The Synergistic Effect of an ATP-Competitive Inhibitor of mTOR and Metformin on Pancreatic Tumor Growth

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ABSTRACT

Background: The mechanistic target of rapamycin complex 1 (mTORC1) is a nutrient-sensing pathway and a key regulator of amino acid and glucose metabolism. Dysregulation of the mTOR pathways is implicated in the pathogenesis of metabolic syndrome, obesity, type 2 diabetes, and pancreatic cancer. Objectives: We investigated the impact of inhibition of mTORC1/mTORC2 and synergism with metformin on pancreatic tumor growth and metabolomics. Methods: Cell lines derived from pancreatic tumors of the KPC (KrasG12D/+; p53R172H/+; Pdx1-Cre) transgenic mice model were implanted into the pancreas of C57BL/6 albino mice (n = 10/group). Two weeks later, the mice were injected intraperitoneally with daily doses of 1) Torin 2 (mTORC1/mTORC2 inhibitor) at a high concentration (TH), 2) Torin 2 at a low concentration (TL), 3) metformin at a low concentration (ML), 4) a combination of Torin 2 and metformin at low concentrations (TLML), or 5) DMSO vehicle (control) for 12 d. Tissues and blood samples were collected for targeted xenometabolomics analysis, drug concentration, and cell signaling. Results: Metabolomic analysis of the control and treated plasma samples showed differential metabolite profiles. Phenylalanine was significantly elevated in the TLML group compared with the control (+426%, P = 0.0004), whereas uracil was significantly lower (–38%, P = 0.009). The combination treatment reduced tumor growth in the orthotopic mouse model. TLML significantly decreased pancreatic tumor volume (498 ± 104 mm³; 37%, P < 0.0004) compared with control (1326 ± 134 mm³; 100%), ML (853 ± 67 mm³; 64%), TL (745 ± 167 mm³; 54%), and TH (665 ± 182 mm³; 50%) (ANOVA and post hoc tests). TLML significantly decreased tumor weights (0.66 ± 0.08 g; 52%) compared with the control (1.28 ± 0.19 g; 100%) (P < 0.002). Conclusions: The combination of mTOR dual inhibition by Torin 2 and metformin is associated with an altered metabolomic profile and a significant reduction in pancreatic tumor burden compared with single-agent therapy, and it is better tolerated. Curr Dev Nutr 2020;4:nzaa131.

Keywords: metabolomics, phenylalanine, KPC mouse model, pancreatic cancer, glycolysis, tricarboxylic acid cycle, xenometabolomics, antidiabetic drug, metformin

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Abbreviations: AMPK, AMP-activated protein kinase; ECL, enhanced chemiluminescence; IACUC, Institutional Animal Care and Use Committee; IS, internal standard; KPC, KrasG12D/+; p3R172H/+; Pdx1-Cre; LSIL, Low-Stop-Low; ML, metformin low concentration; mTOR, mechanistic target of rapamycin; mTORC, mechanistic target of rapamycin complex; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; TBST, Tris-buffered saline with Tween 20; TCA, tricarboxylic acid cycle; TH, Torin 2 high concentration; TL, Torin 2 low concentration; TLML, Torin low, metformin low.

Introduction

The mechanistic target of rapamycin (mTOR) is a protein kinase that tightly controls nutrient metabolism and cell proliferation. mTOR is a highly conserved, 289-kDa serine/threonine kinase and a master regulator of cell growth and energy metabolism (1–3). mTOR integrates inputs from nutrients, energy levels, and growth signals and coordinates anabolic cell growth, nutrient metabolism, and inhibits catabolic autophagy (4). As such, mTOR regulates protein, lipid, and nucleotide synthesis, and it inhibits catabolism in normal cell and cancer metabolism.

Pancreatic cancer is the third leading cause of cancer-related death in the United States. This outcome is attributable to its late diagnosis...
and lack of response to current therapies. Furthermore, recent studies have linked both type 2 diabetes and new-onset diabetes to pancreatic cancer as either risk factors or symptoms. Alarmingly, the 5-y survival rate of pancreatic cancer patients was a dismal 8.5% in the United States in 2019, with an incidence (56,770 cases/y) nearly equal to the mortality (45,750 cases/y) (5). Thus, there is an urgent need to develop new strategies for diagnosis and treatment. Rapamycin, a prototype allosteric mTOR inhibitor, acutely inhibits mTOR complex 1 (mTORC1). Chronic administration of rapamycin may inhibit mTORC2 in some cell types (2). However, ATP-competitive mTOR kinase inhibitors block both mTORC1 and mTORC2 complexes. Although the mTOR inhibitors have pharmacological applications, the fundamental value of mTOR chemical knockdown is gaining insight into the mechanistic regulation of nutrient anabolism and catabolism. We hacked the mTOR pathways with drugs to determine their functionality, monitor the inner working of the mTORC1/mTORC2 signaling network, and advance the disease therapeutics. Several lines of evidence have revealed that the mTOR network regulates energy and nutrient sensing, cell growth and proliferation, and cancer metabolism via both transcriptional mechanisms and posttranslational modifications (1, 6–10).

mTOR is a druggable protein that is dysregulated in a multitude of chronic diseases, including type 2 diabetes and pancreatic cancer, and therefore is a promising target for diabetes and pancreatic cancer interventions. mTOR has also been identified as a driver of stem cell growth and pancreatic progenitor cell differentiation (11–13).

In addition, the antidiabetic drug metformin inhibits mTORC1 indirectly by activating AMP-activated protein kinase (AMPK) (14) and has emerged as a potential therapeutic target for the treatment of several types of cancer, including pancreatic ductal adenocarcinoma (PDAC) (15–18). Thus, mTOR is an attractive actionable gene due to its central role in cellular metabolism, pancreatic progenitor cell growth, and tumor metabolism. By nucleating 2 functionally distinct complexes, namely mTORC1 and mTORC2, mTOR plays a central role in pancreatic progenitor cell growth and proliferation and tumor metabolism.

The tumor microenvironment is not a static entity but, rather, functions as a sophisticated network communicating between tumor cells, stroma, immune cells, soluble cytokines, proteases, and components of the extracellular matrix. These complex interactions are critical drivers of tumor growth advantage, differentiation of multipotent stem cells, cancer metabolism and progression, and acquired treatment resistance (19). Mounting evidence has linked glycolytic metabolic disturbances to adverse pancreatic cancer prognosis. The tumor microenvironment can influence the tumor immune-editing process (20) and tumor heterogeneity (21) and thus may lead to therapeutic resistance (22). In this context, PDAC is considered one of the most stroma-rich tumors. Therefore, factors that influence the tumor microenvironment are attractive targets for pancreatic cancer early detection, control, and intervention (23, 24). Moreover, it has been reported that the tumor microenvironment mediates mTOR-induced tumor resistance to therapy (22).

In this study, we surgically implanted pancreatic cancer cells derived from pancreatic ductal adenocarcinoma of genetically engineered triple-mutant [KrasG12D/+; Pdx1-Cre (KPC)] transgenic mice in the pancreas of C57BL/6 albino mice. This orthotopic mouse model provides a physiologically relevant model that emulates human PDAC in the milieu of an intact tumor microenvironment in vivo. This model allowed us to, directly and indirectly, investigate the role of mTOR signaling and the impact of mTOR inhibition on cellular transformation during the progression from premalignancy to PDAC. In addition, because single-agent regimens do not work in pancreatic cancer (23), this study addressed the possible synergistic effects of mTORC1/mTORC2 inhibition by Torin 2 and interactions with metformin. We hypothesized that a combination of an mTOR inhibitor and pharmacological AMPK activator might act synergistically on different targets to overcome cancer cell heterogeneity, thereby reducing drug resistance, which is a significant obstacle in pancreatic cancer therapy. Thus, in this study, we evaluated the synergistic effects of mTOR inhibition and metformin activity on pancreatic cancer tumor size and volume and the associated metabolomics changes.

**Methods**

**Reagents**

Torin 2 (9-(6-aminopyridine-3-yl)-1-(3-trifluromethyl)-phenyl benzoz[h]-1,6)naphthryridin-2 (H) (catalog no. 4248) was obtained from Tocris Bioscience (R & D Systems). Metformin hydrochloride (N,N-dimethylyimidodicarbonimidic diamide hydrochloride) (Tocris; catalog no. 2864) and rapamycin were obtained from Cell Signaling (catalog no. 9904). Other chemicals were obtained from either Sigma or Fisher Scientific. Immobilon-P polyvinylidene difluoride membrane (0.45 µm) and the reagents for enhanced chemiluminescence (ECL) were obtained from Millipore (Immobilon Western chemiluminescent horseradish peroxidase), as we previously reported (25). HPLC-grade methanol, acetonitrile, ammonium acetate, acetic acid, propylene glycol, and PBS (1×) were obtained from Fisher Scientific, as previously described (26). Isoflurane was obtained from Halocarbon Product Corporation.

**Antibodies**

Antibodies against the following proteins were purchased from Cell Signaling: total mTOR (catalog no. 2983), serine P-2481 mTOR (catalog no. 2976), S6 (catalog no. 2217), serine P-473 Akt (catalog no. 4691), Akt (catalog no. 473), total p-Akt (catalog no. 9272), and total phospho-p-Akt (catalog no. 2920). Other chemicals were obtained from either Sigma or Fisher Scientific. HRP-labeled secondary antibodies were obtained from GE Healthcare Life Sciences.

**Orthotopic mouse model**

We used an orthotopic mouse model of pancreatic cancer, which allowed for tumor growth at the natural organ site of the primary tumor and within an intact tumor microenvironment. Pancreatic tumors were harvested after 25 wk under sterile conditions and cultured in DMEM supplemented with 10% FBS, penicillin-streptomycin, and antimitotics. The tumors were cut into small pieces, washed in PBS, and placed in 10-cm dishes in DMEM. Once cells reached 80% confluence, tumor pieces were removed, and cells were trypsinized and seeded at very low density to minimize fibroblast contamination. KPC cell lines were confirmed with sequencing following PCR amplification. The KPC cell lines were characterized and their tumorigenic potential was tested after 40 passages, as previously described (27, 28).
We surgically implanted pancreatic cancer cell lines derived from pancreatic ductal adenocarcinoma of triple-mutant (KPC) genetically engineered mice in the gastric lobe of the pancreas of C57BL/6 albino mice. The abdomen of the mice was opened by a 1-cm incision in the upper medial abdomen. The transplanted KPC adenocarcinoma cell lines (5 × 10^5 cells) in 30 μL PBS were injected in the gastric lobe of the pancreas. The abdomen was closed by using a 2-layer suture with 5–0 Vicryl absorbable suture material and Ethilon sutures. Tumor growth was monitored by palpitation by calipers. After 2 wk, the pancreatic tumors were palpable. At that time, the albino mice were injected intraperitoneally daily with DMSO, Torin 2 (mTORC1/mTORC2 inhibitor) at a low concentration (5 mg/kg) or a high concentration (25 mg/kg), metformin (10 mg/kg), or a combination of Torin 2 (5 mg/kg) and metformin (10 mg/kg). Mice were sacrificed, and pancreatic tumors were dissected, measured, and weighed. Tissues were harvested, and blood samples were collected to conduct the metabolomics, drug tissue distribution, and cell signaling studies. The Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center approved the protocol for the orthotopic mouse model (no. 15-101-12) and the animal protocol for breeding colonies (no. 13-011-04EP). The procedures followed the preapproval protocols, and the number of animals used and euthanized conformed to the numbers approved by IACUC. For this study, the power calculations were as follows: the sample size of n = 50 mice (10/group × 5 groups) achieves 80% power to detect a moderate effect size of 0.3 using 1-way ANOVA with a significance level of 0.05. In addition, 10% extra animals were added to account for surgical error and inevitable losses (n = 10).

Pancreatic tissue lysis and immunoblotting
Pancreatic tissues were weighed, snap-frozen in liquid nitrogen, and stored at −80°C until the time of analysis. The details of the immunoblot analysis have been reported previously (25, 29, 30). Briefly, the tissues were washed twice with ice-cold PBS (pH 7.4) and collected in ice-cold lysis Buffer A containing 10 mM KPO4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate (Na3VO4), 5 μg/mL peptatin A, 10 μg/mL leupeptin, and 40 μg/mL PMSF. Cells were lysed in the presence of the detergent Nonidet P-40. Cell lysates were centrifuged at 13,200 × g for 5 min at 4°C, and the supernatants were collected. Protein concentration was measured with a Bradford assay to normalize the concentration for immunoblotting, as we described previously (25, 29, 30). Briefly, samples were heated at 95°C for 5 min and electrophoresed in SDS-PAGE gels to resolve protein bands according to the molecular weight. The protein bands were transferred to polyvinylidene difluoride membranes in Tobin buffer (24 mM Tris, 192 mM glycine, 10% methanol, and 0.02% SDS). Western blotting was performed by blocking the membranes in Tris-buffered saline with Tween 20 [TBST; 40 mM Tris HCl (pH 7.5), 0.9% NaCl, and 0.1% Tween 20] containing 3% nonfat milk. The membranes were then incubated in TBST with 2% BSA containing either the primary or the secondary horseradish peroxidase-conjugated antibodies. The blots were developed via ECL.

Targeted metabolomics analysis using LC-MS/MS
Metabolites from frozen serum were extracted using 1.5 mL chilled methanol:chloroform:water at a ratio of 8:1.1, and 13C-labeled glycolysis and tricarboxylic acid (TCA) cycle standards were utilized. Following extraction, the samples were sonicated on ice (20% duty cycle and 20% maximum power for 20 s). Briefly, polar metabolites were extracted and analyzed using LC-MS/MS, as described previously (31). The selected reaction monitoring LC-MS/MS method was employed using positive/negative ion polarity switching on a Xevo Tandem Quadrupole-TQ-S mass spectrometer (32). The peak chromatographic areas were integrated using MassLynx 4.1 software (Waters) and normalized to the corresponding tissue protein concentration. The resultant peaks were identified and quantified using MetaboAnalyst 3 (http://www.metaboanalyst.ca) (33).

Quantification and distribution of Torin 2 and metformin in plasma and tissues
Mouse tissues were snap-frozen in liquid nitrogen and stored at −80°C until the time of analysis. Plasma samples were separated from RBCs via centrifugation at 15,000 × g for 5 min at 4°C within 1 h of sample collection and stored at −80°C until analysis. Torin 2 and metformin quantification was performed using LC-MS/MS. For plasma samples, 50 μL of plasma was mixed with 1 mL ice-cold acetone and spiked with 10 μL of internal standard (IS; maraviroc at a final concentration of 25 ng/mL for Torin 2 and indinavir at a final concentration of 50 ng/mL for metformin). Samples were then vortexed for 3 min and centrifuged at 17,000 × g for 10 min at 4°C. Supernatants were dried, reconstituted in 100 μL 50% methanol in H2O, and injected for LC-MS/MS analysis. For tissue samples, ~100 mg of tissues was homogenized in 4 volumes of 90% methanol in H2O. The homogenates (100 μL) were mixed with 90 μL methanol containing IS and 10 μL of 50% methanol in H2O containing no IS. Samples were then vortexed for 3 min and centrifuged at 17,000 × g for 10 min. Then, 100 μL of supernatants was collected and mixed with 50 μL H2O before LC-MS/MS analysis. Plasma and tissue standards were prepared at a final concentration of 0.5–1000 ng/mL.

For LC-MS/MS analysis, 10 μL of the samples prepared above was injected onto an LC-MS/MS Waters Acquity UPLC system coupled to an Applied Biosystems 4000 Q TRAP quadrupole linear ion trap hybrid mass spectrometer (Applied Biosystems, MDS Sciei) (26, 34). Chromatographic separation was performed using a phenyl column for Torin 2 and HILIC column for metformin (2.1 × 100 mm, 1.7 μm; Waters). Mobile phase A consisted of 7.5 mM ammonium bicarbonate (pH 7), and mobile phase B was methanol. The flow rate was 0.25 mL/min. For Torin 2 separation, the initial mobile phase composition was 60% B for the first 4.75 min, increased to 95% B over 0.5 min, and then held constant for 1.05 min. Mobile phase B was then reset to 60% over 0.1 min, and the column was equilibrated for 1 min before the next injection. For metformin separation, the initial mobile phase composition was 50% B for the first 4.25 min, increased to 95% B over 0.25 min, and then held constant for 1 min. Mobile phase B was then reset to 50% over 0.25 min, and the column was equilibrated for 1 min before the next injection. The mass spectrometer was operated in the positive ion mode using multiple reaction monitoring. The following transitions were monitored: m/z 433.2 → 373.2 for Torin 2, m/z 130 → 71.1 for metformin, m/z 514.2 → 280 for the IS maraviroc, and m/z 614.4 → 421.2 for the IS indinavir (26, 34).

Data processing and statistical analysis
Metabolites were identified based on retention time, which is based on the solubility of the analyte in the stationary phase and m/z spectrum.
match to injections of known standards. The data are expressed as the mean ± SE. ANOVA was performed using SPSS software (IBM) to determine whether the treatment groups differed with respect to a given outcome compared with the control group. If the overall ANOVA was significant, then a secondary post hoc statistical test was employed to identify the pairs of groups that differed. Statistical significance was assigned as P < 0.05 if an overall statistical significance was detected between groups.

Results

**mTORC1/mTORC2 inhibitor (Torin 2) and metformin exhibit a combinatorial effect on pancreatic tumor growth**

After 12 d of daily metformin and Torin 2 injections, Torin 2 at low and high concentrations led to a significant decrease in pancreatic tumor weight and volume, and a combination of the drug with metformin augmented these effects (Figure 1, Table 1). However, a high dose of Torin 2 was associated with lower survival compared with the control, a low dose of Torin 2, and the combination of Torin 2 and metformin. Only 3 of 10 mice/group tolerated the high dose of Torin 2 compared with 8 mice on the TLML treatment, 6 mice on metformin, 5 mice on TL, and the control group (18).

The TLML combination significantly decreased tumor volume (498 ± 104 mm³; 37%; P < 0.0004) compared with the control group (1326 ± 134; 100%); ML (853 ± 67; 64%), TL (745 ± 167; 54%) and TH (665 ± 182; 50%) (ANOVA and post hoc protected t tests). However, a high concentration of Torin 2 was toxic to the animals (only 3 of 10 mice survived). In addition, TLML significantly decreased (0.66 ± 0.08 g; 52%) tumor weights compared with the control (1.28 ± 0.19; 100%) (P < 0.002). Torin 2 synergistically acted with metformin and led to a significant decrease in pancreatic cancer tumor size and volume (Figure 1, Table 1) compared with either agent alone. All treatment groups were significantly different from the DMSO control.

**mTORC1/mTORC2 inhibition modulates cell signaling**

Torin 2 (TH and TL) and the combination of Torin 2 and metformin (TLML) inhibited mTORC1 signaling based on analysis of the phosphorylation of the mTORC1 downstream target ribosomal protein S6. In contrast, metformin (ML) alone did not affect the phosphorylation state (Figure 2). In addition, Torin 2 inhibited mTORC2, as shown by the level of Akt phosphorylation at serine 473, which is selectively phosphorylated and activated by mTORC2 (Figure 2). mTORC2 also activates other members of the AGC kinase family, such as glucocorticoid inducible kinase 1 and protein kinase C. We also quantified the concentrations of Torin 2 in blood and tissue by LC-MS/MS.

**Dual mTORC1/mTORC2 inhibition decreases concentrations of glycolysis and TCA cycle intermediates**

A heat map of the metabolites was constructed based on the relative metabolite analysis (Figure 3). Metabolomics analysis of the control and treated plasma samples (Torin 2 and metformin) showed significantly different metabolite profiles compared with the control group. The concentrations of both blood glucose and lactate decreased significantly upon treatment with Torin 2 and combined treatment with Torin 2 and metformin (Figure 4). The metabolite concentrations of some glycolysis intermediates, such as 3-phosphoglycerate and the TCA cycle intermediates citrate and α-ketoglutarate, were decreased after treatment with Torin 2 at a high concentration (TH), Torin 2 at a low concentration (TL), metformin (ML), and combined Torin 2 low/metformin low (TLML) compared with the DMSO control group, as shown in Figure 4. Thus, the dual mTOR inhibitor may synergize with metformin to decrease the electron donors NAD⁺ and FAD in the TCA cycle.

**Dual mTORC1/mTORC2 inhibition and amino acid metabolism**

We leveraged the use of the MetaboAnalyst platform and machine learning to link our experimental data to publicly available genomic and
metabolic databases such as the global KEGG and BioCyc metabolic networks. This approach allowed us to transform the raw LC/MS data to a comprehensive and integrative global metabolomic analysis. As such, we were able to identify the metabolic pathways altered by the combination inhibition of mTOR by ATP competitive catalytic inhibitor and metformin. These affected pathways included amino acids, nucleotides, and glucose metabolism (Figure 5A).

Our data from the orthotopic pancreatic cancer mouse model also showed that treatment with Torin 2 (TL and TH) or with a combination of metformin and Torin 2 (TLML) significantly increased phenylalanine concentrations (4-fold) in the plasma of mice orthotopically implanted with pancreatic tumor cells derived from KPC mice (Figure 5). However, tyrosine, which is derived from phenylalanine, was not elevated; on the contrary, its concentration showed a decreasing trend that did not reach statistical significance ($P = 0.07$). This finding suggests that phenylalanine may be metabolized to its byproducts phenylpyruvate and phenylacetate or that it enters the TCA cycle via acetoacetate or fumarate. The finding that phenylalanine is elevated when both mTORC1 and mTORC2 are inhibited is novel in the current study; perturbation of nucleotide metabolism is a new finding as well. In addition, TL and TH increased the threonine concentration by 2-fold ($P = 0.0002$), tryptophan by 1.6-fold ($P = 0.01$), and lysine by 0.25-fold ($P = 0.04$). However, the TLML combination had no effect on these amino acids, whereas ML alone increased the amino acid asparagine.

The mTORC1/mTORC2 inhibitor and metformin combination facilitates tissue availability of the drugs
Torin 2 and metformin concentrations were quantified via LC-MS/MS in several tissues, including muscle, spleen, kidney, liver, lung, and plasma (Figure 6).

As shown in Figure 6, our data indicate that Torin 2 and metformin were differentially distributed in different organs. The muscles retained the highest concentrations of both drugs. The accumulation of Torin 2 in the muscle after treatment with a low concentration (TL) was $360 \pm 104$ ng/g tissue and that with Torin 2 administration at a high concentration (TH) was $197 \pm 112$ ng/g, whereas the combination of Torin 2-low and metformin-low (TLML) treatment significantly elevated Torin 2 accumulation ($2447 \pm 806$ ng/g; $P = 0.001$). Because the tissue concentrations of both drugs in the combination TLML group are much higher than ML or TL alone, these data suggest a synergism between Torin 2 and metformin that is more than an additive effect. The data also indicate that a low concentration of Torin 2 is more effective than a high concentration, possibly due to the toxicity of the higher dose.

Similarly, when metformin was measured in the muscle, low metformin administration led to an accumulation of metformin of $29.9 \pm 18.6$ ng/g, and the combination of Torin 2 and metformin led to an accumulation of $1081 \pm 335$ ng/g. Taken together, these findings suggest a synergistic effect rather than an additive effect of the Torin 2 and metformin combination. A similar pattern was observed in the
FIGURE 3 The heat map and PLS-DA analysis show that the control and the mTORC1/mTORC2-treated mice have distinctive metabolomic profiles. An orthotopic pancreatic cancer mouse model was generated from KPC cells. The mice were injected intraperitoneally daily with Torin 2, metformin, both, or DMSO control for 12 d. Plasma was collected, and targeted metabolomics was performed using LC-MS/MS. Comparison of the control and TLML metabolite distinction between the 2 groups. (A) Comparison of the control and treated orthotopic KPC tumor metabolomes via principal component analysis. (B) Heat map analysis showing differences in the TCA cycle and glycolysis metabolites between the control group and the TLML group. mTORC, mechanistic target of rapamycin complex; PLS-DA, partial least square-discriminant analysis; TCA, tricarboxylic acid; TLML, Torin 2 and metformin at low doses.

spleen. However, the difference did not reach the level of significance due to high variability in the amount of Torin 2 accumulation in the spleen (TLML: 1333.5 ± 936 ng/g).

Discussion

The mTOR pathway is critical for nutrient metabolism. It integrates nutrition status, energy level, and environmental cues to coordinate cellular metabolism and physiological homeostasis. We targeted the mTOR pathway by pharmacological inhibitors to determine the functionality of the mTORC1 and mTORC2 complexes in the disease state. Drugs that inhibit mTOR catalytic functions serve as tools to investigate the molecular mechanism of the protein functions and the metabolic outcome.

As an anabolic kinase, mTORC1 controls energy metabolism and metabolic integration while suppressing the catabolic autophagy. mTORC1 is activated by amino acids, glucose, and fat as a source of energy, and in turn increases protein and nucleotide synthesis, glycolysis, fatty acid synthesis, and esterification. mTORC1 also inhibits the catabolic lysosomal degradation of intracellular components and lysosomal biogenesis, known as autophagy. mTORC2 has been shown to inhibit insulin receptor substrate 1 ubiquitination and thus stabilize insulin receptor substrate and insulin signaling. Rictor, the mTORC2 exclusive partner, was shown to contribute to glucose and lipid homeostasis, as evidenced by conditional tissue-specific deletion of rictor in mouse models (35–37). Also, Sestrin 2 has been shown to regulate glucose metabolism via the mTORC2/Akt pathway (37, 38).

Intriguingly, the mTOR-mediated amino acid sensing in normal cells is also utilized by cancer cells to drive metabolic reprogramming.
FIGURE 4 Dual mTORC1/mTORC2 inhibition decreases concentrations of glycolysis and TCA cycle intermediates: glycolysis and TCA intermediate concentrations in an orthotopic pancreatic cancer mouse model after Torin 2 and metformin treatment. C57BL/6J albino mice were implanted with KPC pancreatic cancer cells and injected intraperitoneally daily with Torin 2 at a high dose, Torin 2 at a low dose, metformin at a low dose, both Torin 2 and metformin at low doses, or DMSO control for 12 d. Mice were killed, plasma was collected, and targeted metabolomics was performed using LC-MS/MS. (A) Glucose, (B) 3-phosphoglycerate, (C) galactose, (D) α-ketoglutarate, (E) lactate, and (F) citrate. ANOVA was performed using SPSS software (IBM) to determine whether the treatment groups differed with respect to a given outcome compared with the control group. If the overall ANOVA was significant, then a post hoc test was employed to identify the pairs of groups that differed. Statistical significance was assigned as \( P < 0.05 \) if an overall statistical difference was detected between groups. The values are means ± SEs (n = 3–7). Means with different letters were significantly different: a < b (\( P < 0.05 \)). ML, metformin at low dose; mTORC, mechanistic target of rapamycin complex; TCA, tricarboxylic acid; TH, Torin 2 at high dose; TL, Torin 2 at low dose; TLML, Torin 2 and metformin at low doses.

It is therefore critical to identify the metabolite readout of mTOR inhibition to provide insight into mTOR regulation of protein synthesis and metabolic integration.

mTORC1 regulates amino acid metabolism via several amino acid sensors. For example, leucine is sensed via Sestrins, GATOR2, and CASTOR sensors (39). Leucine recruits mTORC1 to the lysosomal surface for activation (40). CASTOR also senses arginine via GTP-RagA and GDP-RagC heterodimerization (41, 42). Furthermore, arginine is sensed via SLC38A9 lysosomal transporter, which mediates the efflux of arginine from the lysosome (43). Glutamine signaling is relayed via the Arf-1-rag-independent mechanism and drives the glutaminolysis pathway (44). Methionine is sensed via SAMTOR (S-adenosylmethionine sensor upstream of mTORC1), which is a GATOR1/KICSTOR-interacting protein. As such, S-adenosylmethionine binds to SAMTOR and interrupts SAMTOR–GATOR1 interaction, which is a negative regulator of mTORC1. The knowledge gap regarding amino acid metabolism stems from 2 possibilities. First, it is not known whether the previously mentioned sensors are specific to 1 amino acid or can cross-talk and detect related amino acids. It is known that some amino acids are more potent in mTORC1 activation than others, so it is possible that these amino acid sensors are specific to individual amino acids. Second, it is not known whether amino acid sensing is tissue-specific, whether all amino acids are sensed by mTORC1, or whether there are mTORC1-independent pathways. Here, we report for the first time that phenylalanine (but not tyrosine) concentrations are significantly elevated when mTORC1 and mTORC2 are inhibited in the pancreas, suggesting that concentrations of phenylalanine and its metabolic channeling may be regulated by mTOR. This novel observation warrants further research to understand the basic physiology and biology and to identify the scientific bases for disease management.

Pancreatic cancer is the third leading cause of cancer death in the United States and has a high mortality rate despite current therapies. Thus, there is an urgent public health need to develop new strategies to combat this devastating disease. In this regard, the protein kinase mTOR is an attractive candidate due to its fundamental role in metabolism and its deregulation in pancreatic diseases, including diabetes and pancreatic cancer. We investigated the impact of inhibition of mTOR complexes by Torin 2, an ATP-competitive inhibitor that inhibits both mTORC1 and mTORC2 complexes. We also evaluated possible synergistic effects of Torin 2 and the antidiabetic drug metformin on targeted metabolomics and pancreatic tumor size and volume. We tested the hypothesis that inhibitors of mTOR complexes will
synergize with AMPK activators to augment tumor responsiveness to treatments.

In this study, we orthotopically implanted pancreatic cancer cells derived from triple-mutant KPC mice. The KPC genetically engineered mouse is a model of PDAC, which undergoes spontaneous pancreatic cancer transformation. This unique KPC model activates a Kras knock-in allele (KrasG12D) and inactivates the tumor suppressor p53R172H/+, to conditionally target the pancreas via PDX1, which is required for pancreatic islet β-cell development, using the Cre–LoxP system. This Kras single point mutation is sufficient to initiate ductal premalignant
pancreatic intraepithelial neoplasia (PanIN) transformation (24). The subsequent loss of p53 [Lox–Stop–Lox (LSL)–p53<sup>fl/fl</sup> mice] aids Kras in promoting tumor invasion in a time- and tissue-specific manner (45). The presence of the Cre–recombinase allows for the deletion of the LSL transcripation termination sequence and thereby enables the expression of the oncogenic protein Kras. Thus, this KPC mouse model faithfully mimics pancreatic cancer in humans in terms of progression from intraepithelial neoplasia (premalignant) to malignant adenocarcinoma.

These mice develop PanIN at 10 wk, progress to PanIN grade II and III by 25 wk, and advance to PDAC at 50 wk (46). Thus, the KPC transgenic mouse genetic model recapitulates the progression of PanIN to pancreatic cancer in humans.

Given that mTORC1/mTORC2 inhibitors were injected daily for 12 d, the significant changes in tumor weight and volume cannot be entirely explained by transient posttranslational mTOR kinase-mediated phosphorylation of downstream targets (Figure 2). Thus, these findings suggest additional long-term mTOR functions via the transcriptional and epigenetic machinery. Indeed, a possible role of mTOR in transcriptional regulation has been proposed (8, 47). Rohde and Cardenas (48) reported that in yeast, TOR regulates gene expression through acetylation in response to nutrients.

Taken together, our data obtained in an orthotopic pancreatic cancer mouse model indicate that mTOR inhibition has a favorable impact on pancreatic cancer outcome, evidenced by decreased pancreatic tumor weight (62% reduction in TLML compared with control) and tumor volume (49% reduction in TLML compared with control) (Figure 1) and the associated changes in glucose and energy metabolism (Figure 4). In humans, researchers conducted an untargeted metabolomics analysis in serum from patients and found 9 metabolites that might differentiate between PDAC and control (49, 50), including ceramide and phospholipids. Other investigators employed an NMR approach to identify a metabolite signature of pancreatic cancer (51). They reported elevated concentrations of triglycerides, leucine, isoleucine, and creatinine in patients with pancreatic cancer compared with the control group. However, these studies did not address the mechanistic underpinning of these observations. We reasoned that our results of changes in targeted metabolomics that were altered by mTOR inhibition could explain such findings. mTOR signaling could be an essential contributor to the development of pancreatic cancer. Thus, changes in mTOR signaling could serve as an early diagnostic biomarker and a therapeutic target.

Although insulin resistance, hyperinsulinemia, increased BMI, and type 2 diabetes are risk factors for pancreatic cancer and are linked to aberrant PI3K-Akt-mTOR signaling, whether they are causal targets of intervention remains unclear because it is difficult to distinguish cause and effect in observational studies. A recent Mendelian randomization study using genetic variants as an unconfounded proxy for exposure failed to show a causal association between type 2 diabetes and the development of pancreatic cancer (52)—a finding that refutes the results of observational studies (53). Instead, pancreatic cancer may lead to the development of type 2 diabetes, generating a reverse causality.

Metformin is a biguanide drug that is considered the first-line of treatment in type 2 diabetes, and it has been employed as an antineoplastic agent (54, 55). Metformin functions via several mechanisms. First, it inhibits complex 1 of the respiratory chain in the mitochondria, leading to uncoupling of oxidative phosphorylation. Second, it increases reactive oxygen species concentrations and reduces mitochondrial transmembrane potential, thereby hampering the self-renewal capacity of cancer stem cells (56, 57). Third, in diabetes-associated cancer, metformin decreases hyperlipidemia and hyperinsulinemia (58). Epidemiological studies have shown that treatment with metformin is associated with decreased cancer risk and/or improved survival. As a member of the biguanide family, metformin activates AMPK and decreases gluconeogenesis (59, 60). Metformin, which also indirectly inhibits mTOR via activation of AMPK as well as AMPK-independent mechanisms,
has emerged as a potential therapeutic agent for the treatment of pancreatic cancer and diabetes-associated cancer. Intriguingly, studies conducted in xenograft models revealed that metformin decreases pancreatic cancer growth in a dose-dependent manner (61, 62). The impact of metformin on mTORC1 inhibition indirectly via AMPK activation is notably different from that of the rapamycin binding domain or the catalytic active-sites mTOR inhibitors (62). This observation suggests that metformin may augment the effects of mTOR inhibition by an additional mechanism. In our study, we found that metformin reduced tumor volume to a lesser extent than Torin 2 and that a combination of metformin and Torin 2 at low doses led to a synergistic reduction in tumor weight and volume (Figure 1).

The metabolomics data revealed that some glycolysis and TCA cycle intermediates were decreased with administration of Torin 2 at a high concentration (TH), Torin 2 at a low concentration (TL), and combined Torin 2 low/metformin low (TLMN) treatment compared with the DMSO control group, as shown in Figure 4. These findings suggest decreased ATP production via the mitochondrial TCA cycle. We also noted that the lactate concentration was reduced in all treatment groups, indicating decreased ATP production via aerobic glycolysis (Warburg effect). Our results also showed that Torin 2, together with metformin, significantly altered phenylalanine, lysine, glucose (hexose), and nucleotide (pyrimidine) metabolism (Figure 5). It is possible that this metabolomic profile can be utilized for early detection, diagnosis, monitoring, and targeted therapy. As such, metabolomic profiling, which provides a snapshot of metabolic alterations, can be utilized as a metabolic laboratory test to improve clinical outcomes.

Our data suggest a synergistic interaction between the highly conserved mTOR pathway and the well-known antidiabetes drug metformin in pancreatic cancer. We found that muscles isolated from the orthotopic pancreatic cancer model mice in the combined TLMN group accumulated Torin 2 and metformin (6-fold for Torin 2 and 36-fold for metformin) compared with accumulated concentrations in the TL or ML mice (Figure 6). Similarly, Wang and colleagues (63) reported that metformin acted synergistically with everolimus (a rapamycin analog) in inhibition of breast cancer growth and abrogation of S6 phosphorylation. In agreement with our data, other investigators reported that rapamycin synergized with metformin to inhibit pancreatic cancer growth in SW1990 pancreatic cancer cell lines and a xenograft mouse model of pancreatic cancer (16). Intriguingly, metformin, which activates the AMPK pathway, indirectly inhibits mTOR signaling and has been investigated as an antitumor agent (64).

In conclusion, our data indicate that mTORC1/mTORC2 inhibition by Torin 2 reduced glycolytic intermediate and TCA metabolite pools and increased phenylalanine concentrations in mice. mTOR inhibition by ATP competitive catalytic inhibitors also altered amino acid and nucleotide metabolism. This dual mTOR inhibitor may synergize with metformin to decrease the electron donors NAD+ and FAD in the TCA cycle, which may lead to reduced energy production. The combination of dual mTOR inhibition by Torin 2 and metformin treatment was associated with a significant reduction in pancreatic tumor size and weight, altered concentrations of glycolysis and TCA cycle intermediates compared with single-agent treatment, and was better tolerated in mice. Metabolomics profiling can be employed for risk assessment, early detection, monitoring, as well as personalized targeted therapy to improve clinical outcomes. The findings suggest that a combination of drugs may act synergistically to provide better tumor reduction and might be a better treatment approach than single-agent therapies.

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