimulates TSC2 function via phosphorylation on Ser^{1345} (69), leading to accumulation of RHEB-GDP (the inactive form) and thereby to inhibition of mTORC1; (ii) AMPK directly phosphorylates RAPTOR (on Ser^{722} and Ser^{792}), which disrupts its association with mTOR, leading to dissociation of mTORC1 (70); (iii) metformin has also been proposed to inhibit mTORC1 via AMPK-independent pathways, targeting RAG GTPases or cyclin D1 but these effects were elicited at very high concentrations. Direct effects of metformin at clinically relevant doses are of great significance because they imply that this drug will be a useful anticancer agent not only for patients with T2DM but also for nondiabetic patients.

Although metformin inhibits the mTORC1/S6K axis, its effects on feedback loops regulating AKT and ERK activation are very different from rapalogs, active-site mTOR inhibitors, and dual PI3K/mTOR inhibitors (25). For example, metformin, in contrast to rapamycin, did not overstimulate AKT phosphorylation on Ser^{473} although both rapamycin and metformin strongly inhibited the mTORC1/S6K axis. Although the precise mechanism explaining this difference is not fully understood, it is relevant that AMPK directly phosphorylates IRS-1 on Ser^{794}, a site that interferes with PI3K activation (71, 72). In addition, mTORC2 phosphorylates AKT not only at Ser^{473} but also at the turn motif site (Thr^{450}) of AKT required for its proper folding (41, 42). A recent study demonstrated that a high level of cellular ATP levels is required to maintain the integrity of mTORC2-mediated phosphorylation of AKT on the turn motif Thr^{450} site (73). Because metformin inhibits mitochondrial ATP production, it is conceivable that a small decline in ATP levels induced by this biguanide interferes with the integrity of mTORC2 and with its ability to phosphorylate AKT at Ser^{473}, another mechanism that would prevent AKT overactivation even when the mTORC1/S6K axis is suppressed.

An important difference between the effects of metformin and mTOR inhibitors is that metformin inhibited rather than overactivated MEK/ERK in response to growth factors (25). A plausible mechanism underlying the inhibitory effect metformin on MEK/ERK activation is suggested by a recent study showing that AMPK phosphorylates BRAF at Ser^{729} (74). The phosphorylation of this site promotes the association of BRAF with 14–3–3 proteins and disrupts its interaction with the KSR1 scaffolding protein leading to decrease in the activity of the MEK/ERK pathway. Interestingly, ERK signaling was not decreased by AMPK in BRAF-mutant tumors (74). Collectively, these studies *in vitro* imply that metformin has considerable advantages in promoting mTOR inhibition without unleashing feedback loops that oppose its antiproliferative effects, though these effects are likely depend on cell context and oncogenic mutations. A number of clinical trials in progress, combining metformin with established anticancer agents, will determine whether metformin is useful in cancer therapeutics. Because AMPK appears to prevent tumor development rather than to inhibit advanced malignancies, it is conceivable that metformin will be more useful in chemoprevention rather than in a therapeutic setting.

Concluding Remarks and Clinical Implications

One of the first indications that cells can be stimulated to reinitiate DNA synthesis through different molecular pathways that act in a combinatorial manner was obtained from studies using multiple growth factors in Swiss 3T3 fibroblasts (75). Subsequent studies substantiated the concept that multiple parallel pathways that cross-talk and converge on key signaling nodes lead to proliferation of both normal and cancer cells. A corollary of this concept is that release of negative feedback loops and consequent compensatory overactivation of promitogenic pathways in response to signal inhibitors can circumvent the mitogenic block imposed by targeting only one pathway. Consequently, the elucidation of the network of feedback loops that regulate signal transduction outputs of complex signaling networks has emerged as an area of fundamental importance for the rational design of effective anticancer combinations of inhibitors. In recent years, it has become apparent that most signaling pathways are controlled by negative feedback loops that fine tune the signaling network and that in many cases, the success of therapies targeting one pathway is thwarted by the compensatory overactivation of upstream pathways that remodeled the network.

Here, we discussed that inhibition of mTOR or PI3K/mTOR induces rapid signaling in a variety of cancer cells through compensatory overactivation of pro-oncogenic and prosurvival pathways mediated by unleashing feedback inhibition of upstream signaling (Figs. 2 and 3). Specifically, rapamycin triggers PI3K activation and AKT phosphorylation at Thr^{308} and Ser^{473} via suppression of mTORC1/S6K phosphorylation of IRS-1 and SIN1. In

some cancer cells, rapalogs also induce MEK/ERK via PI3K-dependent pathway that could involve RAC/PAK1. Active-site mTOR inhibitors induce PI3K activation and AKT phosphorylation at Thr[308] but not at Ser[473] most likely via suppression of GRB10-mediated feedback inhibition of insulin/IGF receptors and/or suppression of PKCα negative regulation of EGFR. In turn, dual PI3K/mTOR inhibitors promote robust overactivation of the MEK/ERK pathway, most likely via suppression of GRB10- and mTORC2-mediated feedback loops.

Chronic exposure to the same agents initiates a program of FOXO-mediated transcriptional derepression leading to increased expression of multiple TRKs, including HER3, IGFR, and insulin receptor. The distinction between short-term and long-term consequences in response to inhibitors is important for defining strategies to overcome drug resistance, including the dosing schedule of the drug and the pathways that should be cotargeted for optimal response. It is reasonable to propose that rapid overactivation of ERK signaling will be important in mediating resistance when strong but intermittent inhibition of mTOR is used. In this case, inhibition of mTOR should be associated with MEK inhibition. Reciprocally, slow FOXO-mediated transcriptional derepression of TRK expression is likely to be important when mTOR or dual PI3K/mTOR inhibitors are administrated to produce constant inhibition of these targets. In this case, inhibitors of EGFR and HER2 (e.g., lapatinib) and/or IGFR might be the drugs of choice for combinatory therapy (i.e., mTOR or dual PI3K/mTOR inhibitors with either lapatinib or IGFR inhibitor).

Although metformin inhibits stimulation of the mTORC1/S6K axis *in vitro*, its effects on feedback loops regulating AKT and ERK activation are very different from rapalogs, active-site mTOR inhibitors or dual PI3K/mTOR inhibitors. Metformin, in contrast to rapamycin, did not overstimulate AKT phosphorylation on Ser[473]. An important difference between the effects of metformin and active-site mTOR inhibitors is that metformin inhibited rather than overactivated the MEK/ERK pathway in several cell types in response to growth factors. Collectively, these studies imply that metformin has considerable advantages in promoting mTOR inhibition without unleashing feedback loops that oppose its antiproliferative effects, though these effects are likely to depend on cell context and oncogenic mutations.

In conclusion, the elucidation of the feedback loops that regulate the outputs of signaling networks has emerged as an area of fundamental importance for the rationale design of effective anticancer combinations of inhibitors. Here, we reviewed pathways in cancer cells that undergo compensatory overactivation in response to inhibitors that suppress feedback inhibition of upstream signaling. Developing appropriate strategies or regimens that maximize the effect on tumor cells and spare normal cells will be also therapeutically important. This article highlights the importance of discovering signaling feedbacks to anticipate mechanisms of tumor resistance to new drugs.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

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Chapter VI.

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Pancreatic ductal adenocarcinoma is one of the most lethal human diseases, with overall 5-year survival rate of only 5%. As we mentioned in the chapter I, so far therapies that target either elements of the MAPK pathway or PI3K/AKT/mTOR pathways have been disappointing in pancreatic cancer clinical trials. (1) (2) (3, 4) (5) In order to have significant impact in clinical outcomes, a detailed understanding of the signaling mechanisms that promote survival, proliferation and invasiveness and the complex feedback mechanisms that mediate drug resistance are key to the development of novel and effective target-therapy strategies for this devastating disease. This thesis dissertation investigates the different strategies to target the PI3K/AKT/mTOR pathway. Although all the inhibitors tested are capable to inhibit signaling downstream of mTOR as anticipated, we identified a novel negative feedback loop leading to over-activation of ERK by active-site mTOR inhibitors and dual PI3K/mTOR inhibitors. More importantly, this work suggests possible strategies to abrogate the activation of this feedback loop, consequently avoiding one of the mechanisms leading to treatment resistance.

In chapter II, we demonstrate that treatment of PANC-1 or MiaPaCa-2 pancreatic cancer cells with either rapamycin (an allosteric inhibitor of mTOR, part of the first generation of mTOR inhibitors) or active-site mTOR inhibitors (second generation of mTOR inhibitors) suppressed S6K and S6 phosphorylation induced by insulin and the GPCR agonist neurotensin. Rapamycin caused a striking increase in Akt phosphorylation at Ser^{473} while the active-site inhibitors of mTOR (KU63794 and PP242) completely abrogated Akt phosphorylation at this site. Conversely, active-site inhibitors of mTOR caused a marked increase in ERK activation whereas rapamycin did not have any stimulatory effect on ERK

activation. The results imply that first and second generation of mTOR inhibitors promote over-activation of different pro-oncogenic pathways in PDAC cells, suggesting that suppression of feedback loops should be a major consideration in the use of these inhibitors for pancreatic adenocarcinoma therapy. In contrast, metformin, which inhibit mTOR activity differently (see chapter II and IV), abolished mTORC1 activation without over-stimulating Akt phosphorylation on Ser^{473} and prevented mitogen-stimulated ERK activation in PDAC cells. Metformin induced a more pronounced inhibition of proliferation than either KU63794 or rapamycin while, the active-site mTOR inhibitor was more effective than rapamycin. Thus, the effects of metformin on Akt and ERK activation are strikingly different from allosteric or active-site mTOR inhibitors in PDAC cells, though all these agents potently inhibited the mTORC1/S6K axis. In this chapter, we verified that a class I PI3K inhibitor (called A66) was unable to abrogate ERK over-activation. We therefore suggest that the over-activation of ERK by active-site mTOR inhibitors was independent of PI3K kinase. This finding was of special interest, as in other tumor types, over-activation of ERK with the use of mTOR inhibitors was linked to PI3K activity. (6)

In chapter III, we studied the third generation of inhibitors, so called dual PI3K and mTOR kinase inhibitors. Their dual activity is based on the structural similarities of the catalytic domain of mTOR and the p110 subunit of PI3K, providing the potential advantage of targeting the pathway at two levels (suppressing mTOR in both the mTORC1 and mTORC2 complexes, and PI3K). (7) Although these inhibitors are well suited to prevent activation of PI3K/AKT caused by suppression of mTORC1/S6K, much less is known about negative feedback loops impinging on other pro-oncogenic pathways (e.g. MEK/ERK) and/or concerning mTORC2 instead mTORC1. In this chapter, we showed

that the dual PI3K/mTOR inhibitors, including NVP-BEZ235, PKI-587 and GDC-0980 also induce MEK/ERK pathway over-activation in human pancreatic adenocarcinoma cell lines.

To confirm that this phenomenon was independent of PI3K activity we performed mechanistic studies assessing PI3K activity in single cells. Such studies verified that dual PI3K/mTOR inhibitors act through a PI3K-independent pathway. Doses of dual PI3K/mTOR inhibitors that enhanced MEK/ERK activation coincided with those that inhibited mTORC2-mediated AKT phosphorylation on Ser^{473}, suggesting a role of mTORC2. To investigate further, we performed knockdown of Rictor (a key component of the mTORC2 complex) via transfection of siRNA and detected a marked attenuation of the enhancing effect of NVP-BEZ235 on ERK phosphorylation. We propose that dual PI3K/mTOR inhibitors suppress a novel negative feedback loop mediated by mTORC2 thereby leading to enhancement of MEK/ERK pathway activity in pancreatic cancer cells. We also demonstrated that MEK inhibitors, such as U126 or PD0325901, prevented ERK over-activation induced by dual PI3K/mTOR inhibitors. Additionally, the combination of NVP-BEZ235 and PD0325901 caused a more pronounced inhibition of cell growth than that produced by each inhibitor individually. These results suggest that combinatorial therapy might be a more effective approach to treat this disease.

In chapter IV, we explored alternative strategies for targeting mTOR. We studied berberine (a phytochemical extracted from a variety of medicinal plants) in comparison to metformin as both compounds have been described to have anti-diabetic (8, 9) and anti-cancer effects. (10-12) We demonstrated that berberine inhibited mTORC1 activity in PDAC cells, as shown by monitoring the phosphorylated state of S6K at Thr^{389} and the phosphorylation of

S6. Furthermore, berberine also inhibited ERK activation in PDAC cells. The inhibitory effects of berberine on mTORC1 and ERK were elicited at doses that hampered mitochondrial function, reduced intracellular levels of ATP and activated AMPK within intact pancreatic adenocarcinoma cells. Furthermore, berberine dose-dependently inhibited mTORC1 (phosphorylation of S6K at Thr^{389} and S6 at $Ser^{240/244}$) and ERK activation in PDAC cells stimulated by insulin and neurotensin or fetal bovine serum. Knockdown of α_1 and α_2 catalytic subunit expression of AMPK reversed the inhibitory effect produced by treatment with low concentrations of berberine on mTORC1, ERK and DNA synthesis in PDAC cells. However, at higher concentrations, berberine inhibited mitogenic signaling (mTORC1 and ERK) and DNA synthesis through an AMPK-independent mechanism. Similar results were obtained with metformin used at doses that induced either modest or pronounced reductions in intracellular ATP levels, which were virtually identical to the decreases in ATP levels obtained in response to berberine. Therefore in this chapter, we propose that berberine and metformin inhibit mitogenic signaling in pancreatic cancer cells through dose-dependent AMPK-dependent and independent pathways.

In chapter V, we reviewed the pathways that undergo compensatory over-activation in response to PI3K/AKT/mTOR or MAPK pathway inhibition and underscore the importance of unintended pathway activation in the development of drug resistance to clinically relevant inhibitors of such pathways. This chapter highlights the importance of discovering signaling feedbacks to anticipate mechanisms of tumor resistance to new drugs and gives insights of how investigators could develop strategies that can overcome treatment resistance. The majority of our work has been done investigating short courses of inhibitors exposure to cell lines. Chronic effects of mTOR inhibition have been described by others. (13-18) The

distinction between short-term and long-term consequences in response to inhibitors are complemental for defining strategies to overcome drug resistance, including the dosing schedule of the drug and the pathways that should be co-targeted for optimal response.

Future directions:

The characterization and kinetics of ERK over-activation following the use of active-site mTOR or dual PI3K/mTOR inhibitors, as well the mechanistic understanding of such phenomena, could translate to patients in several aspects: a) guide the development of drug combinations for patients with pancreatic cancer; b) guide timing of treatment e.g.: is sequential or intermittent treatment better than chronic treatment with drug combinations; c) guide development of new drugs based on novel feedback loops/targets identified here.

Our work investigated the effects of targeting mTOR in pancreatic cell lines. Logically, one of the next steps would be assess the effects of such inhibitors *in vivo*. Studies in the literature demonstrated that chronic mTOR inhibition will lead to *in vivo* ERK activation, (19, 20) however, characterization of acute versus chronic effects of mTOR inhibition *in vivo* is little understood, particularly in pancreatic cancer. Therefore, we could characterize the acute and chronic effects of mTOR pathway inhibition using different generations of inhibitors, including rapamycin, NVP-BEZ235 and GDC-0980, using xenograft nude mouse model. (21) Additionally, would be of interest to assess the effects of drug withdrawal after chronic exposure. Naturally, studying combination of these inhibitors in combination with MEK inhibitors would of interest. Additionally, based on our data showing that metformin and berberine cause inhibition of ERK in addition to mTORC1 inhibition, we suggest to study the combination of active-site or dual PI3K/mTOR inhibitors and such drugs both in vitro and in vivo models.

Our work is extremely relevant clinically. In fact, there are several trials currently studying dual PI3K/mTOR inhibitors in human subjects, including in patients with pancreatic cancer. It would be of significant value if one could assess the status of activation of the AKT, mTOR and ERK by assessing the phosphorylation status of these proteins tissue biopsies of patients by immunohistochemistry pre and post inhibitors treatment. So far, it appears that only pre inhibitors tissue samples from patients that have participated on related trial have been collected for future analysis. Although they would have value as predictors of treatment response, the examination of post treatment samples would help to understand treatment failure and mechanisms of drug resistance.

Mechanistically, we were the first to describe that ERK over-activation with the use of dual PI3K/mTOR inhibitors is linked to mTORC2. Although the mechanism by which that occurs is not understood, we hypothesize that mTORC2 could repress ERK over-activation through two mechanisms: "directly"; by protein-protein interaction that inhibit RAS activity or "indirectly", by modulation of downstream substrates. To explore this hypothesis, we could assess in detail if AKT and/or PKCα, downstream substrates of mTORC2, (22, 23) are responsible for this negative feedback regulation. If AKT is responsible for repressing ERK over-activation, we would anticipate that the use of AKT inhibitors will unleash ERK over-activation. We also can argue that mTORC2-mediated phosphorylation of PKCα, required for its activity, inhibits EGFR by phosphorylation of EGFR at Thr 654 (24) (25) and that this inhibition contributes to feedback loop of EGFR-mediated Ras/MEK/ERK activation. Therefore, we could expose cells to dual PI3K/mTOR and PKCα inhibitors (including Ro 32-0432) and determine whether ERK over-activation in response to dual PI3K/mTOR inibition is abrogated. Alternatively, we could investigate whether the dual PI3K/mTOR

inhibitors affect the protein-to-protein interaction in the mTORC2 complex, composed by the key elements mTOR, Rictor and Sin1. Sin1 is known to contain a Raf-like Ras-binding domain (RBD) and a pleckstrin homology (PH) domain, both considered functional in the human Sin1 protein. (26) In other cell lines Sin1 co-localizes with KRAS at the cell membrane and inhibits its activity. (26) Thus, the disruption of the complex and consequently the Sin1-RAS interaction could unleash the MAPK pathway downstream. Consequently we could first assess if dual PI3K/mTOR inhibitors affect the integrity of the mTORC2 complex by preventing mTOR to bind to Rictor and/or Sin1. We could also test the complex formation between mTOR and Rictor and Sin1 by co-immunoprecipitation in the presence and absence of the PI3K/mTOR inhibitors. If the inhibitors indeed disrupt the mTORC2 complex, we could evaluate if Sin1 and Ras interact (e.g. by co-immunoprecipitation and co-localization) and assess the impact of PI3K/mTOR inhibition on Ras-GTP levels and RAF-kinase activity (27). Understanding the mechanism by which mTORC2 leads to ERK over-activation has significant translational importance as can lead to the development of more specific inhibitors.

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