Published in final edited form as:

Mol Cancer Ther. 2011 November ; 10(11): 2104–2114. doi:10.1158/1535-7163.MCT-11-0498.

Inactivation of Mirk/Dyrk1b Kinase Targets Quiescent Pancreatic Cancer Cells *

Daina Z. Ewton¹, Jing Hu¹, Maria Vilenchik², Xiaobing Deng¹, Kin-chun Luk², Ann Polonskaia², Ann F. Hoffman², Karen Zipf², John F. Boylan², and Eileen A. Friedman^{1,3}

¹Pathology Department, SUNY Upstate Medical Univ., Syracuse, NY

²Discovery Oncology, Discovery Chemistry and Discovery Technologies, Hoffmann-La Roche, Nutley, NJ

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 re-enter the cell cycle. Earlier studies demonstrated that levels of the serine/threonine kinase Mirk/dyrk1B are elevated up to 10-fold in quiescent G0 tumor cells, that Mirk uses several mechanisms to block cell cycling, and that Mirk increases expression of antioxidant genes which lower 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 prior 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.

Keywords

quiescence; pancreatic cancer; Mirk; Dyrk1B; gemcitabine

Introduction

Quiescent cancer cells are relatively insensitive to most chemotherapeutic drugs and radiation and can cause tumor recurrence when they re-enter 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 (about 8µm diameter) while cycling tumor cells are usually much larger (20–30µm). Since G0 cells have 2N DNA but total RNA levels lower than G1 or S phase cells, they are readily identified by

*This work was supported by National Institutes of Health Grant 5R21CA135164 (to E.F.), the Jones/Rohner Foundation (E.F.), and Hoffmann La Roche.

Address correspondence to: Eileen Friedman, Ph.D., SUNY Upstate Medical University, Department of Pathology, 2305 Weiskotten Hall, 750 East Adams Street, Syracuse, New York 13210; Tel: 315-464-7148; FAX 315-464-8419; friedmae@upstate.edu.

2-parameter flow cytometry (2). Tumor cells could either enter an irreversible G0 state prior to undergoing apoptosis, terminal differentiation or senescence, or could 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 prior studies, cultures of serum-starved cancer cells were enriched in G0 cells (60–90%) (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 ROS levels (2), (5). Oncogenes such as mutant ras, the rapid growth of tumor cells, and metabolic stress all raise ROS levels (6). Mirk depletion increased ROS levels in each of 4 lines of ovarian cancer cells, enabling low doses of the chemotherapeutic drug cisplatin (which by itself increases ROS levels) to raise cellular ROS to toxic levels (5). Pharmacological 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 difluorodeoxycytidine 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, while reduction of ROS by the free radical scavenger N-acetylcysteine decreased the growth inhibition caused by gemcitabine (7). Moreover, when 10 pancreatic cancer cell lines were surveyed for ROS levels, the lines with higher endogenous ROS levels were more sensitive to the cytotoxic effects of gemcitabine, suggesting that gemcitabine further increases the already high endogenous ROS levels of tumor cells to toxic levels (7). In patients the response rate to gemcitabine is less than 20% because of drug resistance caused by various mechanisms including reduced gemcitabine metabolism (reviewed in (8)), and decreased delivery of gemcitabine because of poor perfusion of the tumor because of its thick desmoplastic stroma (9). Stratagems to increase delivery of gemcitabine in animal models by inhibiting stromal hedgehog signaling have shown some success (9). Thus identifying methods to increase gemcitabine toxicity towards pancreatic cancer cells is timely. In the following study we tested whether inactivation of Mirk kinase in quiescent cells using a novel small molecule inhibitor increased pancreatic cancer cell sensitivity to gemcitabine.

Methods & Materials

Materials

Affinity-purified polyclonal antibody to the Mirk unique C-terminus was described previously (10). Antibodies to activated phosphorylated γ H2AX, LC3 β , cleaved caspase 3, Dyrk1A, cleaved PARP, BrdU and the senescence-associated β -galactosidase assay kit were from Cell Signaling. Gemcitabine (gemzar) was from Eli Lilly. All other reagents including cisplatin were from Sigma. The human cancer cell lines were obtained from ATCC, with new stocks obtained in 2010 and not further authenticated, and maintained in DMEM supplemented with 2 mM glutamine, antibiotics and 10% heat-inactivated fetal bovine serum. SU86.86, Panc1, AsPc1, SU86.86/shMirk and Panc1/shMirk cells were negative for mycoplasma (Sigma test kit).

Mirk kinase inhibitor

The Mirk kinase inhibitor was identified from a Roche generic kinase inhibitor library by screening an Ambit kinase library (KINOMEScan) of 317 kinases. Compound A was the most selective inhibitor among the various hits when compared by a Caliper kinase profiling assay. A series of analogs of compound A was synthesized. These analogs were assayed for IC₅₀ on purified Dyrk1B and Dyrk1A using a synthetic substrate, FITC-RRRFRPASLRGPPK, and for EC₅₀ and EC₉₀ on SW620 colon carcinoma cells (K. Luk,

manuscript in preparation), and the most active analog was used in the current studies. Structure of the Mirk kinase inhibitor is shown in Fig.2A. This analog had an EC₅₀ of 1.9+/-0.2 μM on SW620 cells and an IC₅₀ of 68+/-48 nM on Mirk/dyrk1B and 22+/-8 nM on Dyrk1A. To determine specificity, this Mirk kinase inhibitor was used at the much higher concentration of 10 μM in a kinase assay (Profiler Pro kit, Caliper Life Sciences) and inhibited the activities of DYRK1A, ABL, FLT3, and MARK1, respectively, 88%, 64%, 56% and 73%. The Mirk kinase inhibitor was inactive against the other kinases in the Caliper assay panel: AKT1, AKT2, AMPK, AurA, BTK, CAMK2, CAMK4, CDK2, CHK1, CHK2, CK1d, c-Raf, c-TAK1, Erk1, Erk2, FGFR1, FYN, GSK3β, HGK, IGF1R, INSR, IRAK4, KDR, LCK, LYN, MAPKAPK2, MAPKAPK5, MET, MSK1, MST2, p38α, p70S6K, PAK2, PIM2, PKA, PKCβ, PKCζ, PKD2, PKG1α, PRAK, ROCK2, RSK1, SGK1, SRC, SYK.

Induction of quiescence

Panc1 or SU86.86 pancreatic cancer cells were plated at 4×10⁵ per 60 mm dish in growth medium (DMEM+10% FBS) and allowed to attach overnight. Media was changed to DMEM+0.2%FBS (tetracycline negative FBS if doxycycline-inducible constructs were utilized) and cells were cultured for 2 to 4 days to become quiescent, with 1 μg/ml doxycycline added to induce the short hairpin (sh)Mirk constructs to deplete Mirk, as needed.

RNA interference

Pancreatic cancer cell pools SU86.86/shMirk, Panc1/shMirk and SU86.86/sh-Luc stably expressed doxycycline inducible shRNAs either to Mirk mRNA sequences starting at bp530 in exon 4, Mirk mRNA sequences starting at bp1699 in exon 11 or to the non-mammalian luciferase gene, respectively. Synthetic RNAi duplexes to Mirk were from Invitrogen. The RNAi 5'-GTGGTGAAAGCCTATGATCAT-3' targeted a sequence in Mirk exon 5. Transfections were performed as in (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 performed using 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, two parameter cell cycle analyses was performed 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 performed on a FACScan flow cytometer (Becton Dickinson). After a 1 hr BrdU pulse (10 μM), harvested cells were fixed with 70% ethanol, treated with an anti-BrdU-FITC conjugated antibody (BD Biosciences), and costained with 0.01 mg/ml of PI (BD Biosciences). PI and BrdU were excited at 488 nm. 3 ×10⁴ cells/sample were analyzed at a rate of 100–200 cells/sec. 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 two parameter flow cytometry. Tumor cell lines that contained an active Mirk kinase were studied, SU86.86, Panc1, AsPc1, SW620 (2), as Mirk is activated by oncogenic K-ras signaling (12). Quiescence was induced in SU86.86 pancreatic cancer cells by 3 days of serum starvation, accumulating 50–60% of cells in G0, while in cultures maintained in log phase growth only about 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). Analysis of parallel cultures by one parameter flow cytometry for DNA content only after propidium iodide staining, demonstrated 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, 50–60% 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% (1/194) and 1.8 % (3/163) 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% (125/189) 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.1). 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 cells were first made quiescent and then treated with gemcitabine (Fig.1B right). After 24 hours, the fraction of G0 cells increased compared to cultures in normal growth medium. However, most gemcitabine-treated cells under quiescent culture conditions initially accumulated at the G1/S phase boundary, probably at early S phase checkpoints, as described by others (15), (16). Loss of cycling cells and appearance of sub G0/G1 apoptotic cells were seen with 3–5 days gemcitabine treatment (Fig.1B, left). When the culture was already quiescent, then treated with gemcitabine for 1–5 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).

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 (18),(4). 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 two 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 lower panel). Cycling cells were treated with the inhibitor for 24 hours followed by a 1 hour BrdU pulse. Flow cytometry analysis demonstrated that the fraction of S phase cells was increased 29% by the Mirk kinase inhibitor in SW620 cells, while 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 the 10% of pancreatic cancers with an amplified Mirk gene (22), while 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&C). In parallel cultures without inhibitor, about half the Panc1 or AsPc1 cells accumulated in G0, while 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 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 two 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 short hairpin RNA construct to exon 11. Even though Mirk depletion required the turnover of the rather stable Mirk protein ($t_{1/2} > 5$ hrs), 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, lowest panel 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% $\pm$ 4% of the cells out of G0. However, about twice as many cells left G0, 29% $\pm$ 5%, if the quiescent cultures were switched to fresh DMEM +0.2% FBS also containing the Mirk kinase inhibitor (Fig.2D). Since Mirk phosphorylates at least three 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 3), which reduce superoxide levels, and the ferroxidase ceruloplasmin that prevents the formation of hydroxyl ions, probably through its transcriptional co-activator 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 demonstrates 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 (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.2). Death occurred by apoptosis, at least in part, because after treatment with the Mirk kinase inhibitor, activation of caspase 3 was seen in 20 cell lines derived from either 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 (γ H2AX) 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 two days while being made quiescent by serum-starvation (Fig. 3B). In Panc1 cells a sharp 8-fold increase in double-strand DNA breaks identified by γ H2AX and increased cleavage of apoptotic proteins PARP and caspase 3 occurred when the inhibitor concentration reached 0.25 μ M, with a concomitant loss in activation of the survival protein Akt. Increased levels of γ H2AX 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 μ M) increased autophagy was seen, with more cleavage of LC3 β -II. No alteration in the amount of Mirk protein was observed with the inhibitor, as expected, since the inhibitor functions by binding to Mirk kinase. In AsPc1 cells Mirk kinase inhibitor treatment also led to DNA breaks identified by phosphorylated γ H2AX and to apoptosis as measured by increased levels of the cleaved PARP and caspase 3, with the maximum effect at 0.25–0.5 μ M (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 short hairpin

RNA 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 γ H2AX increased (Fig.4A&B, quiescent lanes). The γ H2AX 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 γ H2AX marked lesions remained after 48 hours (Fig.4A, 0 drug). Gemcitabine treatment is known to create γ H2AX by the indirect mechanism of replication fork collapse (26). As the positive control, gemcitabine-treated cycling Mirk-depleted cells had higher γ H2AX levels than undepleted cells. Doxycycline itself had no effect on γ H2AX formation when a doxycycline-inducible shRNA to the unexpressed luciferase gene was examined (Supplementary Fig.4). 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 (27), (16). When quiescent Panc1 cells, either undepleted or Mirk-depleted, were allowed to re-enter cycle by a switch to growth medium containing the mitotic inhibitor nocodazole, the undepleted cells traversed one cell cycle to arrest in G2+M, while the Mirk-depleted cells were unable to completely traverse S phase indicating Mirk depletion alone in quiescent cells caused DNA damage. When quiescent Panc1 cells, undepleted or Mirk-depleted, were allowed to re-enter cycle by a switch to growth medium containing gemcitabine, many undepleted cells arrested throughout S phase at checkpoints as expected (Fig.4C, lowest panel). 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 to undepleted cells, when both were released from quiescence (Supplementary Fig.3). Thus Mirk depletion in quiescent tumor cells led to DNA damage that was unrepaired when cells re-entered the cell cycle, sensitizing them to additional DNA damage by gemcitabine.

The Mirk kinase inhibitor sensitizes cells to gemcitabine and cisplatin

While the lowest concentration of Mirk kinase inhibitor that optimally induced DNA damage and apoptosis in quiescent Panc1 cells was 0.25 μ M, the lower concentration of 0.12 μ M 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 μ M caused a small growth arrest, decreasing cell numbers by only 25%, while addition of Mirk kinase inhibitor up to 0.12 μ M decreased cell number by MTT metabolism to 65% of control values. After 4 days, 0.3 μ M gemcitabine reduced viable cell numbers 3 fold, while addition of Mirk kinase inhibitor further reduced cell number about 2 fold (Fig.5A). Similarly, addition of Mirk kinase inhibitor to 0.1 μ M gemcitabine for 2 days decreased cell number by MTT metabolism 4 fold, from 16% to 65% of control values, and after 4 days increased cell kill measured by direct cell counting about 2 fold. Thus pharmacological 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 towards Panc1 cells and SW620 colon carcinoma cells. The EC₅₀ of cisplatin towards Panc1 cells was decreased from about 4 μ M to 0.7 μ M, while the EC₅₀ of cisplatin towards SW620 cells was decreased from about 2.5 μ M to 0.4 μ M (Fig.5B). Thus the small molecule Mirk kinase inhibitor increased gemcitabine or cisplatin toxicity an average of about 5-fold in two 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 two pancreatic cancer cell lines to gemcitabine was tested 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 about a 2-fold loss of Panc1 cell viability, a statistically significant difference with $p=0.0051$ by student's paired t test (Fig.6A). Trypan blue exclusion assays showed that induced depletion of Mirk in quiescent SU86.86 cells led to over a 3-fold increase in killing by $0.3 \mu\text{M}$ to $10 \mu\text{M}$ gemcitabine, a statistically significant difference with $p=0.0064$ by student's paired t test (Fig.6B). Similar results occurred by depleting Mirk by targeting a different sequence (Supplementary Fig.4). Placing anchorage-dependent fibroblasts in suspension culture can induce quiescence (3), and loss of adhesion along with serum-starvation increased the fraction of G0 pancreatic cancer cells to about 70%. Quiescent SU86.86 cells and Panc1 cells in suspension reformed small clumps within a day and were killed by lower concentrations of gemcitabine than adherent cells (Supplementary Fig.5). Thus Mirk depletion, like Mirk kinase inhibition, increased the killing of Panc1 and SU86.86 pancreatic cancer cells by gemcitabine.

DISCUSSION

In the current study pharmacological 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, since Mirk is most highly expressed in quiescent pancreatic cancer cells, targeting Mirk may enable gemcitabine to kill these out of cycle cells in two 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, pharmacological 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 the current 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-strand 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 since 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). Since 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

The abbreviations used are

ROS	reactive oxygen species
RNAi	RNA interference
FBS	fetal bovine serum
PARP	poly(ADP-ribose) polymerase
SOD	superoxide dismutase

Acknowledgments

We wish to thank Dr. Dave Heimbrook for helpful discussions.

REFERENCES

- Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. *Science*. 2011; 331:1559–1564. [PubMed: 21436443]
- Deng X, Ewton DZ, Friedman E. Mirk/Dyrk1B maintains the viability of quiescent pancreatic cancer cells by decreasing ROS levels. *Cancer Res*. 2009; 69:3317–3324. [PubMed: 19351855]
- Coller H, Sang L, Roberts JM. A new description of cellular quiescence. *PLOS Biology*. 2006; 4:329–349.
- Jin K, Ewton D, Park S, Hu J, Friedman E. Mirk regulates the exit of colon cancer cells from quiescence. *J Biol Chem*. 2009; 284:22916–22925. [PubMed: 19542220]
- Hu J, Friedman E. Depleting Mirk kinase increases cisplatin toxicity in ovarian cancer cells. *Genes & Cancer*. 2010; 1:803–811. [PubMed: 21113238]
- Trachootham D, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H, et al. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by β -phenylethyl isothiocyanate. *Cancer Cell*. 2006; 10:241–252. [PubMed: 16959615]
- Donadelli M, Costanzo C, Beghelli S, Scupoli MT, Dandrea M, Bonora A, et al. Synergistic inhibition of pancreatic adenocarcinoma cell growth by trichostatin A and gemcitabine. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2007; 1773:1095–1106.
- Kim M, Gallick G. Gemcitabine resistance in pancreatic cancer: picking the key players. *Clin Cancer Res*. 2008; 14:1284–1285. [PubMed: 18316544]
- Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science*. 2009; 324:1457–1461. [PubMed: 19460966]
- Lee K, Deng X, Friedman E. Mirk Protein Kinase Is a Mitogen-activated Protein Kinase Substrate That Mediates Survival of Colon Cancer Cells. *Cancer Res*. 2000; 60:3631–3637. [PubMed: 10910078]
- de Magalhães J, Chainiaux F, Remacle J, Toussaint O. Stress-induced premature senescence in BJ and hTERT-BJ1 human foreskin fibroblasts. *FEBS Lett*. 2002; 523:157–162. [PubMed: 12123824]
- Jin K, Park S-J, Ewton D, Friedman E. The survival kinase Mirk/dyrk1B is a downstream effector of oncogenic K-ras. *Cancer Res*. 2007; 67:7247–7255. [PubMed: 17671193]

13. Dimri G, Campisi J. Molecular and cell biology of replicative senescence. *Cold Spring Harb Symp Quant Biol.* 1994; 59:67–73. [PubMed: 7587128]
14. Amaravadi RK. Autophagy-induced tumor dormancy in ovarian cancer. *J Clin Investigation.* 2008; 118:3837–3840.
15. Cappella P, Tomasoni D, Faretta M, Lupi M, Montalenti F, Viale F, et al. Cell cycle effects of gemcitabine. *Int J Cancer.* 2001; 93:401–408. [PubMed: 11433406]
16. Morgan MA, Parsels LA, Parsels JD, Mesiwala AK, Maybaum J, Lawrence TS. Role of Checkpoint Kinase 1 in Preventing Premature Mitosis in Response to Gemcitabine. *Cancer Res.* 2005; 65:6835–6842. [PubMed: 16061666]
17. Hu J, Nakhla H, Friedman E. Mirk/dyrk1B and p130/Rb2 mediate quiescence in ovarian cancer cells. *International Journal of Cancer.* 2011; 129:307–318.
18. Zou Y, Ewton D, Deng D, Mercer S, Friedman E. Mirk/dyrk1B Kinase Destabilizes Cyclin D1 by Phosphorylation at Threonine 288. *J Biol Chem.* 2004; 279:27790–27798. [PubMed: 15075324]
19. Deng X, Ewton DZ, Li S, Naqvi A, Mercer SE, Landas S, et al. The Kinase Mirk/Dyrk1B Mediates Cell Survival in Pancreatic Ductal Adenocarcinoma. *Cancer Res.* 2006; 66:4149–4158. [PubMed: 16618736]
20. Janumyan Y, Cui Q, Yan L, Sansam CG, Vanlentin M, Yang E. G0 function of BCL2 and BCL-xL requires BAX, BAK, and p27 phosphorylation by Mirk, revealing a novel role of BAX and BAK in quiescence regulation. *J Biol Chem.* 2008; 283:34108–34120. [PubMed: 18818203]
21. Litovchick L, Florens LA, Swanson SK, Washburn MP, DeCaprio JA. DYRK1A protein kinase promotes quiescence and senescence through DREAM complex assembly. *Genes & Development.* 2011; 25:801–813. [PubMed: 21498570]
22. Karhu R, Mahlamaki E, Kallioniemi A. Pancreatic adenocarcinoma- genetic portrait from chromosomes to microarrays. *Genes Chromosomes Cancer.* 2006; 45:721–730. [PubMed: 16688744]
23. Kuuselo R, Savinainen K, Azorsa DO, Basu GD, Karhu R, Tuzmen S, et al. Intersex-like (IXL) Is a Cell Survival Regulator in Pancreatic Cancer with 19q13 Amplification. *Cancer Res.* 2007; 67:1943–1949. [PubMed: 17332321]
24. Mercer S, Friedman E. Mirk/Dyrk1B: a multifunctional dual-specificity kinase involved in growth arrest, differentiation and cell survival. *Cell Biochemistry and Biophysics.* 2006; 45:303–315. [PubMed: 16845176]
25. Bonner WM, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, et al. GammaH2AX and cancer. *Nat Rev Cancer.* 2008; 8:957–967. [PubMed: 19005492]
26. Ewald B, Sampath D, Plunkett W. H2AX phosphorylation marks gemcitabine-induced stalled replication forks and their collapse upon S-phase checkpoint abrogation. *Mol Cancer Ther.* 2007; 6:1239–1248. [PubMed: 17406032]
27. Karnitz LM, Flatten KS, Wagner JM, Loegering D, Hackbarth JS, Arlander SJH, et al. Gemcitabine-Induced Activation of Checkpoint Signaling Pathways That Affect Tumor Cell Survival. *Mol Pharmacol.* 2005; 68:1636–1644. [PubMed: 16126823]
28. Azorsa DO, Gonzales IM, Basu GD, Choudhary A, Arora S, Bisanz KM, et al. Synthetic lethal RNAi screening identifies sensitizing targets for gemcitabine therapy in pancreatic cancer. *J Transl Med.* 2009; 7:43. [PubMed: 19519883]
29. Deng X, Mercer SE, Shah S, Ewton DZ, Friedman E. The Cyclin-dependent Kinase Inhibitor p27Kip1 Is Stabilized in G0 by Mirk/dyrk1B Kinase. *J Biol Chem.* 2004; 279:22498–22504. [PubMed: 15010468]
30. Leder S, Czajkowska H, Maenz B, de Graaf K, Barthel A, Joost H-G, et al. Alternative splicing variants of the protein kinase DYRK1B exhibit distinct patterns of expression and functional properties. *Biochem J.* 2003; 372:881–888. [PubMed: 12633499]
31. Becker W, Friedman E. Dyrk1b. *UCSD-Nature Molecule Pages.* 2008 doi:10.1038/mp.a000797.01.

A Pancreatic Cancer Cells Undergo Reversible Arrest in G0

B G0 Cells Remain After Gemcitabine Treatment

FIGURE 1. Pancreatic cancer cells can enter a reversible quiescent G0 state, and G0 cells are enriched in cultures after gemcitabine treatment

A. *upper panel*: 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 released for 24 hours into fresh growth medium containing 100 ng/ml nocodazole to arrest cycling cells in G2+M, with analysis only for DNA content following propidium iodide staining. The quiescent-> released culture contains no nocodazole. *lower panel*: similar experiment with cells designated as G0 (arrow), G1, S, G2+M and >4DNA content (G0 gold; G1 dark blue; S purple; G2+M red) as measured by two component analysis by flow cytometry after staining DNA with Hoechst dye and RNA with Pyronin Y. RNAase treatment eliminated the Pyronin

Y binding. *right panel* : Cultures of log and quiescent SU86.86 cells were analyzed by western blotting for cleaved caspase 3 forms of 19 and 17 kDa, and the autophagy markers forms I and II of LC3 β .

B. (*left panel*) Cycling SU86.86 cells (8% G0, arrow) were placed in DMEM+0.2% FBS to induce quiescence and concurrently treated with 1 μ M gemcitabine for 1–5 days with analysis by Hoechst/Pyronin staining so G0 cells were detected (20% G0, 60% in G1 and at G1/S boundary; the original data was in color: G0 gold and G1 dark blue, so the G1 cells are rendered a darker gray than the G0 cells when the figure was presented as shades of gray) or by propidium iodide staining which allows apoptotic sub-G0/G1 cells to be quantitated (*, with 3%, 9%, 18% and 25% apoptotic sub-G0/G1 cells, respectively after days 0, 3, 4, or 5 days treatment with 1 μ M gemcitabine). (*right panel*) SU86.86 cells were placed in suspension culture to make cells quiescent. Cultures were trypsinized to single cells, washed, pooled, and 10⁵ resuspended in 20 ml DMEM +0.4% BSA per non-tissue culture 100 mm plates. One set of cultures was treated with 0.3 μ M gemcitabine for 5 days with viable cell numbers determined daily by exclusion of trypan blue dye (n=3).

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 μ M Mirk kinase inhibitor for 24 hours with a 1 hour pulse of BrdU, before analysis by flow cytometry after propidium iodide staining of DNA. Lower panel: 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 shown.

B. Analysis by two parameter flow cytometry. Insets on right show the DNA distribution by Hoechst staining. *top panel*: Panc1 cells in log phase growth. *middle panel*: quiescent Panc1 cells. Inset shows DNA profile of cultures released for 48 hr into growth medium plus 100 ng/ml nocodazole. *lowest panel*: Log phase Panc1 cells were switched to DMEM+0.2%FBS and cultured for 3 days with 0.25 μ M 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 3 days.

C. Upper: Log phase AsPc1 cells cultured in DMEM+0.2%FBS for 2 days. lower: parallel cultures treated with 0.25 μ M 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 μ M Mirk kinase inhibitor before analysis by two parameter flow cytometry as in Fig.1A. The fractions of cells in G0 are listed. One of duplicate experiments with similar results.

A Mirk Kinase Inhibitor Increases ROS, as does Mirk Depletion

B Induction of DNA Breaks and Apoptosis by Mirk Kinase Inhibitor in Quiescent Cells, Enhanced by Gemcitabine

FIGURE 3. Mirk kinase inhibitor increases ROS levels and leads to damaged DNA

A. Panc1 cells were made quiescent in DMEM+0.2%FBS containing 0.1 to 2.5 μM Mirk kinase inhibitor for 3 days and then assayed for release of superoxides by incubation with dihydroethidium, which is oxidized by superoxide to 2-hydroxyethidium (left) or by incubation with CM- H_2DCFA (right), which reacts with hydroxyl radicals and other ROS species. Data from duplicate experiments averaged (mean $\pm$ SE).

B. Quiescent Panc1 cells were treated 2 days with 0.06–1.0 μM Mirk kinase inhibitor with 0.1 μM gemcitabine added to parallel cultures. Lysates were examined by western blotting for histone H2AX phosphorylation, Akt phosphorylation, levels of Mirk and actin, the apoptotic markers cleaved PARP and cleaved caspase 3 of 17 and 19 kDa, and the autophagy markers forms I and II of LC3 β . right panel: Similar study with AsPc1 cells.

FIGURE 4. Mirk depletion, by either induction of a stably transfected short hairpin RNA to Mirk exon 11 or by transient transfection of RNAi duplexes to exon 5, induce double strand DNA breaks in quiescent tumor cells. Mirk depletion allows Panc1 cells to escape quiescence and increases the S phase checkpoints induced by gemcitabine

A. Panc1-shMirk cells were depleted of Mirk by doxycycline induction of shRNA to Mirk exon 11. Cells were released from quiescence by culture in growth medium containing 0–3 μ M gemcitabine. After 24 and 48 hours, lysates were examined as in Fig.3B.

B. Quiescent Panc1 cells were depleted of Mirk by synthetic RNAi duplexes to a sequence in exon 5. Cells were released into growth medium containing 0–1 μ M gemcitabine for 24 hours, then lysates were examined as in “A”.

C. Panc1/sh-Mirk pancreatic cancer cells depleted of Mirk (+DOX) in DMEM+0.2%FBS for 3 days and then switched to growth medium containing 0.3 μ M gemcitabine, with 100 ng/ml nocodazole or nocodazole alone. After 48 hours, DNA content was determined by flow cytometry after propidium iodide staining.

FIGURE 5. The Mirk kinase inhibitor enhances the toxicity of gemcitabine in quiescent pancreatic cancer cells, and enhances the toxicity of cisplatin for cycling cancer cells

A. (upper panels) Quiescent Panc1 cells were treated 2 days with 0.06–2 μ M Mirk kinase inhibitor with 0.3 μ M (left panel) or 0.1 μ M (right panel) gemcitabine added to one set of parallel plates. Cell growth measured by metabolism of MTT, $n=3$ per point, mean $\pm$ SE. (lower panels) Quiescent Panc1 cells were treated 4 days with 0.03–0.5 μ M Mirk kinase inhibitor with 0.3 μ M (left panel) or 0.1 μ M (right panel) gemcitabine added to one set of parallel plates. Cell growth measured by counting viable cell numbers after trypan blue exclusion, mean $\pm$ SD shown if SD $> 5\%$. One of duplicate experiments with similar results shown.

B. Cycling SW620 colon carcinoma cells and Panc1 cells were treated with a range of cisplatin concentrations and 0.6 μ M Mirk kinase inhibitor for 4 days in triplicate in 96 well plates. Cell growth was analyzed by metabolism of MTT, and normalized to 0% kill for the inhibitor alone, and to 100% for the maximum effect on growth.

A Gemcitabine More Toxic in Quiescent Panc1 Cells Following Mirk Depletion

B Quiescent SU86.86 Cells Exhibit Increased Sensitivity to Gemcitabine Following Mirk Depletion

FIGURE 6. Depletion of Mirk by targeting two different exons enhances the toxicity of gemcitabine in two different lines of pancreatic cancer cells made quiescent

A. Mirk was depleted by shRNA directed to exon 11 for 3 days in quiescent Panc1 cells, then were treated for 4 days with gemcitabine in fresh medium. Cell number quantitated by crystal violet staining, with all values plus doxycycline statistically different from uninduced values by students paired t test with $p=0.0051$.

B. Quiescent SU86.86/sh-Mirk cells depleted of Mirk by shRNA to exon 4 for 4 days with gemcitabine added. Trypan blue exclusion assays were performed ($n=3$), with $p=0.0064$ by students paired t test. Mean $\pm$ SD if $SD > 5\%$.