Glycogen Synthase Kinase-3 Inhibition Sensitizes Pancreatic Cancer Cells to Chemotherapy by Abrogating the TopBP1/ATR-Mediated DNA Damage Response

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Abstract

**Purpose:** Pancreatic ductal adenocarcinoma (PDAC) is a predominantly fatal common malignancy with inadequate treatment options. Glycogen synthase kinase 3β (GSK-3β) is an emerging target in human malignancies including PDAC.

**Experimental Design:** Pancreatic cancer cell lines and patient-derived xenografts were treated with a novel GSK-3 inhibitor 9-ING-41 alone or in combination with chemotherapy. Activation of the DNA damage response pathway and S-phase arrest induced by gemcitabine were assessed in pancreatic tumor cells with pharmacologic inhibition or siRNA depletion of GSK-3 kinases by immunoblotting, flow cytometry, and immunofluorescence.

**Results:** 9-ING-41 treatment significantly increased pancreatic tumor cell killing when combined with chemotherapy. Inhibition of GSK-3 by 9-ING-41 prevented gemcitabine-induced S-phase arrest suggesting an impact on the ATR-mediated DNA damage response. Both 9-ING-41 and siRNA depletion of GSK-3 kinases impaired the activation of ATR leading to the phosphorylation and activation of Chk1. Mechanistically, depletion or knockdown of GSK-3 kinases resulted in the degradation of the ATR-interacting protein TopBP1, thus limiting the activation of ATR in response to single-strand DNA damage.

**Conclusions:** These data identify a previously unknown role for GSK-3 kinases in the regulation of the TopBP1/ATR/Chk1 DNA damage response pathway. The data also support the inclusion of patients with PDAC in clinical studies of 9-ING-41 alone and in combination with gemcitabine.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), which constitutes 93% of pancreatic cancers, is predicted to be the second leading cause of cancer-related deaths in the United States by 2030 (1, 2). The 5-year relative survival rate of all stages combined PDAC patients is less than 10% (3). As a standard therapy for locally advanced and metastatic pancreatic cancer, gemcitabine has a 5.4% partial response rate (4) and the great preponderance of initially sensitive tumors develop overt chemoresistance within weeks (5). FOLFIRINOX (folinic acid, fluorouracil, irinotecan, and oxaliplatin) and Nab-paclitaxel in combination with gemcitabine represent modest improvements over single-agent gemcitabine (6, 7). Novel approaches are thus urgently needed for patients with PDAC as are mechanism-based discovery of new therapeutic strategies to overcome chemotherapy resistance (8).

Glycogen synthase kinase-3 (GSK-3) α and β are highly conserved serine-threonine kinases initially described as key enzymes in regulating glycogen metabolism, with critical roles in Wnt/β-catenin signaling, immune regulation, and maintenance of stem cell identity (9, 10). We have previously shown that GSK-3β expression is regulated by oncogenic KRas signaling and its overexpression together with nuclear accumulation correlated with moderately and poorly differentiated pancreatic tumors (11–13). We found that GSK-3β promoted cell proliferation and survival through the regulation of NF-kB–dependent gene transcription (12). Consistent with this growth promoting effect of GSK-3β in PDAC, pharmacologic inhibition or genetic depletion of GSK-3β limited pancreatic cancer cell viability in vitro and suppressed tumor growth in vivo (11, 14, 15). Using a genetically engineered mouse model we demonstrated that GSK-3β contributes to KRas-driven tumor-promoting pathways...
although GSK-3 expression of genes involved in DNA repair (23). Interestingly, sensitizes pancreatic cancer cells to gemcitabine with altered gnosti cally enhances the anticancer effect of gemcitabine by promoting the ubiquitin-dependent proteasome degradation of TopBP1, a key molecule that is required for the degradation of TopBP1. As previously uncharacterized for GSK3β, the ATR-Chk1 DNA damage response pathway. Treatment with the GSK-3 inhibitor 9-ING-41 sensitized PDAC cells to gemcitabine, as well as liposomal irinotecan in vivo. As 9-ING-41 has recently entered clinical studies, our data highlight not only a novel mechanism of action for 9-ING-41, but also provide a compelling rationale for the inclusion of patients with PDAC in clinical studies of 9-ING-41 in combination with gemcitabine/abraxane or MM398. These data also support the study of 9-ING-41 with other agents that induce an ATR-mediated DNA damage response.

**Materials and Methods**

Cell culture, reagents, and treatments

All the chemicals were obtained from Sigma unless otherwise specified. BxPC3, HupT3, Panc01, CFPAC-1, I.3.6 were obtained from ATCC. Panc01 and CFPAC were maintained in DMEM medium. BxPC3 and HupT3 were maintained in RPMI1640 medium. 1.3.6 cells were maintained in MEM medium and supplemented with 1% nonessential MEM amino acids. Pancreatic cancer patient-derived xenografts (PDX) cell lines including 6741, 5160, 6413, and 4041 were developed from PDAC tissue resections that had been established in nude mice as described previously (28) and were maintained in DMEM-F12 medium. GSK-3β-null mouse embryonic fibroblasts (MEF) and matching wildtype MEFs were a kind gift from Dr. Jim Woodgett (Ontario Cancer Institute, Toronto, ON, Canada) and maintained in DMEM medium. All culture media were supplemented with 10% FBS, 1% 1-glutamine and 1% penicillin streptomycin. Cells were counted and plated 24 hours before treatment. Mycoplasma Detection Kit was used for detecting mycoplasma contamination. The latest testing was performed on April 30, 2019. All cells used in the described experiments were collected within 5 passages. The GSK-3 inhibitor Bio (Selleckchem), 9-ING-41 (Actuate Therapeutics Inc.), gemcitabine (Eli Lilly), Irinotecan liposomal formulation (JRT-IP; obtained from the Mayo Pharmacy) and MG132 (Sigma-Aldrich) were also used in this study.

**MTS and clonogenic assay**

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega). Briefly, 5,000 cells/well were seeded in a 96-well culture plates and incubated in culture medium with or without indicated drug treatments for 48 hours. Medium was removed, and fresh medium was added to each well along with 1:20 dilution of MTS solution. After 2 hours of incubation, the plates were analyzed with a microplate reader at a wavelength of 490 nm (Molecular Devices). To assess for potential drug synergy, the combination index (CI) was calculated using CalcuSyn (Biosoft). For clonogenic assays, cells were collected and seeded in 6-well plates at 1,500 cells/ml. After a 4-hour incubation, which allowed cells to attach, culture medium with or without indicated vehicle or drug treatments were added. Forty-eight hours later, supernatant in the wells were aspirated and washed with PBS (NaCl 0.137 M, KCl 2.7 mmol/L, Na₂HPO₄ 8.1 mmol/L, KH₂PO₄ 1.5 mmol/L, pH 7.4) to remove residual drug. Fresh medium was then added to allow colony formation. Colonies were grown until visible and counted after staining with Coomassie brilliant blue R (42% methanol, 16.8% acetic acid, 1 mg/ml Brilliant blue R).

**Subcutaneous and orthotopic pancreatic cancer animal model**

The evaluation of 9-ING-41 in combination with gemcitabine therapy in PDX pancreatic tumor model was carried out by A. Ugolkov and A.P. Mazar in the Center for Developmental Therapeutics, Northwestern University, Evanston, IL, and approved by the Institutional Animal Care and Use Committee of Northwestern University, as described previously (29). The pancreatic PDX tumor model PCF379419 was transplanted subcutaneously into the flanks (left and right side) of nude mice (Jackson Laboratory). Three weeks after tumor transplantation, mice were randomized into 4 groups (n = 3/group) and treated with: Vehicle (DMSO),
Gemcitabine (10 mg/kg in week 1 and 5 mg/kg in week 2 and 3), 9-ING-41 (40 mg/kg), or both Gemcitabine and 9-ING-41 twice a week for 3 weeks by intraperitoneal injection. For orthotopic pancreatic cancer animal models performed at Mayo Clinic, 6- to 8-week-old NSG male mice were procured from Charles River Laboratories and housed in the institutional animal facilities and were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. To establish an orthotopic pancreatic tumor model, approximately 1 million 6,741 PDX cells suspended in 100 μL PBS containing 20% matrigel were slowly injected orthotopically into the head pancreas. In the orthotopic studies, 9-ING-41 was diluted in PEG400/Tween80/Ethanol (PTE) at a ratio of 75:8:17. Prior to injection, an equal volume of saline was used to further dilute the sample. Three weeks following tumor cell implantation, mice were randomly divided into 4 groups (n = 5) and treated with: Vehicle (vehicle consisted of PTE). Gemcitabine (10 mg/kg), 9-ING-41 (40 mg/kg), or both Gemcitabine (10 mg/kg) and 9-ING-41 (40 mg/kg) twice a week for 4 weeks by intraperitoneal injection. In the combination group, gemcitabine was given 1 hour following 9-ING-41 injection. Tumor size was measured with calipers and tumor volume was calculated using the formula 1/2(length x width x width).

Cell apoptosis and necrosis analysis
Apoptosis and necrosis of pancreatic cancer cells were measured as previously described (30). Briefly, the treated pancreatic cancer cells were detached by trypsinization and stained with annexin V labeled with APC (BD Bioscience) and PI (20 μg/mL; Sigma) for 15 minutes. Cells (50,000 per condition) were then analyzed as previously described (30). Brieﬂy, the treated pancreatic cancer cells were detached by trypsinization and stained with annexin V labeled with APC (BD Bioscience) and PI (20 μg/mL; Sigma) for 15 minutes. Cells (50,000 per condition) were then analyzed as previously described (30). Brieﬂy, the treated pancreatic cancer cells were detached by trypsinization and stained with annexin V labeled with APC (BD Bioscience) and PI (20 μg/mL; Sigma) for 15 minutes. Cells (50,000 per condition) were then analyzed as previously described (30). Brieﬂy, the treated pancreatic cancer cells were detached by trypsinization and stained with annexin V labeled with APC (BD Bioscience) and PI (20 μg/mL; Sigma) for 15 minutes. Cells (50,000 per condition) were then analyzed as previously described (30). Brieﬂy, the treated pancreatic cancer cells were detached by trypsinization and stained with annexin V labeled with APC (BD Bioscience) and PI (20 μg/mL; Sigma) for 15 minutes. Cells (50,000 per condition) were then analyzed as previously described (30).
Results
9-ING-41 reduces growth of PDAC cells and sensitizes them to gemcitabine in vitro
The novel small-molecule ATP-competitive GSK-3 inhibitor 9-ING-41 has been shown to inhibit various human cancer cells growth in vitro and significantly increase tumor-killing effect when combined with chemotherapies in resistant glioblastoma and breast cancer (27, 29, 32, 33). To examine its antitumor proliferation effect on pancreatic cancer cells, 5 previously described PDAC cell lines (30) and 3 recently developed pancreatic cancer PDX (28) cell lines were plated and treated with 9-ING-41 in increasing nanomolar concentrations (50 to 2,000 nmol/L). Growth suppression was observed in all tested cell lines using a colorimetric, MTS assay after 48 hours (Fig. 1A). We next tested the effect of 9-ING-41 in combination with gemcitabine. Although 9-ING-41 alone inhibited 6741 proliferation at both 48 and 72 hours, it also synergistically sensitized 6741 (Fig. 1B) and 5160 (Supplementary Fig. S1A) to gemcitabine as determined by calculating the combination index. To further investigate the cancer cell killing and chemo-sensitizing effect of 9-ING-41, we

Figure 1.
9-ING-41 treatment synergizes with gemcitabine to abrogate PDAC cell proliferation and colony formation in vitro. A, The indicated PDAC cell lines were seeded in 96-well plates and treated with DMSO or increasing concentration of 9-ING-41 (nmol/L) for 48 hours. Cell proliferation was determined by MTS assay. Data were quantified as percentage of control and expressed as mean ± SEM; n = 6. B, The 6741PDX-derived cell line was plated and treated with 1 μmol/L 9-ING-41 alone or with increasing concentrations of gemcitabine (nmol/L) for 48 and 72 hours. Cell proliferation was determined by MTS assay. Data were quantified as percentage of control and expressed as mean ± SE. *, P < 0.05 gemcitabine and 9-ING-41 combinations versus gemcitabine alone. #, P < 0.05 gemcitabine and 9-ING-41 combination versus 9-ING-41; n = 6. C, L3.6 and 6741 PDAC cells were seeded in 6-well plate and treated with DMSO or increasing concentration of 9-ING-41 (nmol/L) for 48 hours. Supernatant was then removed and remaining cells were allowed to form colonies. Colony number from triplicate samples were counted and expressed as mean ± SEM. *, P < 0.05 9-ING-41 versus DMSO. D, Clonogenic assay was carried out as described in C but 200 nmol/L 9-ING-41 was added together with increasing concentration of gemcitabine. Colony number from triplicate samples were counted and expressed as mean ± SEM. *, P < 0.05 gemcitabine and 9-ING-41 combination versus gemcitabine alone.
utilized L3.6 and 6741 in a clonogenic assay (Supplementary Fig. S1B and S1C). L3.6 and 6741 colony numbers decreased in a dose-dependent manner following 9-ING-41 treatment (Fig. 1C). When combined with increasing doses of gemcitabine, 9-ING-41 could substantially reduce colony number compared with gemcitabine alone (Fig. 1D). Previous studies have shown that 9-ING-41 treatment inhibited the proliferation of ovarian cancer cell lines by induction of apoptosis (27). Therefore, we examined cell apoptosis/necrosis by annexin V/PI staining in 9-ING-41-treated pancreatic cancer cells. As shown in Supplementary Fig. S2A and S2B, combination of both 9-ING-41 and gemcitabine decreased the number of live cells and increased the population of necrotic cells. Immunoblotting results further confirmed the phenotype of significant cell death in the combination drug group (Supplementary Fig. S2C). Taken together, these data suggest that 9-ING-41 can suppress cell proliferation and sensitize PDAC cells to gemcitabine in vitro.

The combination of 9-ING-41 and gemcitabine limits tumor growth in vivo

To better understand the antitumor effect of 9-ING-41 alone and in combination with gemcitabine in vivo, we first tested 9-ING-41 using the PDAC PDX model PCF379419. As shown in Fig. 2A, the PDX tumor expanded aggressively in the vehicle and 9-ING-41-treated animals, whereas monotherapy with gemcitabine suppressed, but did not completely block tumor growth. In contrast, the combination treatment with 9-ING-41 and gemcitabine caused a profound decrease in tumor growth, ending with notable regression after 3 weeks of treatment (Fig. 2A and B).

We next evaluated the effect of 9-ING-41 using an orthotopic tumor mouse model (34). The 6741 PDAC cell line was implanted in the head of the pancreas and allowed to grow until tumors were palpable. Mice were then randomized into treatment groups and treated twice a week for 4 weeks (Fig. 2C).

Two days following the last round of therapy, orthotopic tumors were isolated and tumor weight and volume were measured. Although we did observe a statistically significant inhibition of tumor growth with monotherapy treatment in the orthotopic model when compared with vehicle, consistent with the subcutaneous model, we observed a greater reduction in tumor weight and tumor volume in animals that received combination therapy when compared with either vehicle or monotherapy (Fig. 2D). Finally, we orthotopically implanted 6741 and 3 additional PDX-derived tumor cell lines (4535, 4636, and 4911) and
assessed survival following individual or combination drug treatments. In addition to using gemcitabine, we also used liposomal-formulated irinotecan (IRT-LP) to assess whether 9-ING-41 would also show increased efficacy when combined with this recently approved therapy for PDAC. Following implantation of the tumors, mice were monitored for tumor growth and then randomized and treated twice a week for 4 weeks (Supplementary Fig. S3A). Following the 4-week treatment, animals were monitored and euthanized when IACUC endpoints were met. All 4 vehicle-treated animals succumb to their tumors within 1-week following treatment, whereas animals treated with 9-ING-41, gemcitabine, or IRT-LP monotherapy survived slightly longer and varied by cell line and their sensitivity to gemcitabine or IRT-LP (Supplementary Fig. S3B). Combining 9-ING-41 with either gemcitabine or IRT-LP significantly extended survival compared with the monotherapy treatment in all 4-cell line models examined (Supplementary Fig. S3B). Taken together, these in vivo studies suggest that patients with PDAC may benefit from the combination of 9-ING-41 with existing chemotherapies.

**GSK3 inhibition impairs gemcitabine induced Chk1 activation in PDAC cells**

We next sought to understand the mechanism by which 9-ING-41 could sensitize PDAC cells to gemcitabine. Because gemcitabine induces the DDR pathway through activation of ATR, we initially investigated the phosphorylation of Chk1 (an ATR target) at S345 following gemcitabine treatment. As expected, gemcitabine treatment induced a time-dependent increase in Chk1 S345 phosphorylation in all cell lines examined (Fig. 3A). Consistent with our previous study (26, 27), 9-ING-41 increased the inhibitory phosphorylation of GSK-3 at serine 9 in pancreatic cancer cells (Supplementary Fig. S4A). We next investigated whether treatment with 9-ING-41 or a tool compound GSK-3 inhibitor, Bio, could impair...
gemcitabine-induced phosphorylation of Chk1. Significantly, a 2-hour pretreatment with either 9-ING-41 or Bio abrogated the gemcitabine-induced phosphorylation of Chk1 at both S317 and S345 (Fig. 3B and C). Although it has been shown that Chk1 is a negative regulator of polo-like kinase 1 (PLK1; ref. 35), we only detected a slight change in PLK1 phosphorylation (Supplementary Fig. S4B). Consistent with activation of Chk1 induced by gemcitabine, cell-cycle analysis by PI staining showed significantly increased G1-S and decreased G2-M population in gemcitabine-treated group, whereas GSK-3 inhibition partially abolished the cell-cycle arrest (Supplementary Fig. S5A). To further evaluate whether GSK-3 inhibition restored cell-cycle progression, we monitored EdU incorporation into cells actively synthesizing DNA. Although neither vehicle nor GSK-3 inhibitor treatment affected EdU incorporation, as expected, treatment with gemcitabine led to decreased EdU incorporation in all 3 PDAC cell lines tested (Fig. 3D and E; Supplementary Fig. S5C and S5D). In contrast, pretreatment with either GSK-3 inhibitor prevented the gemcitabine-induced S-phase arrest (Fig. 3D and E; Supplementary Fig. S5C and S5D). Taken together, these data indicate that GSK-3 inhibition abrogates the activation of the ATR-Chk1 DDR leading to S-phase arrest.

GSK-3β regulates the ATR–Chk1 signaling pathway

Because 9-ING-41 and Bio are not totally selective for GSK-3β or GSK-3α, we next sought to determine which of these 2 kinases participated in the activation of the ATR–Chk1 pathway. To accomplish this, we depleted GSK-3β or GSK-3α in PDAC cell lines using siRNA and examined the phosphorylation of Chk1 following gemcitabine treatment. As can be seen in Fig. 4A, depletion of either GSK-3 kinase led to a reduction in gemcitabine-induced Chk1 phosphorylation, with GSK-3β depletion having a more pronounced effect. Because the effect on Chk1 phosphorylation was impacted more by GSK-3β depletion, we next constructed stable GSK-3β knockdown L3.6 and 6741 cells (Fig. 4B). Similar to the siRNA knockdown results, depletion of GSK3β showed a significant effect on Chk1 phosphorylation following gemcitabine treatment when compared with shVector control cells (Fig. 4B). Consistent with these results, GSK-3β

Figure 4.

GSK-3β regulates ATR-dependent phosphorylation of Chk1 in response to gemcitabine treatment. A, PDAC cell lines were depleted of GSK-3α or GSK-3β using siRNA and then treated with gemcitabine (500 nmol/L) for 2 hours. Cell lysates were prepared and immunoblotted with the indicated antibodies. Average signal intensity of pS345 Chk1 was analyzed and expressed as mean ± SEM. *, P < 0.05 gemcitabine versus DMSO. #, P < 0.05 siGSK-3 versus siNT after gemcitabine; n = 3. B, L3.6 and 6741 PDAC cell lines stably depleted of GSK-3β were treated with gemcitabine (500 nmol/L) for 2 hours. Protein lysates were prepared and immunoblotted as indicated. Average signal intensity of pS345 Chk1 was analyzed and expressed as mean ± SEM. *, P < 0.05 gemcitabine versus DMSO in shVector cells. #, P < 0.05 shGSK-3β versus shVector cells after gemcitabine; n = 3. C, WT or GSK-3β KO MEFs were left untransfected or transfected with vector control or WT GSK-3β and treated with gemcitabine (500 nmol/L) for 2 hours. Protein lysates were prepared and immunoblotted as indicated. D and E, L3.6 cells were left uninfected or were infected with a control lentivirus or one that stably depletes GSK-3β and re-expresses a nontargetable kinase-dead or constitutively active GSK-3β cDNA. Cells were then treated with gemcitabine (500 nmol/L) for 2 hours, protein lysates were obtained and immunoblotted as indicated.
knockout mouse embryonic fibroblasts (MEF) transfected with an empty Flag-Vector exhibited remarkable reduction of phosphorylated Chk1 after gemcitabine treatment compared with wild-type (WT) MEF cells (Fig. 4C). Significantly, re-expression of Flag-GSK-3β in GSK-3β knockout cells rescued Chk1 phosphorylation (Fig. 4C). Finally, L3.6 cells engineered to be stably depleted of GSK-3β and expressing either kinase-dead or constitutively active GSK-3β were assessed for gemcitabine-induced activation of Chk1. As can be seen in Fig. 4D and E, stable knockdown of GSK-3β impacted gemcitabine-induced phosphorylation of Chk1, which was not rescued by re-expression of kinase-dead GSK-3β, but was substantially restored in cells expressing constitutively active GSK-3β. Altogether, these data provide genetic evidence that GSK-3β, and to some extent GSK-3α, regulate the gemcitabine-induced DDR signaling pathway leading to ATR-Chk1 activation.

GSK-3 contributes to Chk1 activation through stabilization of TopBP1

ATR-dependent phosphorylation of Chk1 during DNA replication stress depends upon several other signaling proteins including ATR interacting protein (ATRIP), and the trimeric Rad9-Hus1-Rad1 (9-1-1) clamp and topoisomerase ll binding protein (TopBP1; ref. 36). To determine the mechanism by which GSK-3 inhibition impacts ATR-Chk1 activation, we examined the protein levels of TopBP1, ATR, and ATRIP following GSK-3 inhibitor treatment. As can be seen in Fig. 5, A and B, expression of TopBP1 (Fig. 5C). Finally, using the L3.6 reconstituted cell line we found that constitutively active but not kinase-dead GSK-3β could rescue TopBP1 protein levels (Fig. 5D). Taken together, these data suggest that GSK-3 kinase activity is required to stabilize the TopBP1 protein.

9-ING-41 decreases pS317 Chk1 levels in gemcitabine-treated animals

We next assessed whether 9-ING-41 could reduce phospho-Chk1 levels in tissues from gemcitabine-treated animals. Initially,
we performed EdU incorporation and stained 6741 cells with anti-p-Chk1 (pS317) that had been treated with control, gemcitabine, 9-ING-41, or combination therapy. Consistent with our immunoblotting and flow cytometry data, phosphorylation of Chk1 at S317 was induced in response to gemcitabine together with dramatic loss of EdU positive cells as compared with DMSO or 9-ING-41-treated cells (Fig. 6A and B). Significantly, 6741 cells treated with the combination of 9-ING-41 and gemcitabine showed diminished Chk1 phosphorylation and restored EdU incorporation (Fig. 6A and B). We next examined the utility of the pS317 Chk1 antibody in our tissues harvested from the orthotopic model. Although the tissue staining showed an overall increase in background pS317 staining, gemcitabine treatment led to increased pS317 nuclear staining when compared with vehicle- and 9-ING-41-treated mice (Fig. 6C and D). In contrast, animals treated with combination therapy showed lower levels of nuclear pS317 staining. Collectively, these results support the overall mechanism that 9-ING-41 treatment impairs ATR-mediated activation of the DNA damage response leading to Chk1 phosphorylation.

Discussion

In this study, we have found that the combination of gemcitabine with the clinically relevant small molecule GSK-3 inhibitor, 9-ING-41, impacts PDAC tumor growth in vitro and in vivo and significantly prolongs survival of mice bearing orthotopic tumors. Mechanistically, we identify a previously unknown role for GSK-3β kinase activity, and to a lesser extent GSK-3α, in the regulation of the ATR–Chk1 DDR signaling pathway through the stabilization of the critical adaptor molecule TopBP1 (Fig. 7). These findings suggest that 9-ING-41 should be studied in combination with gemcitabine or liposomal-formulated irinotecan for first-line therapy in patients with PDAC. Moreover, our data indicate that 9-ING-41 may overcome gemcitabine resistance in pancreatic cancer.

Although GSK-3β has sometimes been proposed to act as a tumor suppressor in various cancer types through its ability to phosphorylate pro-oncogenic molecules, for example c-Jun, c-Myc, cyclin D1, and β-catenin, leading to their proteasomal degradation (41), we and others have previously demonstrated that GSK-3β is overexpressed in many human malignancies including PDAC, and can be targeted for therapeutic intervention (11, 27, 29, 32, 33, 42). Indeed, in pancreatic cancer, GSK-3 has been implicated in the initiation of pancreatic cancer precursor lesions (16), resistance to chemotherapy (23) and overexpression correlated with reduced survival (21, 30, 12). Herein, we show that the combination of 9-ING-41 with gemcitabine can significantly enhance the survival and tumor killing effect in vivo. Recently, we have also shown that 9-ING-41 can overcome chemoresistance in breast cancer (33), impair tumor growth in renal cell cancer (32), neuroblastoma (43), and glioblastoma (29) suggesting that this clinically-relevant compound could be paired with other chemotherapy to treat several different human malignancies.

The DNA damage response pathway is a signaling network that senses different types of damage and coordinates a response that includes activation of transcription, cell-cycle control, apoptosis, senescence, and DNA repair (44). ATR along with its regulator ATRIP sense single-stranded DNA (ssDNA) such as the ssDNA present at stalled replication forks induced by gemcitabine (45). Chk1 is one of the established substrates for ATR that initiates a

Figure 6. 9-ING-41 reverses Chk1 phosphorylation induced by gemcitabine treatment. A, 6741 cells were grown on coverslips, treated with DMSO, 9-ING-41 (5 μmol/L), gemcitabine (500 nmol/L), or the combination of 9-ING-41 and gemcitabine and pulsed with EdU 1 hour prior to fixation. Fixed cells were subsequently stained with anti-pS317 Chk1 antibodies and detected with an Alexa Fluor 568 conjugated donkey-anti-rabbit secondary (red) and EdU-488 (green). DNA was visualized following Hoechst staining (blue). B, The normalized MFI of nuclear pS317 Chk1, EdU-488, and the percentage of EdU-positive cells were evaluated by ImageJ and expressed as mean ± SEM. *P < 0.05 gemcitabine versus DMSO. #, P < 0.05 gemcitabine and 9-ING-41 versus gemcitabine alone. n = 200 cells per treatment group. C, Immunofluorescence staining of pS317 Chk1 (red) and Hoechst (blue) from orthotopic 6741 PDX tumor tissue sections treated as described in Fig. 2D. D, The normalized MFI of pS317 Chk1 within the nucleus was evaluated by ImageJ and expressed as mean ± SEM. *, P < 0.05 gemcitabine versus Vehicle. #, P < 0.05 gemcitabine and 9-ING-41 versus gemcitabine. n = 200 cells per treatment group.
secondary wave of phosphorylation events that impact signaling networks leading to cell-cycle arrest and DNA repair (46). Several studies have shown that cancer cells lacking ATR or Chk1 are vulnerable to chemotherapeutics including gemcitabine and cytarabine highlighting the possibility that inhibiting the ATR–Chk1 signaling pathway may sensitize tumor cells or overcome resistance to chemotherapies that induce this DNA damage checkpoint (47, 45). Recently, several studies using ATR or Chk1 inhibitors in combination with gemcitabine provided direct evidence that targeting ATR-Chk1 signaling could sensitize PDAC cells to gemcitabine (48, 49). Herein, we observed synergetic tumor killing when 9-ING-41 was combined with gemcitabine or IRF-LP. Surprisingly, we found that GSK-3 inhibition or genetic ablation of GSK-3β indicated phosphorylation. Dashed rectangle for GSK-3β indicates phosphorylation. Circle with red P indicates phosphorylation. Circle with red X indicates TopBP1 degradation.

Figure 7.
Proposed model by which GSK-3 inhibition by 9-ING-41 disrupts the TopBP1/ATR/Chk1 pathway. In response to gemcitabine-induced DNA replication stress, TopBP1/ATR/ATRIP (not shown) complexes are recruited to stalled replication forks where ATR can fully activate Chk1 leading to cell cycle arrest and DNA repair. In the presence of 9-ING-41, TopBP1 protein levels are destabilized thus abrogating the full activation of ATR leading to reduced Chk1 phosphorylation and ultimately impaired cell-cycle arrest and likely DNA repair. Circle with red P indicates phosphorylation. Dashed rectangle for GSK-3β indicates phosphorylation. Circle with red X indicates TopBP1 degradation.

ever-growing list of proteins whose function/stability are regulated by GSK-3β.

In summary, our study identified a heretofore-unknown role for GSK-3β in the regulation of ATR-mediated DDR checkpoint signaling through the stabilization of TopBP1. Moreover, this study provides valuable preclinical data for the inclusion of patients with PDAC in studies of 9-ING-41 given in combination with chemotherapy.

Disclosure of Potential Conflicts of Interest
D.D. Billadeau and A. Ugolkov have ownership interests (including patents) in and are consultants/advisory board members for Actuate Therapeutics, Inc. D.M. Schmitt is an employee of and has ownership interests (including patents) at Actuate Therapeutics, Inc. F.J. Giles has ownership interests (including patents) at Actuate Therapeutics, Inc. A. Koszikowski has ownership interests (including patents) at Actuate Therapeutics, Inc. and StarWise Therapeutics. A.P. Mazar is a consultant/advisory board member for Actuate Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Other (invented drug used in the paper): A.P. Koszikowski
Other (his lab did all the early development work and advanced 9-ING-41 into the clinic. They provided input on all studies with regard to drug formulation, use, delivery and doses): A.P. Mazar

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