Inactivation of Mirk/Dyrk1b Kinase Targets Quiescent Pancreatic Cancer Cells

Daina Z. Ewton, Jing Hu, Maria Vilenchik, Xiaobing Deng, Kin-chun Luk, Ann Polonskaia, Ann F. Hoffman, Karen Zipf, John F. Boylan, and Eileen A. Friedman

Abstract

A major problem in the treatment of cancer arises from quiescent cancer cells that are relatively insensitive to most chemotherapeutic drugs and radiation. Such residual cancer cells can cause tumor regrowth or recurrence when they reenter the cell cycle. Earlier studies showed that levels of the serine/theronine kinase Mirk/dyrk1b are elevated up to 10-fold in quiescent G0 tumor cells. Mirk uses several mechanisms to block cell cycling, and Mirk increases expression of antioxidant genes that decrease reactive oxygen species (ROS) levels and increase quiescent cell viability. We now show that a novel small molecule Mirk kinase inhibitor blocked tumor cells from undergoing reversible arrest in a quiescent G0 state and enabled some cells to exit quiescence. The inhibitor increased cycling in Panc1, AsPc1, and SW620 cells that expressed Mirk, but not in HCT116 cells that did not. Mirk kinase inhibition elevated ROS levels and DNA damage detected by increased phosphorylation of the histone protein H2AX and by S-phase checkpoints. The Mirk kinase inhibitor increased cleavage of the apoptotic proteins PARP and caspase 3, and increased tumor cell kill several-fold by gemcitabine and cisplatin. A phenocopy of these effects occurred following Mirk depletion, showing drug specificity. In previous studies Mirk knockout or depletion had no detectable effect on normal tissue, suggesting that the Mirk kinase inhibitor could have a selective effect on cancer cells expressing elevated levels of Mirk kinase. Mol Cancer Ther; 10(11); 2104–14. ©2011 AACR.

Introduction

Quiescent cancer cells are relatively insensitive to most chemotherapeutic drugs and radiation and can cause tumor recurrence when they reenter the cell cycle. In addition, metastatic cancer cells in the bloodstream may experience a period of dormancy or quiescence while they adapt to their new microenvironment (1). Quiescent tumor cells degrade their polyribosomes, thus blocking translation and reducing total RNA and protein content. These shrunk tumor cells may be able to enter the bores of capillaries (approximately 8 μm diameter) whereas cycling tumor cells are usually much larger (20–30 μm). Because G0 cells have 2N DNA, but total RNA levels lower than G1 or S-phase cells, they are readily identified by 2-parameter flow cytometry (2). Tumor cells could either enter an irreversible G0 state before undergoing apoptosis, terminal differentiation, or senescence, or enter a reversible, terminal differentiation suppressed, true quiescent G0 state or dormant state from which they could resume cycling, like quiescent fibroblasts (3). In our previous studies, cultures of serum-starved cancer cells were enriched in G0 cells (60%–90%; refs. 2, 4) and had up to 10-fold higher protein levels of the activated kinase Mirk/dyrk1b and the CDK inhibitor p27kip1, as well as 16-fold higher levels of the retinoblastoma protein family member p130/Rb2 that sequesters the E2F4 transcription factor, thus preventing entry into cycle. Mirk induction of a set of antioxidant genes kept G0 cells viable by reducing their reactive oxygen species (ROS) levels (2, 5). Oncogenes such as mutant ras, the rapid growth of oncologic inhibition of Mirk kinase could prevent some antioxidant genes from being induced, raising ROS levels, and thus increasing sensitivity to drugs such as gemcitabine, which also raise ROS levels. The difluoro-deoxyctydine gemcitabine is used for treatment of pancreatic cancer. ROS production plays a role in the drug’s cytotoxicity, as gemcitabine doubled the levels of ROS in T3M4 pancreatic cancer cells, whereas reduction of ROS by the free-radical scavenger N-acetylcysteine decreased...
Materials and Methods

Materials
Affinity-purified polyclonal antibody to the Mirk unique C terminus was described previously (10). Antibodies to activated phosphorylated pH2AX, LC3β, cleaved caspase 3, Dyrk1A, cleaved PARP, bromodeoxyuridine (BrdU), and the senescence-associated β-galactosidase (β-gal) were from Cell Signaling. Gemcitabine (Gemzar) was from Eli Lilly. All other reagents including cisplatin (11). 

RNA interference
Pancreatic cancer cell pools SU86.86/shMirk, Panc1/shMirk, and SU86.86/sh-Luc stably expressed doxycycline-inducible short hairpin RNAs (shRNAs) to Mirk mRNA sequences starting at base pair 1,699 in exon 4, Mirk mRNA sequences starting at base pair 1,699 in exon 11, and the nonmammalian luciferase gene, respectively. Synthetic RNA interference (RNAi) duplexes to Mirk were from Invitrogen. The RNAi 5'GTGGTGAAAGCCCTATGATCAT-3' targeted a sequence in Mirk exon 5. Transfections were done as in ref. 2.

Assay of senescence
Confluent cultures of late-passage human diploid fibroblasts, strain BJ, were assayed for senescence-associated β-galactosidase activity at pH 6.0 by histochemistry (11).

Quantitation of Western blots
Densitometry analysis of scanned autoradiographs was carried out by UN-SCAN-IT gel software (Silk Scientific).

Flow cytometry
For analysis of DNA content only, cells were fixed with 70% ethanol and then treated with RNase A, before a minimum of 10,000 propidium iodide (PI) stained cells were analyzed by the LSR II. For determination of DNA and RNA content to distinguish G0 from G1 cells, 2-parameter cell-cycle analyses were carried out on cells fixed in ice cold 70% ethanol, then washed and Hoechst 33258 added to bind to DNA and block DNA staining by Pyronin Y, followed by Pyronin Y exactly as detailed (2). Separate and simultaneous analyses of intracellular DNA content and BrdU uptake were carried out on a FACScan flow cytometer (Beckton Dickinson). After a 1-hour BrdU
**A**

**PI for DNA Only**

- Log
- Quiescent
- Quies->Release
- Release->Noco

**Hoechst/Pyronin Y for DNA + RNA**

- Log
- Quiescent
- Quies->Noco

**Autophagy and apoptosis**

- Caspase 3
- Actin
- LC3

**B**

**Hoechst/Pyronin Y for DNA + RNA**

- Control
- +Gemcitabine 1 day
- +Gemcitabine 2 days
- +Gemcitabine 4 days

**PI for DNA only**

- Control
- +Gemcitabine 3 days
- +Gemcitabine 4 days
- +Gemcitabine 5 days

**Viable cells in 10 mL by dye exclusion**

- Days treated

- Control
- +Gemcitabine

**DNA content**

- 2N
- 4N
- apop

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**Molecular Cancer Therapeutics**

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pulse (10 μmol/L), harvested cells were fixed with 70% ethanol, treated with an anti-BrdU-FITC conjugated antibody (BD Biosciences), and stained with 0.01 mg/mL of PI (BD Biosciences). PI and BrdU were excited at 488 nm. A total of 3 × 10^4 cells/sample were analyzed at a rate of 100 to 200 cells/s. Data were analyzed by FlowJo software.

Results

Pancreatic cancer cells can enter a reversible G0 quiescent phase where they exhibit low levels of apoptosis and senescence

Quiescent cells degrade their polyribosomes allowing G0 cells to be distinguished from G1 cells by their 2N DNA content, but lower RNA content, by 2-parameter flow cytometry. Tumor cell lines that contained an active Mirk kinase—SU86.86, Panc1, AsPC1, SW620—were studied (2), as Mirk is activated by oncogenic K-ras (3). Quiescence was induced in SU86.86 pancreatic cancer cells by 3 days of serum starvation, accumulating 50% to 60% of cells in G0, whereas in cultures maintained in log phase growth only approximately 5% of cells were in G0 (Fig. 1A). The arrest was reversible, because only 1% of cells remained in G0 after quiescent cultures were placed in fresh growth media containing 10% FBS for 24 hours. Most cells had traversed the cell cycle and were either arrested in G2 + M by the mitotic inhibitor nocodazole or had slipped through mitotic arrest to a 4N + state (Fig. 1A). Only after PI staining, analysis of parallel cultures by 1-parameter flow cytometry for DNA content showed similar findings that under serum deprivation most cells have a 2N DNA content (G0 or G1); most quiescent cells released into growth medium moved through the cell cycle to be arrested by nocodazole in G2 + M, and few apoptotic sub-G0–G1 cells were seen. Similarly, 50% to 60% of Panc1 pancreatic cancer cells were accumulated in G0 by 3 days of serum starvation and the arrest was reversible when the quiescent cells were released into fresh growth medium (Fig. 2B, insets). To further define the quiescent state, biochemical markers for apoptosis, senescence, and autophagy were examined. Few sub-G0–G1 SU86.86 cells or Panc1 cells were seen by either cytometric analysis in quiescent cultures, consistent with only a 2-fold increase in the low level of the apoptotic protein cleaved caspase 3, (Fig. 1A, right). Next, measurement of senescence-associated β-galactosidase activity at pH 6.0 (13) by histochemistry showed only 0.5% (1 of 194) and 1.8% (3 of 163) senescent cells in cultures of quiescent SU86.86 cells and quiescent Panc1 cells, respectively. The positive control was confluent, late-passage BJ human diploid fibroblast cultures containing 66% (125 of 189) senescent BJ fibroblasts. Autophagy has been convincingly associated with ovarian cancer cell dormancy as cells reduce their rRNA and protein levels (14). Panc1 cells and SU86.86 cells in log phase growth exhibited low levels of the autophagic marker LC3-β-II, which were strongly increased when cells became quiescent (Fig. 1A, right; controls for autophagy induction in Supplementary Fig. S1). Thus, few quiescent cells were lost by apoptosis or senescence, but cells underwent autophagy to reduce their size and metabolic activity.

G0 cells are maintained after many cycling cells are killed by gemcitabine

Quiescent cancer cells in vivo are a clinical problem because of lack of sensitivity to chemotherapeutic drugs. To test whether the quiescent G0 cancer cells found in tissue culture had this property and so modeled the in vivo state, SU86.86 cells were either treated with gemcitabine at the same time as they were made quiescent by culture in low serum medium (Fig. 1B, left), or first made quiescent and then treated with gemcitabine (Fig. 1B, right). After 24 hours, the fraction of G0 cells increased compared with cultures in normal growth medium. However, most gemcitabine-treated cells remained quiescent (Fig. 1B, right). When the culture was already quiescent, then treated with gemcitabine for 1 to 5 days, the G0 fraction increased and dead sub-G0 cells accumulated as the number of viable cells decreased (Fig. 1B, right). Thus, SU86.86 cell cultures that remained after gemcitabine treatment contained G0 quiescent cells. Entry into quiescence may be a general stress response as ovarian cancer cells starved of FBS or nutrients accumulated in G0 (17).

Figure 1. Pancreatic cancer cells can enter a reversible quiescent G0 state, and G0 cells are enriched in cultures after gemcitabine treatment. A, top, SU86.86 pancreatic cancer cells in log phase growth, cultured in DMEM + 0.2% FBS for 3 days to accumulate G0 quiescent cells, and quiescent cells (Quiex) released for 24 hours into fresh growth medium containing 10% FBS to induce cycling cells in G2 + M, with analysis only for DNA content following PI staining. The quiescent released culture contains no nocodazole. Bottom, similar experiment with cells designated as G0 (arrow), G1, S, G2 + M, and 4N DNA content (G0, gold; G1, dark blue; S, purple; G2 + M, red) as measured by 2 component analysis by flow cytometry after staining DNA with Hoechst dye and RNA with Pyronin Y. RNAase treatment eliminated the Pyronin Y binding. Right, cultures of log and quiescent (qui) SU86.86 cells were analyzed by Western blotting for cleaved caspase 3 forms of 19 and 17 kDa, and the autophagy markers formed I and II of LC3-II, which were strongly increased when cells became quiescent (Fig. 1A, right; controls for autophagy induction in Supplementary Fig. S1). Thus, few quiescent cells were lost by apoptosis or senescence, but cells underwent autophagy to reduce their size and metabolic activity.

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Characterization of Mirk kinase inhibitor in cells

Mirk kinase phosphorylates several different cell-cycle regulators to maintain cells in quiescence. Mirk destabilizes cyclin D isoforms by phosphorylation at a known ubiquitination site, preventing passage through G1 (4, 18). Mirk phosphorylates and stabilizes the CDK inhibitor p27, which is elevated in quiescent cells (2, 19, 20). Both Mirk/dyrk1B and Dyrk1A phosphorylate LIN52-ser28, which is required for the assembly of the DREAM complex consisting of p130/Rb2 and bound E2F4 and DP1, as well as the core proteins LIN9, LIN37, LIN52, LIN54, and RBBP4 (21). Assembly of the DREAM complex is necessary for cells to enter quiescence. As a result, depletion of Mirk allowed SU86.86 pancreatic cancer cells to escape quiescence by increasing phosphorylation of p130/Rb2, causing loss of sequestered E2F4 and thus disassembly of the DREAM complex (2). The Mirk kinase inhibitor should have a similar effect if it targets Mirk within cells. The effect of the Mirk kinase inhibitor on cell cycling was compared in 2 colon cancer cell lines, SW620 cells which express Mirk/dyrk1B kinase and Dyrk1A and, as a control for off-target effects, HCT116 cells which only express Dyrk1A (Fig. 2A, Western blot, bottom). Cycling cells were treated with the inhibitor for 24 hours followed by a 1-hour BrdU pulse. Flow cytometry analysis showed that the fraction of S-phase cells was increased 29% by the Mirk kinase inhibitor in SW620 cells, whereas no change was seen in parallel cultures of HCT116 cells, which do not express Mirk/dyrk1B (Fig. 2A). Although the inhibitor was effective against each kinase in vitro, Mirk/dyrk1B, not Dyrk1A, was responsible for the change in the cycling of these tumor cells.

The Mirk kinase inhibitor blocked accumulation of quiescent cancer cells

The Mirk role in allowing cycling tumor cells to enter a reversible quiescent state was compared in Panc1 and AsPc1 cells, which express active Mirk kinase (19). Panc1 cells represent 10% of the pancreatic cancers with an amplified Mirk gene (22), whereas the Mirk gene is not amplified in AsPc1 cells (23). The Mirk kinase inhibitor was added to log phase cultures at the time when they were switched to fresh DMEM (+ 0.2% FBS (Fig. 2B and C). In parallel cultures without inhibitor, approximately half of the Panc1 or AsPc1 cells accumulated in G0, whereas in cultures treated with the Mirk kinase inhibitor only 14% of Panc1 cells and 29% of AsPc1 cells were found in G0. In 4 replicate experiments the Mirk kinase inhibitor blocked these cancer cells from remaining in G0 during serum starvation. Treatment with the Mirk kinase inhibitor, like Mirk depletion (2), increased amounts of cyclin D1 by 3-fold (data not shown), consistent with cells escaping into cycle. Thus, Mirk kinase activity was necessary for Panc1 and AsPc1 pancreatic cancer cells to accumulate in a quiescent state. In contrast, treatment of either of the 2 strains of human diploid fibroblasts with the Mirk kinase inhibitor at a range of concentrations did not increase their cycling (Hu and Friedman, manuscript in preparation). Diploid fibroblasts express very low levels of Mirk like most other normal tissues (10), in contrast to the elevated levels of Mirk found in most pancreatic cancers (19).

Mirk depletion in Panc1 cells also reduces the fraction of quiescent cells

If the increase in cycling caused by the Mirk kinase inhibitor in cancer cells was due to off-target effects, Mirk depletion would not alter the fraction of G0 cells. Panc1/shMirk cells were serum-starved; at the same time they were depleted of Mirk by induction of the shRNA construct to exon 11. Even though Mirk depletion required the turnover of the rather stable Mirk protein (t1/2 > 5 hours), Mirk depletion, like Mirk kinase inhibitor treatment, increased the fraction of cells in S and G2 + M phases, showing that the increase in cell cycling was not due to off-target effects (Fig. 2B, bottom, insets).

Inhibition of Mirk kinase allows some quiescent tumor cells to enter cycle

Panc1, AsPc1, and SU86.86 cultures were serum-starved to accumulate G0 cells. In each line, addition of fresh nutrients plus very limited FBS (0.2%) was enough to move a mean of 15% ± 4% of the cells out of G0. However, approximately twice as many cells left G0, 29% ± 5%, if the quiescent cultures were switched to fresh DMEM + 0.2% FBS also containing the Mirk kinase inhibitor (Fig. 2D). Because Mirk phosphorylates at least 3 cell-cycle regulators, each of which mediates arrest in G0, inhibition of one or more of these activities may be sufficient to move cells into cycle.

DNA damage in quiescent pancreatic cancer cells is increased either by treatment with the Mirk kinase inhibitor or by Mirk depletion through targeting either exon 11 or exon 5

Mirk increases expression of the antioxidants superoxide dismutases (SOD2 and SOD3), which reduce superoxide levels, and the ferroxidase ceruloplasmin that prevents the formation of hydroxyl ions, probably through its transcriptional coactivator functions (24). As a result, Mirk depletion increased total ROS (2). Similarly, treatment of Panc1 cells by the Mirk kinase inhibitor led to a dose-dependent increase in total ROS, which activate the DCFA fluorochrome (Fig. 3A, right), and an increase in superoxide ions, which activate the dihydroethidium fluorochrome (Fig. 3A, left). The increase in superoxides by the Mirk kinase inhibitor shows its specificity in intact cells. Mirk increases expression of SOD2 and SOD3, but none of the other 4 kinases that are blocked by the Mirk kinase inhibitor in vitro have any reported antioxidant activity (Materials and Methods). Increased ROS levels can lead to cell death, and time-lapse studies of Mirk kinase inhibitor treated Panc1 cells showed flattening and intracellular vesicle formation within 40 hours, with marked cell loss by 86 hours (Supplementary Fig. S2). Death occurred by apoptosis.

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Figure 2. The Mirk kinase inhibitor exhibits specificity by increasing cycling only of cancer cells expressing Mirk/Dyrk1B and blocks many Panc1 and AsPc1 cells from entering quiescence, while allowing some cancer cells to escape quiescence. A, SW620 (Dyrk1A and Mirk/dyrk1B expressing) and HCT116 (only Dyrk1A expressing) cells treated with 1 μmol/L Mirk kinase inhibitor for 24 hours with a 1-hour pulse of BrdU, before analysis by flow cytometry after PI staining of DNA. Bottom, lysates of SW620 cells and HCT116 cells were immunoblotted for Mirk/Dyrk1B and the related kinase Dyrk1A. Dyrk1B migrates as a 69/65 kDa dimer and was not detected in HCT116 cells. Structure of the Mirk kinase inhibitor is shown. B, analysis by 2-parameter flow cytometry. Insets on right show the DNA distribution by Hoechst staining. Top, Panc1 cells in log phase growth. Middle, quiescent Panc1 cells. Inset shows DNA profile of cultures released for 48 hours into growth medium plus 100 ng/mL nocodazole. Bottom, log phase Panc1 cells were switched to DMEM + 0.2% FBS and cultured for 3 days with 0.25 μmol/L Mirk kinase inhibitor; right, Panc1/sh-Mirk pancreatic cancer cells depleted of Mirk while being made quiescent by culture in DMEM + 0.2% FBS for 2 days. Bottom, parallel cultures treated with 0.25 μmol/L of Mirk kinase inhibitor for 2 days. Mirk kinase inhibitor treatment for 3 days left 10% of cells in G0. D, quiescent Panc1, AsPc1, and SU86.86 cells were switched to fresh DMEM + 0.2% FBS with a parallel set also treated with 0.25 μmol/L Mirk kinase inhibitor before analysis by 2-parameter flow cytometry as in Fig. 1A. The fractions of cells in G0 are listed. One of the duplicate experiments with similar results is shown.
at least in part, because after treatment with the Mirk kinase inhibitor, activation of caspase 3 was seen in 20 cell lines derived from pancreatic, colon, or breast cancers (data not shown).

ROS are known to damage cellular components such as DNA. Histone protein H2AX molecules within the chromatin at a double-stranded DNA break site become phosphorylated on a serine near the carboxyl terminus. Many phosphorylated H2AX (γH2AX) molecules are found at such a break, creating a focal site for accumulation of proteins involved in DNA repair and chromatin remodeling within a short time of DNA damage (25). Panc1 cells and AsPc1 cells were treated with a range of concentrations of the Mirk kinase inhibitor for 2 days while being made quiescent by serum starvation (Fig. 3B). In Panc1 cells, a sharp 8-fold increase in double-stranded DNA breaks identified by γH2AX and increased cleavage of apoptotic proteins PARP and caspase 3 occurred when the inhibitor concentration reached 0.25 μmol/L, with a concomitant loss in activation of the survival protein Akt. Increased levels of γH2AX were also seen at nuclear foci in Mirk kinase inhibitor treated cells by fluorescence microscopy (not shown). The addition of gemcitabine together with the Mirk inhibitor resulted in more intense H2AX phosphorylation indicating that the inhibitor sensitizes Panc1 cells to gemcitabine (Fig. 3B, right lanes).

At more elevated levels of the Mirk inhibitor (0.5 μmol/L) increased autophagy was seen, with more cleavage of LC3-II. No alteration in the amount of Mirk protein was observed with the inhibitor, as expected, because the inhibitor functions by binding to Mirk kinase. In AsPc1 cells, Mirk kinase inhibitor treatment also led to DNA breaks identified by phosphorylated γH2AX and to apoptosis as measured by increased levels of the cleaved PARP and caspase 3, with the maximum effect at 0.25 to 0.5 μmol/L (Fig. 3B).

To test the possibility that the double-stranded DNA breaks and apoptosis seen after treatment of quiescent cancer cells with the Mirk kinase inhibitor were due to off-target effects, their analysis was repeated after depletion of Mirk. Panc1 cells were made quiescent while being depleted of Mirk by doxycycline induction of a stably transfected shRNA construct to exon 11 (Fig. 4A) or by transient transfection of synthetic RNAi duplexes to Mirk exon 5 (Fig. 4B). When Mirk was depleted in G0 cells the low levels of γH2AX increased (Fig. 4A and B, quiescent lanes). The γH2AX levels were 2- to 5-fold higher when the Mirk-depleted cells were released from quiescence by culture in fresh growth medium for 24 hours, and some unrepaired γH2AX marked lesions remained after 48 hours (Fig. 4A, 0 drug). Gemcitabine treatment is known to create γH2AX by the indirect mechanism of replication
fork collapse (26). As the positive control, gemcitabine-treated cycling Mirk-depleted cells had higher $\gamma$H2AX levels than undepleted cells. Doxycycline itself had no effect on $\gamma$H2AX formation when a doxycycline-inducible shRNA to the unexpressed luciferase gene was examined (Supplementary Fig. S4). Thus, inhibiting Mirk kinase or depleting it in quiescent tumor cells led to increased DNA damage, which could be increased by gemcitabine treatment.

DNA damage activates a signaling pathway through ATR that arrests cells at S-phase checkpoints (16, 27). When quiescent Panc1 cells, either undepleted or Mirk-depleted, were allowed to reenter cycle by a switch to growth medium containing the mitotic inhibitor nocodazole, the undepleted cells traversed one cell cycle to arrest in G2 + M, whereas the Mirk-depleted cells were unable to completely traverse S-phase indicating that Mirk depletion alone in quiescent cells caused DNA damage. When quiescent Panc1 cells, undepleted or Mirk-depleted, were allowed to reenter cycle by a switch to growth medium containing gemcitabine, many undepleted cells arrested throughout S-phase at checkpoints as expected (Fig. 4C, bottom). In contrast, the Mirk-depleted Panc1 cells arrested at very early S-phase checkpoints, suggesting more DNA damage. Similarly, gemcitabine induced more S-phase checkpoints in a second pancreatic cancer cell line, Mirk-depleted SU86.86 cells compared with undepleted cells, when both were released from quiescence (Supplementary Fig. S3). Thus, Mirk depletion in quiescent tumor cells led to DNA damage that was unrepaired when cells reentered the cell cycle, sensitizing them to additional DNA damage by gemcitabine.

**The Mirk kinase inhibitor sensitizes cells to gemcitabine and cisplatin**

Although the lowest concentration of Mirk kinase inhibitor that optimally induced DNA damage and apoptosis in quiescent Panc1 cells was 0.25 $\mu$mol/L, the lower concentration of 0.12 $\mu$mol/L was sufficient when gemcitabine was added at the same time, suggesting an additive effect (Fig. 3B). Thus, treatment with the Mirk kinase inhibitor should increase the toxicity of gemcitabine. Two assays were used: metabolism of MTT to measure growth arrest after 2 days of exposure and viable cell number by dye exclusion analysis after 4 days of treatment. Gemcitabine at 0.3 $\mu$mol/L caused a small growth arrest, decreasing cell numbers by only 25%, whereas addition of Mirk kinase inhibitor up to 0.12 $\mu$mol/L decreased cell number by MTT metabolism to 65% of control values. After 4 days, 0.3 $\mu$mol/L gemcitabine reduced viable cell numbers by 3-fold, whereas addition of Mirk kinase inhibitor further reduced cell number by approximately 2-fold (Fig. 5A). Similarly, addition of Mirk kinase inhibitor to 0.1 $\mu$mol/L gemci-
tabine for 2 days decreased cell number by MTT metabolism by 4-fold, from 16% to 65% of control values, and after 4 days increased cell kill measured by direct cell counting by approximately 2-fold. Thus, pharmacologic inhibition of Mirk kinase increased the tumor cell kill by gemcitabine. Similar studies tested whether the Mirk kinase inhibitor would potentiate the toxicity of cisplatin toward Panc1 cells and SW620 colon carcinoma cells. The EC50 of cisplatin toward Panc1 cells was decreased from approximately 4 to 0.7 μmol/L, whereas the EC50 of cisplatin toward SW620 cells was decreased from approximately 2.5 to 0.4 μmol/L (Fig. 5B). Thus, the small-molecule Mirk kinase inhibitor increased gemcitabine or cisplatin toxicity by an average of approximately 5-fold in 2 different tumor cell lines. These results are consistent with our earlier studies in which Mirk depletion enhanced the sensitivity of ovarian cancer cells to cisplatin (5).

**Mirk depletion sensitizes quiescent pancreatic cancer cells to killing by gemcitabine**

To confirm the specificity of the Mirk kinase inhibitor targeting in intact cells, the capacity of Mirk depletion to sensitize 2 pancreatic cancer cell lines to gemcitabine was tested by using cell viability assays to ensure that cell death, not growth arrest, was measured. Quiescent Panc1/shMirk cells were depleted of Mirk while being treated over 4 days with gemcitabine. Depletion of Mirk led to approximately a 2-fold loss of Panc1 cell viability, a statistically significant difference with P = 0.0051 by the Student paired t test (Fig. 6A). Trypan blue exclusion assays showed that induced depletion of Mirk in
Discussion

In this study pharmacologic inhibition of Mirk kinase sensitized pancreatic cancer cells to gemcitabine and cisplatin, increasing cell kill up to 5-fold. This drug sensitization was also seen following depletion of Mirk using different targets of RNA interference and thus confirmed the correct targeting by the kinase inhibitor within cells. Significantly, because Mirk is most highly expressed in quiescent pancreatic cancer cells, targeting Mirk may enable gemcitabine to kill these out of cycle cells in 2 ways: by damaging them in G0 by increasing their ROS levels and then by forcing them back into cycle before repair. In support of this hypothesis, pharmacologic inhibition of Mirk kinase blocked most cycling Panc1 and AsPc1 cells from becoming quiescent in response to suboptimal growth conditions, and moved some pancreatic cancer cells out of the quiescent state. Mirk transcriptional coactivator activity increases expression of antioxidant genes, thus reducing ROS levels and increasing quiescent tumor cell viability (2). In this study, inhibition of Mirk kinase in quiescent cells increased their ROS levels. Mirk induces SOD2 and SOD3, which reduce superoxides, and the Mirk kinase inhibitor increased superoxide levels, again showing inhibitor specificity. ROS damage DNA, and a marker of double-stranded DNA breaks, phosphorylated histone H2AX, was elevated in quiescent Panc1, SU86.86, and AsPc1 pancreatic cancer cells in which Mirk kinase was inhibited or Mirk was depleted. Loss of the Mirk capacity to increase antioxidant gene expression would lead to more ROS and thus to more DNA damage. Targeting Mirk may appear controversial because a recent survey of the kinome by a synthetic lethal RNAi screening strategy revealed several kinases, but not but Mirk/dyrk1B, capable of sensitizing MiaPaCa cells to gemcitabine (28). Because Mirk is not expressed in MiaPaCa cells, the omission is understandable. MiaPaCa cells model the 10% of resected human pancreatic adenocarcinomas that do not express Mirk protein (19). In addition, most screening studies use cycling cells, and Mirk protein levels and kinase activities are highest in cells in G0 and quite low in cycling cells (29).

Mirk is an unusual kinase in that it is widely expressed at very low levels in most normal adult tissues suggesting a noncritical function (10). In support of this interpretation, depletion of the already low levels of Mirk in fibroblasts caused no detectable decrease in viability as measured by clonogenic assays (12), or by dye exclusion tests (17). Mirk levels are elevated in skeletal muscle and testes, but embryonic knockout of the Mirk/dyrk1B gene resulted in viable, fertile mice with normal skeletal muscle development, so other genes must compensate for loss of Mirk (30, 31). In contrast, the Mirk gene is amplified in a subset of pancreatic cancers, Mirk expression is upregulated in other pancreatic cancers (19), and Mirk/dyrk1B mediates survival in pancreatic cancer cells (19). This difference in sensitivity to Mirk depletion suggests that targeting Mirk in pancreatic cancers might limit most effects to the cancer cells themselves.
Disclosure of Potential Conflicts of Interest

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References


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# Molecular Cancer Therapeutics

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