Inhibition of MLK3 Decreases Proliferation and Increases Antiproliferative Activity of Epidermal Growth Factor Receptor (EGFR) Inhibitor in Pancreatic Cancer Cell Lines

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Abstract: Pancreatic adenocarcinoma is associated with advanced presentation and poor survival. Currently approved therapies have minimal effect on patient survival. Pancreatic adenocarcinomas have a high incidence of activated K-RAS, which may confer resistance to epidermal growth factor receptor (EGFR) inhibitors. Mixed lineage kinase-3 (MLK3) is a MAP3K that activates multiple MAPK pathways. The role of MLK3 in the pathophysiology and resistance to therapy of pancreatic adenocarcinoma has not been investigated. MLK3 is over expressed in pancreatic cancer cell lines compared to an immortalized pancreatic epithelial cell line. The requirement of MLK3 for cell proliferation and survival of pancreatic cancer cell lines, PANC-1 and MiaPaCa-2, was investigated using RNA interference (siRNA) and MLK inhibitor, K252a, alone or in conjunction with the EGFR inhibitor, Compound 56. Ablation of expression of MLK3 via siRNA-mediated gene silencing and pharmacological inhibition of MLK3 by K252a each decreased cell viability in both pancreatic cancer cell lines, with a concurrent decrease in the activation of ERK, JNK and AKT. Concomitant inhibition of EGFR and MLK3 induced apoptosis, as evidenced by increased cleavage of PARP and caspase-3. These results suggest that MLK3 plays an important role in survival and proliferation of pancreatic cancer cell lines and that inhibition of MLK3 may enhance the therapeutic efficacy of EGFR inhibitors in the treatment of pancreatic cancer.

Keywords: pancreatic cancer, MLK3, EGFR signalling, K-RAS, kinase inhibitor, proliferation

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**Introduction**

Pancreatic adenocarcinoma constitutes about 90% of pancreatic cancers in humans. Pancreatic cancer is the fourth leading cause of cancer deaths in the United States.\(^1\) Around the globe, over 200,000 individuals will be afflicted by this disease annually. Each year in the United States, nearly 38,000 individuals are diagnosed with pancreatic cancer and over 34,000 succumb to this condition, with a median survival of about 6 months. The 5-year overall survival rate for pancreatic cancer patients is only 5%, due to the advanced stage at presentation and the lack of effective treatments.\(^2\) Novel diagnostic and therapeutic strategies are desperately needed to improve the survival of these patients. Recent data suggest that the molecular portrait of pancreatic cancer is rather complex, involving multiple genetic abnormalities and epigenetic changes.\(^3\) Targeted therapy directed against critical, aberrant signaling pathways represents a promising area of cancer research. However, such therapies have not yet been efficacious, perhaps due to activation of other compensatory signaling pathways that promote proliferation and/or survival. It is important to identify additional signaling pathways that may be exploited to increase the effectiveness of targeted agents for the treatment of pancreatic cancer.

Despite the promise of targeted therapeutic approaches in pancreatic cancer, currently approved therapies, including epidermal growth factor receptor (EGFR) inhibitors, have minimal effect on patient survival.\(^4\) EGFR overexpression is detected in up to 90% of pancreatic tumors, including pancreatic adenocarcinoma.\(^5\) EGFR regulates intracellular signaling processes via the RAS/Raf/MAP kinase pathway, the phosphatidylinositol-3-phosphate kinase (PIP Kinase/PI-3K)/AKT pathway, and the JAK/STAT pathway.\(^6\) Greater than 90% of human carcinomas of the exocrine pancreas have oncogenic, activated K-RAS.\(^7\) Constitutive signaling initiated by mutated K-RAS may bypass inhibition of EGFR, resulting in resistance to EGFR inhibitors, as has been shown in colorectal cancer.\(^8,9\)

An interesting family of proteins, which might regulate effector pathways of K-RAS, are the mixed-lineage kinases (MLKs).\(^10\) MLKs are serine/threonine protein kinases that function as MAPK kinase kinases (MAP3K), to activate c-Jun amino-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 pathways.\(^10-13\) As shown in Figure 1, MLK3 exerts its function presumably by directly phosphorylating MKK4/7 (for the JNK pathway) and MKK3/6 (for the p38 pathway).\(^12,14\) MLK3 is proposed to act as a scaffold in B-Raf mediated ERK activation.\(^15\) Silencing of MLK3 using RNA interference was shown to decrease cell proliferation in several cancer cell lines.\(^15\)

Although MLK3 was previously implicated in neuronal apoptosis,\(^16\) there are several lines of evidence to suggest that MLKs, including MLK3, might contribute to cancer progression. Overexpression of wild type MLK3 can transform NIH3T3 cells.\(^17\) In addition, a relatively high level of MLK3 has been noted in breast cancer cell lines as compared with nontumorigenic mammary epithelial cell lines (Gallo lab, in review). Overexpression of an MLK-like protein, MLTK, was shown to be sufficient to induce cell transformation and formation of fibrosarcoma in a nude mouse model.\(^18\) Pharmacological inhibition of MLK also was shown to cause cell cycle arrest in HeLa cells, preventing cell proliferation.\(^19\) These studies suggest that MLK family proteins, including MLK3, might have a role in the cellular transformation leading to a cancer phenotype.

Since MLK3 regulates cellular functions via known effectors of the K-RAS pathway, including JNK, ERK, and p38, we hypothesized that MLK3 might be a potential cancer therapy target in combination with...
EGFR inhibitors in pancreatic cancer (Fig. 1). Herein, we report that MLK3 expression is significantly higher in human pancreatic cancer cell lines compared to a normal pancreatic ductal epithelial cell line. Inhibition of MLK3 expression using RNA interference or MLK3 activity by pharmacological inhibition, led to a decrease in pancreatic cancer cell viability. Combined inhibition of EGFR and MLK3 decreased cancer cell viability, compared to either agent alone. Based on these results, we propose that MLK3 may be a potential target for therapeutics in pancreatic cancer that could be used in combination with conventional chemotherapeutic agents and/or with other targeted therapies including EGFR inhibitors.

Materials and Methods

Cell lines

Two well-established pancreatic cancer cell lines, PANC-1 and MiaPaCa-2 (both with K-RAS mutations) were used. The MiaPaCa-2 cell line was obtained from the American Type Culture Collection (ATCC) and cultured as recommended. PANC-1 cells were kindly provided by Dr. Justin McCormick (Michigan State University) and were maintained under standard culture conditions. HPDE6-E6E7-c7 cells, an immortalized human pancreatic ductal epithelial cell line with near normal genotype and phenotype, were kindly provided by Dr. Ming Tsao (University Health Network, Toronto, Ontario, Canada) and routinely cultured in keratinocyte serum-free (KSF) medium supplemented by epidermal growth factor and bovine pituitary extract. All media were supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml Penicillin, 100 μg/ml streptomycin and 2 mM glutamine (Life Technologies, Inc). All cells were cultured in 5% CO₂ at 37°C. Cells were plated approximately 12 h prior to addition of inhibitor or transfection of siRNA.

Pharmacological agents

K252a ((9S,10R,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-meth yl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-'kl]pyrrolo[3,4-i][1,6] benzodiazocine-10-carboxylic acid methyl ester, C_{27}H_{21}N_{3}O_{5}) (EMD, Darmstadt, Germany) was used to pharmacologically inhibit MLK3. K252a was dissolved in dimethyl sulfoxide (DMSO). A small molecule EGFR inhibitor, compound 56 (CMP56, 4-[(3-Bromophenyl)amino]-6,7-diethoxyquinazoline), was purchased from EMD (Darmstadt, Germany). CMP 56 was dissolved in DMSO at the appropriate concentrations.

Antibodies

EGFR, ERK, phospho-ERK, AKT, phospho-AKT (473), Poly (ADP-ribose) polymerase (PARP), caspase 3, JNK, phospho-JNK antibodies were obtained from Cell Signaling Technology (Boston, MA). Antibody to phospho-EGFR (T1173) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MLK3 antibody was produced previously.

RNA interference (siRNA)

Synthetic double-stranded RNA (dsRNA) oligonucleotides targeting MLK3 (ID#235 and ID#236), silencer negative control and GAPDH were purchased from Ambion (Austin, TX). Transfection of the gene-specific siRNA plus the two controls was performed using the siPORT NeoFX transfection agent (Ambion) according to the manufacturer’s protocol. Transfection complexes consisting of siRNA and transfection agent were dispensed into 6-well plates. A cell suspension (1 x 10⁶ cells/well) was overlayed onto the RNA complexes and incubated 24 h at 37°C according to manufacturer’s instructions at which time fresh growth media was added. Cells were lysed 48–72 h post transfection and Western analysis was performed as described.

Cell treatment

Pharmacological agents were added to culture medium at the indicated final concentrations, combinations and time course. Cells were plated in duplicate in 6-well plates, and transfected as described above. In transfection experiments, medium was supplemented with inhibitor (K252a) 24 h after transfection, for an additional 24 h. Negative controls lacking inhibitor were set up in all experiments. All the experiments were repeated at least twice.

Western blotting

Cells were washed with ice-cold phosphate-buffered saline, and whole cell extracts were prepared in protein lysis buffer (100 mM Tris HCl (pH 7.4), 20% SDS, 10 mM EDTA, 5 mM EGTA) supplemented with protease inhibitor cocktail 1:200 dilution (Sigma-Aldrich, St. Louis, MO) and 2.0 mM sodium orthovanadate.
Proteins were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in 5% milk, the blots were incubated with antibodies using the following dilutions: MLK3 (1:1000), p-EGFR, p-AKT (Santa Cruz), p-ERK, p-JNK, PARP, caspase 3 (1:250) (Cell Signaling) and actin 1:2000, (Sigma-Aldrich, St. Louis, MO). After washing with Tris-buffered saline containing the detergent 0.01% Tween 20, the membranes were incubated with anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugate antibody (Biorad) at a 1:5000 for 1 h at room temperature and developed using an ECL detection kit (GE Healthcare, Piscataway, NJ). Semi-quantitative measurements of western blots were performed using densitometric analysis (ImageJ gel analysis program, http://rsbweb.nih.gov/ij/). The density of the band corresponding to the protein of interest was normalized to that of the actin band.

MTT assays
Cell viability was determined using the MTT assay as per the manufacturer’s (Sigma-Aldrich) instructions. Briefly, cells were seeded in 96-well plates at a density of 1 × 10^5 cells/plate, grown overnight and exposed to CMP56 or K252a for 24–72 h. After the incubation, 10 µl thiazolyl blue tetrazolium bromide (MTT), was added to the wells. After 3 h in a CO₂-incubator, formazan products were solubilized with 100 µl detergent for a total volume of 300 µl and the optical density was measured at 570 nm. To determine the concentration at which CMP56 and/or K252a substantially decreased cell viability, graded concentrations were added to duplicate plates using the formula (C-T/C) × 100, where T is the optical density of the test plate sample and C is control optical density. All points were read as mean ± SD of readings of three samples.

Caspase 3 activity
The PathScan Sandwich Elisa Kit (Cell Signaling Technology) was used to detect levels of cleaved caspase-3. Total caspase-3 antibody was coated onto a micro-well plate and, following incubation with cellular lysates, the total caspase-3 protein was captured. Following extensive washing, a biotinylated cleaved caspase-3 antibody was added to detect the captured cleaved caspase-3 protein. An HRP-linked streptavidin was then used to recognize the bound detection antibody and an HRP substrate was added to develop color. The magnitude of optical density for the developed color was proportional to quantity of cleaved caspase-3 protein.

Statistical analysis
Quantitative results were expressed as mean ± SEM. Statistical analysis was performed using a two-tailed unpaired t test (between two groups) or a one way analysis of variance (ANOVA) with the computer software SPSS. P < 0.05 was considered statistically significant.

Results
Expression of MLK3 in normal and malignant pancreatic cell lines
The expression of MLK3 in cellular lysates derived from both “normal” and malignant pancreatic cell lines was semi-quantitatively measured after western blot analysis using densitometry (Optical Density units).
As shown in Figure 2, the MLK3 protein level is significantly (nearly 3-fold) higher in both PANC-1 (P) and MiaPaCa-2 (M) pancreatic cancer cell lines than in the immortalized pancreatic epithelial cell line (H).

Inhibition of MLK3 expression in pancreatic cancer cell lines
Using RNA interference, we successfully reduced MLK3 expression in both PANC-1 and MiaPaCa-2 cells. As expected, expression of MLK3 protein in MLK3 siRNA-treated pancreatic cancer cell lines was markedly lower than those in negative control cells (Fig. 3A). The reduction of protein expression was specific as oligonucleotides with a single bp alteration or specific for other targets (GAPDH) were ineffective at reducing the level of MLK3 expression (data not shown). As shown in Figure 3A, the reduction in target protein expression was dependent on the concentration of siRNA. As shown in Figures 3B and 3C, there was a statistically significant reduction in MLK3 expression (MLK3: Actin ratio; 1.0 in control cells vs. 0.39 (30 nM concentration) vs. 0.24 (at 90 nM concentration) in the PANC-1 cells (p < 0.01) and 1.0 in control cell vs. 0.26 (10 nM concentration) vs. 0.14 (at 30 nM concentration) (p < 0.01) in MiaPaCa-2 cell line. Based on these results, 30 nM of MLK3 siRNA was used in further experiments for both pancreatic cancer cell lines.

Inhibition of MLK3 expression and activity leads to decreased cell proliferation
In order to understand the role of MLK3 in cell survival and proliferation in pancreatic cancer cell lines, we investigated the fate of cancer cells after inhibiting MLK3, either by decreasing its expression

Figure 3 A B C

Figure 3. RNA interference decreases MLK3 expression in pancreatic cancer cell lines. Treating pancreatic cancer cell lines with increasing concentrations of MLK3 siRNA (C-Negative control, 1 = 10 nM, 2 = 30 nM, 3 = 90 nM) decreased MLK3 protein expression in a concentration dependent manner, assessed by Western blot assays (A). Protein expression was measured semi-quantitatively using densitometry. Intensities of MLK3 in different cell lines are normalized to that of actin in each cell line and ratios are shown as bar diagrams. Figure B shows bar diagram for PANC-1 cells and Figure C shows a bar diagram for MiaPaCa-2 cells.

Figure 4 A B

Figure 4. Inhibition of MLK3 expression or activity decreased cell viability. After incubating cells in standard culture conditions for 24 h, cells were subjected to RNA interference (siRNA) or MLK3 inhibitor K252a treatments (at two different concentrations: 100 nM or 250 nM). MTT assays were performed as described. Cell viability is shown as percent of negative control in PANC-1 cells (A) and MiaPaCa-2 cells (B).
via siRNA or inhibiting its activity using K252a, which effectively inhibits MLK3 activity.\textsuperscript{21} As shown in Figure 4, cell proliferation was reduced significantly by both MLK3 siRNA (p = 0.001) and K252a (p < 0.001). RNA interference decreased cell proliferation by about 20\% in both PANC-1 and MiaPaCa-2 cells. There was a dose-dependent reduction in cell proliferation when pancreatic cells were incubated with K252a for 24 h. A concentration of 250 nM of K252a decreased cell proliferation by about 70\% for PANC-1 cells (Fig. 4A) and by about 50\% in MiaPaCa-2 cell lines compared to control cells (Fig. 4B). These results showed that suppression of MLK3 resulted in a significant decrease in cell viability of pancreatic cancer cells in a dose-dependent manner compared with control cells.

**Inhibition of MLK3 causes pancreatic cancer cell apoptosis possibly through blockade of JNK and ERK pathways**

To better understand the mechanisms underlying decreased cell proliferation due to MLK3 inhibition, we hypothesized the involvement of MAPKs as well as the mitochondrial apoptotic pathway in this process. To test this, the activation status of various MAPKs was assessed by western blotting with appropriate phospho-specific antibodies, after treating the pancreatic cancer cell lines with MLK3 siRNA or K252a. Since EGFR is a well-characterized target in pancreatic cancer, we also studied the effect of treatment of pancreatic cancer cells with the EGFR inhibitor CMP 56 in combination with either MLK3 siRNA or K252a. The results show that RNA interference decreased the expression of MLK3, in a concentration-dependent manner in both pancreatic cancer cell lines, as previously observed. The decrease in the MLK3 expression was accompanied by decreased activation of ERK, JNK and AKT in an MLK3 siRNA concentration-dependent manner (Fig. 5). As shown in Figure 6, inhibition of MLK3 combined with EGFR inhibition caused a dramatic decrease in phosphorylation of AKT and increased cleavage of PARP, compared to either agent alone, suggesting increased apoptotic cell death. In order to confirm this finding, we examined caspase-3 activity using an ELISA assay. There was no statistically significant increase in cleaved caspase-3 activity after treating the pancreatic cancer cell lines with either MLK3 siRNA or K252a alone (Fig. 7). However, treatment of cells with the EGFR inhibitor combined with MLK3 inhibition induced a statistically significant increase in cleaved caspase-3 activity (p < 0.001). These results suggest that MLK3 inhibition alone blocks pancreatic cancer cell proliferation, but combined EGFR and MLK3 inhibition induces apoptosis in pancreatic cancer cell lines.

**Discussion**

Pancreatic cancer is one of the most aggressive forms of cancer. Standard gemcitabine-based regimens result in an overall survival of less than a year.\textsuperscript{22} With advances in understanding the biology of this aggressive cancer, several targeted therapies are under evaluation,\textsuperscript{23} but these efforts are far from clinical use. One important target of recent interest in pancreatic cancer is the EGFR pathway. Preclinical models interrupting EGFR signaling demonstrated a significant antitumor effect.\textsuperscript{24} However, results from clinical trials, thus far, show only a modest benefit for such therapies in patients with pancreatic cancer when anti-EGFR agents were combined with gemcitabine.\textsuperscript{4} This could be secondary to constitutive activation of signaling molecules downstream of EGFR, and/or to redundant and cross talk pathways. It is well known that activating K-RAS gene mutations occur in 95\% of pancreatic cancers\textsuperscript{7} leading to constitutive activation of several signal transduction effectors, including the RAS/Raf/ERK pathway.\textsuperscript{25} To circumvent this constitutively
active pathway downstream of EGFR, we hypothesized that drug combinations inhibiting the effector pathways regulated by the constitutively active K-RAS may be of interest.

Our approach for a combined targeted strategy against proliferative and apoptotic signaling was based on the known molecular effects of EGFR inhibitors in pancreatic cancer and known effects of MLK3 inhibition in cancer cell lines. In a seminal study by Chadee et al., it was noted that MLK3 is important for proliferation in colorectal cancer cells harboring activating K-RAS mutation, but not those with B-Raf mutations. By using RNA interference of MLK3, they demonstrated marked suppression of cell proliferation in several cancer cell lines including the human adenocarcinoma cell line HCT15, which harbors an activating G13D mutation in K-RAS. Similar results were also seen in neurofibroma cell lines that lack functional NF-1, a GTPase for K-RAS. Loss of function of NF-1 leads to constitutive activation of K-RAS. Taken together, these experiments suggest that MLK3 may modulate downstream effectors of K-RAS that are critical for cell proliferation.

The role of MLK3 in pancreatic cancer has not been studied. We tested our hypothesis in well-characterized pancreatic cell lines with known mutations in K-RAS. Both MiaPaCa-2 and PANC-1 cell lines harbor a K-RAS mutation in codon 12, leading to a change from glycine to cysteine (G12C) and aspartate (G12D) respectively, which confer constitutive activation status. Fleming et al reported decreased cell proliferation and increased apoptosis in MiaPaCa-2 and PANC-1 cell lines by using RNA interference targeting mutated K-RAS. This approach is even more relevant since emerging clinical data in colorectal and lung cancer show that K-RAS mutation...
status is important in predicting tumor response to EGFR inhibitors.\textsuperscript{30,31} Hence, we reasoned that inhibition of MLK3 might resensitize pancreatic tumors to EGFR inhibitors. In support of this idea, we observed that simultaneous inhibition of both EGFR and MLK3 promoted apoptosis in pancreatic cancer cell lines.

In the current study, we found that MLK3 was over expressed (~3 fold) in both of these pancreatic cancer cell lines compared to the pancreatic epithelial cell line. Silencing of MLK3 by RNA interference in PANC-1 and MiaPaCa-2 cell lines markedly suppressed cell proliferation. As confirmation of this finding, pharmacological inhibition of MLK3 also led to marked suppression of cell proliferation in both pancreatic cancer cell lines. Interestingly, pharmacological inhibition (concentration dependent) led to more significant suppression of cell proliferation than RNA interference. This could suggest that K252a targets other MLK isoforms and that these other MLK isoforms might compensate for silencing of MLK3. It could also be due to off target effects of K252a. K252a also inhibits other protein kinases such as protein kinase C, cyclic nucleotide-dependent protein kinases\textsuperscript{32} and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II.\textsuperscript{33} When compared to CEP 1347, an MLK-selective inhibitor, K252a has an essentially similar inhibitory profile towards MLK3.\textsuperscript{21} Therefore, we used K252a as the MLK3 inhibitor in our studies. Future studies using MLK3-specific inhibitors are warranted to confirm our results.

To further characterize the downstream molecular events associated with MLK3 inhibition in pancreatic cancer cell lines, additional molecular analyses were performed. In PANC-1 cells, MLK3 silencing led to a decrease in the phosphorylation of JNK, ERK, and AKT. Similar results were seen in the MiaPaCa-2 cell line. Decreased phosphorylation of ERK, JNK and AKT and an increase in apoptosis, as evidenced by significant increase in cleaved caspase-3 activity, was observed upon simultaneous EGFR and MLK3 inhibition (siRNA or K252a). MLK3 inhibition (either by siRNA or K252a) also led to a decrease in the phosphorylation of AKT, which was more robust when MLK3 was inhibited in combination with EGFR inhibition. This could explain the increased effect of the combined drugs on the programmed cell death (apoptosis) in pancreatic cancer. Fleming et al\textsuperscript{29} also noticed only 30% apoptotic cell death in MiaPaCa-2 cell line and no apoptosis in PANC-1 cells, when treated with mutant-specific \textit{K-RAS} siRNA. These findings suggest that inhibition of effectors of mutant \textit{K-RAS} may not be sufficient in inducing cell death (apoptosis) in pancreatic cancer. Our results indicate that combined EGFR and MLK3 inhibition induces pancreatic cancer cell death, likely through inhibition of JNK, ERK and AKT, kinases that are known to function downstream of activated \textit{K-RAS}.

In conclusion, this study suggests that MLK3 might be an interesting target in pancreatic cancer. Further studies will be necessary to evaluate this possibility. We are in the process of assessing the expression of MLK3 in malignant and benign pancreatic tissue from patients. Preclinical and clinical studies are also needed to study the safety and efficacy of the EGFR and MLK3 inhibitors alone or in combination with other conventional chemotherapeutic agents. If further studies confirm these preliminary findings, then the combination of EGFR and MLK3 inhibitors may be one strategy to improve therapeutic efficacy in treating patients with pancreatic cancer.

Disclosures
This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors report no conflicts of interest.

References
Simultaneous inhibition of EGFR and MLK3 in pancreatic cancer


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