Targeting of NAD Metabolism in Pancreatic Cancer Cells: Potential Novel Therapy for Pancreatic Tumors

Claudia C.S. Chini, Anatilde M. Gonzalez Guerrico, Veronica Nin, Juliana Camacho-Pereira, Carlos Escande, Maria Thereza Barbosa, and Eduardo N. Chini

Abstract

Purpose: Here, we describe a novel interplay between NAD synthesis and degradation involved in pancreatic tumor growth.

Experimental Design: We used human pancreatic cancer cells, both in vitro (cell culture experiments) and in vivo (xenograft experiments), to demonstrate the role of NAD synthesis and degradation in tumor cell metabolism and growth.

Results: We demonstrated that pharmacologic and genetic targeting of Nampt, the key enzyme in the NAD salvage synthesis pathway, inhibits cell growth and survival of pancreatic cancer cells. These changes were accompanied by a reduction of NAD levels, glycolytic flux, lactate production, mitochondrial function, and levels of ATP. The massive reduction in overall metabolic activity induced by Nampt inhibition was accompanied by a dramatic decrease in pancreatic tumor growth. The results of the mechanistic experiments showed that neither the NAD-dependent enzymes PARP-1 nor SIRT1 play a significant role on the effect of Nampt inhibition on pancreatic cancer cells. However, we identified a role for the NAD degradation pathway mediated by the NADase CD38 on the sensitivity to Nampt inhibition. The responsiveness to Nampt inhibition is modulated by the expression of CD38; low levels of this enzyme decrease the sensitivity to Nampt inhibition. In contrast, its overexpression decreased cell growth in vitro and in vivo, and further increased the sensitivity to Nampt inhibition.

Conclusions: Our study demonstrates that NAD metabolism is essential for pancreatic cancer cell survival and proliferation and that targeting NAD synthesis via the Nampt pathway could lead to novel therapeutic treatments for pancreatic cancer. Clin Cancer Res; 20(1); 120–30. ©2013 AACR.

Introduction

In a series of seminal studies in the early 1900s, Otto Warburg defined unique metabolic features of cancer cells (1–4). These metabolic changes are critical for tumor cell survival, proliferation, and metastatic potential (1–5). However, it was not until recently that cancer cell metabolism became the focus of intense investigation (1–11).

NAD is a crucial cofactor in redox reactions within metabolic pathways of nearly every cell (7, 12). It has been shown that NAD participates in multiple physiologic processes (7, 13–20). In addition, NAD metabolism appears to have a crucial role in the fate of tumor cells (21–24). Cellular NAD levels are maintained at stable levels via equilibrium between NAD degradation and NAD synthesis. NAD synthesis is mediated by two distinct mechanisms, the salvage and the de novo pathway (7, 12). NAD degradation is mainly regulated by CD38 (13–18), with other enzymes including sirtuins, PARPs, and ADP-ribosyltransferases (ART) playing a complementary role.

In this study, we investigated a novel hypothesis that the interplay between (NAD) synthetic and degrading pathways was involved in the regulation of pancreatic tumorogenesis. We studied how inhibition of Nampt, the rate-limiting enzyme of the salvage pathway, affects NAD levels, metabolism, cellular energy production, and tumorigenesis. In addition, we studied the role of NAD-degrading enzymes in modulating this response.

Materials and Methods

Cell lines

PaTu8988t, Panc-1, SU86.86, Panc04.03, and HPDE cells were either provided by Dr. D. Billadeau or obtained from the American Type Culture Collection (ATCC). Cultures used for experiments were reinitiated every 4 to 6 months from cryopreserved stocks. The pancreatic cancer cell lines possess k-ras and/or p53 mutations that were validated by

Authors’ Affiliation:
Department of Anesthesiology, Mayo Clinic Cancer Center, Mayo Clinic College of Medicine, Rochester, Minnesota

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C.C.S. Chini and A.M.G. Guerrico contributed equally to this work.

Corresponding Author: Eduardo N. Chini, Mayo Clinic, 2nd Street, Rochester, MN 55902. Phone: 507-284-6696; Fax: 507-284-6696; E-mail: chini.eduardo@mayo.edu
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Trypan blue assay. IC50s were calculated using CalcuSyn viability was determined by the standard MTT assay or treated with the drugs for 48 to 72 hours at 37 and 50 keratinocyte medium supplemented with 5 ng/mL of EGF penicillin/streptomycin. HPDE cells were grown in SFM- grown in RPMI medium supplemented with 10% FBS and tomycin (Invitrogen). SU86.86 and Panc04.03 cells were (DMEM) supplemented with 10% FBS and penicillin/streptomycin (pH 6.0), 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 150 mmol/L NaCl, and 2 mmol/L MgCl2.

Reagents and antibodies

Except when specified, all reagents and chemicals were purchased from Sigma Chemical. Antibodies were CD38 (Epitomics), Nampt (Bethyl), NaprT1 (Proteintech), and CD38 siRNAs were a pool of three target-specific siRNAs (sc-45843, Santa Cruz Biotechnology), and a human on-target plus probe (J-009222-05, Dharmacon). Transfections were performed with 50 nmol/L of siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions.

Transfection and Western blots

Panc-1 cells were transfected with Flag or Flag-CD38 vector using Lipofectamine 2000 (Invitrogen). For stable transfections, Panc-1 cells were cotransfected with Flag-CD38/puromycin vector or Flag-vector/puromycin vector and selected with 4 μg/mL of puromycin. Western blot analyses were performed using standard laboratory techniques as described previously (14, 16).

β-Galactosidase staining

Cells were washed in PBS, fixed for 10 minutes with 3% formaldehyde, washed, and incubated for 24 hours at 37°C with β-galactosidase (β-Gal) staining solution: X-Gal 1 mg/mL, 40 mmol/L citric acid, sodium phosphate (pH 6.0), 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 150 mmol/L NaCl, and 2 mmol/L MgCl2.

Soft agar colony-formation assay

Cells were seeded at a density of 10,000 per well in 6-well plates in 0.35% agar over 0.6% bottom agar layer in growth media containing 5% FBS and increasing concentrations of FK866. Cell colonies were grown in a humidified 5% CO2 incubator at 37°C. Colonies measuring 50 μm or more were counted after 7 to 10 days of culture using a cell colony counter (Gelcount, Oxford Optronix). Experiments were repeated 3 times, each in triplicates.

NAD quantification and NADase activity

NAD was measured by an enzymatic cycling assay as extensively described by us previously (14, 16, 18). NADase activity was determined previously by us using etheno-NAD as a substrate (14, 16–18).

Determination of glycolytic intermediates

Nuclear magnetic resonance (NMR) metabolomic analysis was performed with Glucose C13 as a tracer. Briefly, culture media of nontreated and FK866-treated cells were replaced by glucose-free DMEM supplemented with D-[U-13C] 5 mmol/L glucose, and cells were incubated for 1 hour. Metabolic analysis was by one-dimensional 13C spectra of media and cell extracts. Spectra of Panc-1 cells metabolites were acquired with a Bruker DRX 400 MHz using a triple resonance probe (TXI). Spectra processing and analysis were performed using Topspin 2.0 and metabolite assignment was done by chemical shift comparison of known metabolites deposited in the Human Metabolome Database v 1.0.

ATP measurements

ATP levels were measured in tumor tissues using the Aposensor ATP luminescence assay from BioVision, and in cells using the ATPlite Luminescence assay system from PerkinElmer.
Lactate production
Lactate assay was performed using hydrazine/glycine buffer (pH 9.2), 5 mg/mL β-NAD⁺ and 15 U/mL lactate dehydrogenase. NADH formation was monitored at 340 nm (25).

Oxygen consumption of intact cells
Oxygen consumption rates were measured polarographically using high-resolution respirometry (Oroboros-O2K). FK866- or vehicle-treated cells were used to measure oxygen consumption at the same time at 37°C (26). After recording routine (basal) oxygen consumption in serum-free DMEM, oligomycin 2 μg/mL was added to inhibit ATP synthesis (leak respiration), followed by titration with the uncoupler FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) until it reached maximum uncoupled respiration (0.2–2.5 μmol/L). Rotenone (2 μmol/L) was added to inhibit complex I. Oxygen consumption after rotenone (not mitochondria related) was subtracted from all other oxygen consumption measurements. Coupled respiration was calculated as the difference between routine and leak respiration.

Tumor xenograft study
Female athymic nu/nu mice were obtained from the National Cancer Institute (NCI). The experiments were performed under the supervision and approval of the Institutional Animal Care and Use Committee at Mayo Clinic (protocol no. A39511). Subconfluent Panc-1 cells were harvested, and suspensions consisting of single cells with 90% viability were used for s.c. injections in both flanks of 5- to 6-week-old mice [4 × 10⁶ cells in 100 μL of PBS:Matrigel (1:1)/site]. Eleven days after implantation (tumor volume, ~60 mm³), mice were randomized into two groups: (i) untreated control (vehicle; PBS containing 1% hydroxypropyl-β-cyclodextrine and 12% propylenglycol); (ii) FK866 [15 mg/kg, twice daily by intraperitoneal (i.p.) injection]. Tumor volumes were measured weekly with a caliper and calculated using the formula \( V = \frac{4}{3}\pi l \times w \times d \), where \( l \) is the length, \( w \) is the width, and \( d \) is the depth.

Quantification of mRNA
mRNAs from tissue samples were prepared from biospecimens obtained from the Mayo Clinic Tissue Registry under an Institutional Review Board–approved protocol. RNA was isolated from a set of samples from patients with pancreatic adenocarcinoma for which frozen, paired specimens obtained from the Mayo Clinic Tissue Registry (HPDE). We found that Nampt was expressed in all cell lines (Fig. 1A), with higher expression in the cell lines Panc04.03 and SI186.86. Next, we examined whether inhibition of Nampt using FK866 resulted in a reduction in cellular NAD levels (Fig. 1B). The effect of FK866 on intracellular NAD levels was variable between the cells tested, with some cells (e.g., PaTu8988t) very sensitive to Nampt inhibition and other cells, like Su86.86, showing a significant lower sensitivity to FK866 (Fig. 1B). To confirm the role of Nampt in the regulation of cellular NAD levels, we tested the effect of an acute reduction in the enzyme expression via knockdown with siRNA in PaTu8988t and Panc-1 cells. Treatment with Nampt siRNA reduced Nampt expression and NAD levels in these cells (Fig. 1C and D), indicating that the salvage pathway of NAD synthesis plays a key role in maintaining NAD levels in these pancreatic cancer cells.

To determine whether FK866 can cause a decrease in overall metabolism in pancreatic cancer cells, we measured several metabolic parameters. First, we observed that glucose consumption was severely impaired in cells treated with FK866 (Fig. 2A). Nampt is important for several biochemical processes including oxidative-reduction reactions of the glycolytic pathway (27). We observed an accumulation of the glycolytic intermediate fructose 1,6-bisphosphate in cells treated with FK866 (Fig. 2A), indicating that FK866 inhibits glycolytic fluxes and may cause cellular metabolic collapse. To confirm these effects, we measured lactate production in FK866-treated cells and found that FK866 inhibited lactate release, in both PaTu8988t and Panc-1 cells (Fig. 2B). We further investigated the effect of FK866 on mitochondrial oxygen consumption, ATP levels, and the activation of AMP-activated protein kinase (AMPK). FK866 treatment decreased mitochondrial maximum respiratory capacity (Fig. 2C and Supplementary Fig. S1), ATP levels (Fig. 2D), and induced phosphorylation/activation of AMPK in pancreatic cancer cells (Fig. 2D), confirming that Nampt inhibition promotes energy collapse in pancreatic cancer cells.

Statistical analysis
Data were expressed as means ± SD from at least three independent experiments. Data was analyzed using unpaired t test and one-way and two-way ANOVA. Significance was set at \( P < 0.05 \).

Results
Nampt plays a key role in maintaining NAD levels in pancreatic cancer cells
We evaluated Nampt expression in a panel of pancreatic cancer cell lines and a normal pancreatic cell line (HPDE). We found that Nampt was expressed in all cell lines (Fig. 1A), with higher expression in the cell lines Panc04.03 and SI186.86. Nampt enzyme expression via knockdown with siRNA reduced Nampt expression and NAD levels in these cell lines (Fig. 1C and D), indicating that the salvage pathway of NAD synthesis plays a key role in maintaining NAD levels in these pancreatic cancer cells.
Inhibition of Nampt decreases pancreatic cancer cell growth and survival

We examined the role of Nampt in the growth and survival of the pancreatic cancer cells. FK866 causes a dose-dependent inhibition of viability in Panc-1 and PaTu8988t cells (Fig. 3A). We observed a significant difference in the sensitivity of cells to Nampt inhibition, with PaTu8988t cells being nearly 10 times more sensitive than other cells. The least sensitive was the normal pancreatic cell HPDE (Fig. 3 and Supplementary Fig. S2). As the MTT assay determines cell viability via an NAD-dependent mechanism, we further confirmed our findings with two other complementary assays to measure cell growth and viability (Fig. 3).

Panc-1 and PaTu8988t cells were treated with 50 nmol/L FK866 for 72 hours, and viable cells were counted by the Trypan blue exclusion assay. Treatment with FK866 decreased the number of viable cells in both cell lines (Fig. 3B).

We determined whether Nampt can regulate anchorage-independent cell growth, an indicator of malignant behavior. Pancreatic cancer cells were treated with FK866, and colony formation in soft agar was measured. Colony formation was decreased on treatment with FK866 in PaTu8988t cells and to a lesser extent in Panc-1 cells (Fig. 3C).

Furthermore, we knocked down Nampt using two different siRNAs. Nampt siRNAs inhibited cell growth in both Panc-1 and PaTu8988t cell lines (Fig. 3D and Supplementary Fig. S3).

NAMPT inhibition impairs pancreatic tumor growth

We further tested the effect of FK866 in a xenograft animal model of pancreatic tumor. Nude mice were injected with Panc-1 cells, and, 10 days after implantation, the animals were treated with daily i.p. injections of vehicle or 15 mg/kg of FK866. FK866 treatment decreased tumor size when compared with vehicle (Fig. 4A). Tumors from mice treated with FK866 had lower cellular NAD and ATP levels (Fig. 4B), and increased phosphorylation of AMPK (Fig. 4C). During the treatment, no obvious treatment-related toxicity was observed. Body weight and food intake of both groups of animals remained similar (Fig. 4D). We conclude that FK866 inhibits pancreatic tumor growth in vivo, by inducing tumoral metabolic collapse.

The effect of FK866 in pancreatic cancer cell viability is reversed by nicotinamide mononucleotide and nicotinic acid

Because NAD can be synthesized by both the salvage and the de novo pathways in cells, we explored whether the two pathways were involved in the regulation of pancreatic cancer cell growth. In the salvage pathway, Nampt produces nicotinamide mononucleotide (NMN) from nicotinamide. In the de novo pathway, nicotinic acid phosphoribosyltransferase (Napr1) produces nicotinic acid mononucleotide.
(NaMN) from nicotinic acid. The effect of FK866 on cell viability of PaTu8988t was completely reversed by treatment with the NAD precursors NMN and nicotinic Acid (Fig. 5A). In addition, the pattern of expression of Nampt in the different pancreatic cell lines (Fig. 5A). These data suggest that both pathways are involved in the modulation of pancreatic cancer cell growth. However, inhibition of the salvage pathway is sufficient to promote energy collapse and cell death in pancreatic cancer cells.

**SIRT1 and PARP-1 are not involved in the effect of FK866 in pancreatic cancer cells**

As NAD is necessary for both oxi–reduction and non-oxi–reduction reactions, we further explored whether inhibition of some of the nonoxidative NAD-dependent cellular reactions were involved in the decrease in cellular viability induced by Nampt inhibition. Two enzymes, PARP-1 and SIRT1, catalyze crucial nonoxidative NAD-dependent reactions. Surprisingly, neither PARP1 nor SIRT1 inhibition recapitulated or modified the effects of Nampt inhibition on cell viability (Fig. 5B). In addition, transient SIRT1 knockdown increased cell proliferation of PaTu8988T cells (Fig. 5C). The difference in cell proliferation is shown by the decrease in doubling time in SIRT1–siRNA-treated cells compared with nontarget siRNA-transfected cells. The doubling time for nontarget siRNA-transfected cells was 21.7 hours compared with nontarget siRNA-transfected cells. The doubling time for SIRT1–siRNA-transfected cells was 19.2 hours and for SIRT1–siRNA-transfected cells was 19.2 ± 0.6 hours (P < 0.05). Moreover, pharmacologic inhibition of SIRT1 using the synthetic inhibitor EX527 resulted in a small induction of cell growth of both PaTu8988t and SU86.86 cells, by 22% and 35%, respectively (Fig. 5D). Taken together, these results indicate that neither PARP-1 nor SIRT1 inhibition can explain the effects of Nampt inhibition in the pancreatic cancer cells.
Sensitivity of cultured pancreatic cancer cells in vitro to FK866 is modulated by CD38 expression

Decreases in cellular NAD can be achieved by inhibition of NAD synthesis or by an increase in its degradation. The enzyme CD38 is the main NADase in many normal mammalian tissues (13–18). Interestingly, it has been proposed that CD38 expression may be lost during the development of prostate cancer (28). However, the functional role of CD38 in pancreatic cancer cells or any other solid tumor has not been explored. Here, we investigated the role of CD38 in pancreatic NAD metabolism and its role in the effect of the Nampt inhibitor FK866 on cell viability and cell growth.

Most cultured pancreatic cancer cells tested had low levels of CD38 expression (Fig. 6A), except for PaTu8988t, which expresses high amounts of CD38. When we knocked down CD38 in PaTu8988t cells, there was an increase in NAD levels and a decrease in NADase activity (Fig. 6B). Although no significant difference in cell viability was observed after cell knockdown of CD38 (data not shown), the cells that were knocked down for CD38 showed lower sensitivity to FK866 than control cells (Fig. 6B and Supplementary Fig. S4).

In contrast, Panc-1 cells have nearly undetectable levels of CD38 (Fig. 6A). Transient expression of CD38 increased NADase activity, decreased NAD levels, and increased sensitivity to the effect of FK866 on cell viability (Fig. 6C). Expression of CD38 in Panc-1 cells did not affect mitochondrial respiration by itself, but it sensitized the cells to the inhibitory effect of FK866 in mitochondrial function (Supplementary Fig. S5A). In addition, overexpression of CD38 in SU86.86 promoted a decrease in NAD levels (data not shown) and a significant decrease in cell viability compared with vector-transfected cells (Supplementary Fig. S5B and S5C).
To further explore the role of CD38 in NAD metabolism and cell growth, we generated a Panc-1-CD38 stable cell line. This cell line has increased NADase activity and lower NAD levels than the control cell line (Fig. 7A). In addition, the Panc-1-CD38 cells showed cell growth arrest both in vitro as well as in an in vivo xenograft mouse model (Fig. 7A and B). These cells also exhibit senescent markers, as measured by β-gal staining and increased p21 protein levels (Fig. 7C). Moreover, in colony formation assays, Panc-1-CD38 cells showed an increase in sensitivity to the FK866 effect in comparison with the control cells (Fig. 7D), confirming that CD38 has an important effect in regulating the sensitivity to Nampt inhibition.

**Heterogeneous expression of NAD-metabolizing enzymes in pancreatic tumor tissues**

We further explored the expression of Nampt and CD38 in pancreatic cancer tumor samples from patients. There was a significant variability in the expression of CD38 and Nampt in pancreatic cancer samples; however, in general, the expression of both Nampt and CD38 was higher in samples from pancreatic tumors than in normal tissue (Supplementary Fig. S6A and S6B). To compare these results with data from the pancreatic cancer cell lines, we performed real-time PCR in samples from pancreatic cancer cell lines and the control pancreatic cell HPDE. All cells express Nampt and Naprt1, but the expression levels differ between cell lines, with some cancer cells expressing similar levels and others expressing lower levels than the control pancreatic cell (Supplementary Fig. S6C and S6D). In contrast, the only pancreatic cancer cell that expresses high amounts of CD38 is PaTu8988t (Supplementary Fig. S6E). For all the cell lines, the patterns of expression of protein and mRNA had a high correlation with all genes tested (Figs. 1A, 5B, and 6A).

Because we found that the expression of CD38 is low in pancreatic cancer cells in culture, the relatively high
expression of CD38 in the pancreatic tumor tissues may be mediated by either inflammatory cell infiltration or by stromal cells. However, it is also possible that pancreatic cancer cells in vivo may express CD38 differently than in vitro.

Discussion

Pancreatic cancer is one of the top five causes of cancer-related deaths worldwide, with an extremely poor prognosis (~5% survival in 5 years) and a median survival for metastatic disease of approximately 6 months (29). New and effective therapies are urgently needed for this disease. The studies described here clearly show a role for the salvage pathway as a potential therapeutic target in pancreatic cancer and have also identified NAD catabolic pathways that modulate sensitivity to Nampt inhibitors in cancer models.

Recently, it has been proposed that NAD metabolism may be a potential target for the treatment of cancers (6, 12, 21–24, 30). NAD is a crucial cofactor in redox reactions in metabolic pathways of nearly every cell (6, 12). NAD can be found in two states, oxidized (NAD\(^{+}\)) or reduced (NADH). Oxidized NAD is necessary for the initial steps of the glycolytic pathway that are important for cancer cell survival and growth (6, 12). Intracellular NAD metabolism in cancer cells can be manipulated in several different ways. For example, the equilibrium between the oxi–reduction states of NAD (the NAD/NADH ratio) can be shifted one way or the other to either support or inhibit glycolysis (31). Recently, Lu and colleagues have elegantly shown that the enzyme NADP(\(^{+}\)) oxidase (NOX), plays a key role in supporting increased glycolysis in pancreatic cancer cells by oxidizing NAD (31).

Yet another way to modulate NAD-dependent reactions in cancer cells is to modify its total pool via its anabolism and catabolism. To date, the functional role of the NAD anabolic pathway in pancreatic cancer cells has not been investigated. In fact, the only information published about...
In this regard, characterization of the metabolic pathways for synthesis was one of the goals of our study. We describe for the first time that pancreatic cancer cells use the salvage pathway and rely on the enzyme Nampt for NAD synthesis, a finding that showed that colo357 pancreatic cancer cells express Nampt in response to interleukin (IL)-1 treatment (ref. 9). In our experiments, inhibition of Nampt expression in response to interleukin (IL)-1 treatment (ref. 9).

The mechanism by which NAD collapse mediated by Nampt inhibition causes cancer cell death has been proposed to be mediated by inhibition of the glycolytic pathway and/or inhibition of the NAD-dependent deacetylase SIRT1 (7, 8, 22, 27, 32). In our experiments, inhibition of SIRT1 was neither necessary nor sufficient to explain the cellular effects of Nampt inhibition in pancreatic cancer cells.

Tumor cells have a highly active "aerobic glycolysis" known as the Warburg effect (1, 2). Mechanistically, the first reaction in the glycolytic pathway that is dependent on NAD(H) is the conversion of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate catalyzed by GAPDH (27). However, the reaction preceding this step, the interconversion of glyceraldehyde-3-phosphate and fructose 1,6 biphosphate is reversible, and we expected that inhibition of the GAPDH reaction could lead to accumulation of one of these metabolites. Treatment with FK866 leads to a decrease in lactate production, metabolic fluxes, and accumulation of fructose 1,6 biphosphate in pancreatic cancer cells. Our data support a model in which Nampt inhibition promotes cell death via a decrease in NAD that leads to inhibition of glycolytic metabolism, ATP depletion, and energy collapse, and not via SIRT1-dependent mechanisms.

Another goal of our study was to characterize the NAD catabolizing pathways in pancreatic cancer cells. To our knowledge, the mechanisms of NAD degradation in tumor cells have not been well described. In particular, given the emerging interest in developing NAD-targeted therapies for human cancers, it is important to determine the mechanisms that modulate NAD degradation in the setting of inhibition of NAD synthetic pathways.

We observed in cultured pancreatic cancer cells that the expression of the NADase CD38 played a key role on the sensitivity of cells to the Nampt inhibitor FK866. In addition, we observed that CD38 and Nampt expression are quite variable between tumors from patients of pancreatic cancer (Supplementary Fig. S6), and we propose that the relative expression of Nampt and CD38 may play a key role in the response to salvage pathway-targeted therapy and may serve as potential biomarkers for the cellular response to Nampt inhibition.
In conclusion, our data provide the first evidence of the role of NAD anabolism and catabolism in pancreatic cancer cells. Specifically, we demonstrated that pancreatic cancer cells rely on the salvage pathway and that inhibition of the enzyme Nampt causes metabolic collapse and cell death, both in vitro and in vivo. Furthermore, we provided one of the first analyses of NAD catabolism in cancer cells. We believe that further systematic characterization of the anabolism and catabolism of NAD in cancer cells may have potential implications for the development of novel and more rational NAD-targeted therapy for cancer.

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

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References
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