KML001 Enhances Anticancer Activity of Gemcitabine Against Pancreatic Cancer Cells

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Abstract. *Background/Aim: Gemcitabine is a drug commonly used to treat pancreatic cancer but chemoresistance to it is a common clinical issue. KML001 (sodium meta-arsenite) has demonstrated certain antitumor activity. The objective of the study was to evaluate the influence of KML001 on the anticancer activity of gemcitabine against pancreatic cancer cells. Materials and Methods: Cell proliferation, migration, and invasion were assessed, as well as the expression of nuclear factor-kappa B (NF-κB) p65, epidermal growth factor receptor (EGFR), matrix metalloproteinase-2 (MMP2), and vascular endothelial growth factor-C (VEGFC) in pancreatic cancer cells. Results: Treatment with a combination of KML001 and gemcitabine resulted in significant inhibition of cell proliferation, migration, and invasion, and significantly reduced EGFR and MMP2 expression compared to gemcitabine treatment-alone. Conclusion: Combination treatment of gemcitabine and KML001 could be an effective chemotherapeutic treatment for pancreatic cancer.*

Pancreatic cancer is a highly aggressive malignancy associated with low survival rates (1). Many chemotherapeutic regimens have been investigated for advanced unresectable and metastatic pancreatic cancer, but with only minimal improvement in survival and prognosis (2). Gemcitabine has since long been considered a standard systemic chemotherapeutic agent for the treatment of advanced cancer, including pancreatic cancer (3); however, its efficacy in patients with pancreatic cancer is unsatisfactory. Intrinsic or acquired resistance to gemcitabine is thought to be a major reason for its limited efficacy (4, 5). It is necessary to find a solution for overcoming chemoresistance in treating pancreatic cancer.

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KML001 (sodium meta-arsenite, $NaAsO_2$) has demonstrated cytotoxic activity as a result of its binding to telomeric sequences and causing telomere erosion in prostate cancer cells (6). It has also been shown to inhibit telomerase and reverse transcriptase activity and to down-regulate expression of tumorigenesis-linked transcripts such as nuclear factor-kappa B (NFκB) and vascular endothelial growth factor (VEGF) in cancer cells with short telomeres (7). Similarly, a previous study by our group has shown that KML001 inhibits cell proliferation, migration, and invasion in pancreatic cancer cells through suppression of NF-κB and vascular endothelial growth factor-C (VEGFC) (8).

Herein, we investigated whether KML001 enhances the anticancer effects of gemcitabine in pancreatic cancer cells.

Materials and Methods

Cell lines and reagents. The human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). Both MIA PaCa-2 and PANC-1 cells were obtained from Caucasian males diagnosed with pancreatic carcinoma. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), 1 mM L-glutamine, 1 mM minimum essential medium (MEM), 100 μg/ml streptomycin, and 100 U/ml penicillin. KML001 (Kominox®) was from Komipharm International (Shiheung, Korea), with 20 mmol/l stock prepared in phosphate-buffered saline (PBS), and aliquots stored at −20˚C. Stock solutions were stable for over one year. Working concentrations were prepared daily by diluting the stock with DMEM.

Determination of cell proliferation. Dojindo Cell Counting Kit-8 (CCK-8; Dojindo, Gaithersburg, MD, USA) was used to assess cell proliferation. This assay is based on the cleavage of the water-soluble tetrazolium salt WST-8 by mitochondrial dehydrogenase in viable cells (9). Cells were seeded in 96-well plates at 5×10^3 cells in 100 μl of culture medium and allowed to adhere overnight. Cells were then treated with 1 μM gemcitabine either alone or in combination with 20 μM KML001 (herein referred to as the combination treatment) for 24, 48, or 72 h, after which tetrazolium substrate (10 μl) was added and cells were incubated at 37˚C for 1 h. Considering the potential for

cytotoxicity, a KML001 concentration of 20 μM was used for combination treatment because our prior studies have shown that exposure to this concentration exerts a suboptimal inhibition of cell proliferation (8). The absorbance at 450 nm was assessed with a thermomax microplate reader (Tecan, Grödig, Austria). The cell proliferation index (%) was calculated using the following formula: (mean absorbance in all wells of the treatment group) ÷ (mean absorbance in all wells of the control group) ×100.

Cell migration and invasion assay. Cell migration was assessed using 24-well inserts (BD, Franklin Lakes, NJ, USA) with 8-μm pores according to the manufacturer's protocol. MIA PaCa-2 and PANC-1 cells were treated with gemcitabine (1 μM) alone or in combination with KML001 (20 μM) for 48 h. Cells were placed in serum-free culture medium and plated into the upper compartment of a migration chamber. After 24 h of incubation, cells in the upper chamber were removed, fixed in ice-cold methanol, and stained with Wright-Giemsa solution (Polysciences, Warrington, PA, USA). Digital images were obtained from the membranes, and cell areas were selected using the ScanScope CS system (Aperio Technologies, Vista, CA, USA). Migrating cells on each membrane were quantified in five randomly selected fields at ×40 magnification in each membrane, and the average value was defined as a migration or invasion index on three independent membranes. Methods for invasion studies were similar to those of the migration assay with the exception that membranes were Matrigel™-coated invasion chambers (BD Biosciences, Bedford, MA, USA) pre-hydrated in serum-free medium. The relative-fold change calculated for migration and invasion of cells treated with gemcitabine alone or in combination with KML001 was normalized to that of PBS-treated cells and expressed as a percentage of the control, which was assumed to be 100%.

Enzyme-linked immunosorbent assay (ELISA). ELISA kits for measurement of EGFR, MMP2, and VEGFC were purchased from R&D Systems (Minneapolis, MN, USA). MIA PaCa-2 and PANC-1 cells were treated with 1 μM gemcitabine alone or in combination with 20 μM KML001 (combination treatment) for 24 h. After treatment, nuclear protein was extracted using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the instructions from the manufacturer (10). DNA binding activity of NFκB p65 was evaluated using an ELISA kit (TransAM NF-κB Chemi; Active Motif) on nuclear protein extract. The TransAM NFκB kit contains a 96-well plate in which an oligonucleotide containing the NFκB consensus site (5'-GGGACTTTCC-3') has been immobilized. The active form of NFκB contained in nuclear extract specifically binds to this oligonucleotide. The primary antibodies used to detect NF-κB recognize an epitope on subunit p65 that is accessible only when NFκB is activated and bound to its target DNA. A horseradish peroxidase-conjugated secondary antibody was added according to the manufacturer's instructions (10). The culture medium was collected and used to determine secretion levels of MMP2, EGFR, and VEGFC using ELISA. The relative-fold change of NF-κB p65, EGFR, MMP2, and VEGFC activity in cells treated with gemcitabine alone or in combination with KML001 was normalized against the level of control cells. Values in control cells were arbitrarily set to one.

Densitometric and statistical analyses. To assure the validity of the results, each assay was performed in triplicate. Results from three independent experiments were expressed as the mean±standard error of the mean (SEM). Cell proliferation indices for each group were compared using the repeated-measures ANOVA test. Group differences were analyzed using the Kruskall–Wallis test. The *post hoc* analysis for multiples was compared with Tukey test using ranks. A *p*-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using PASW Statistics v. 18.0 (SPSS, Cary, NC, USA).

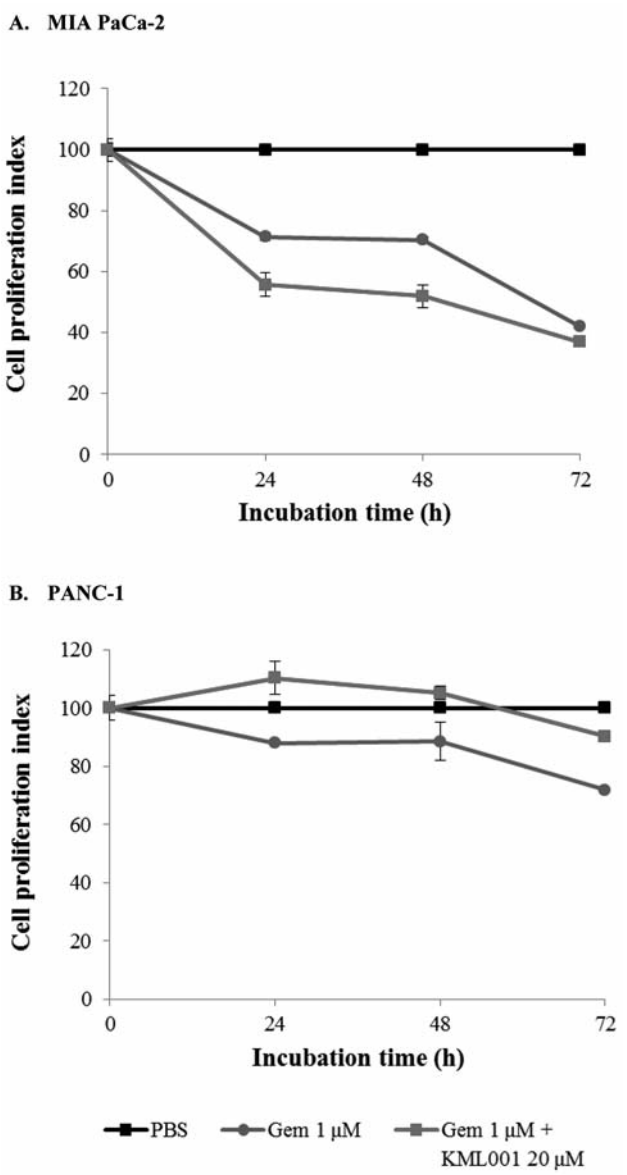

Figure 1. *KML001 enhances the antiproliferative effects of gemcitabine in MIA PaCa-2 cells. MIA PaCa-2 and PANC-1 cells were treated with gemcitabine (1 μM) alone and in combination with KML001 (20 μM) for the indicated time periods prior to estimation of the cell number using the Dojindo Cell Counting Kit-8 (CCK-8) assay. There was a significant reduction in cell proliferation in MIA PaCa-2 cells treated with a combination of gemcitabine and KML001 compared to cells treated with gemcitabine alone ($p<0.001$). The experiment was performed in triplicate. PBS, Control treated with phosphate-buffered saline; Gem, gemcitabine; KML001, sodium meta-arsenite. Data are the mean±standard error of the mean (SEM) of three independent experiments.*

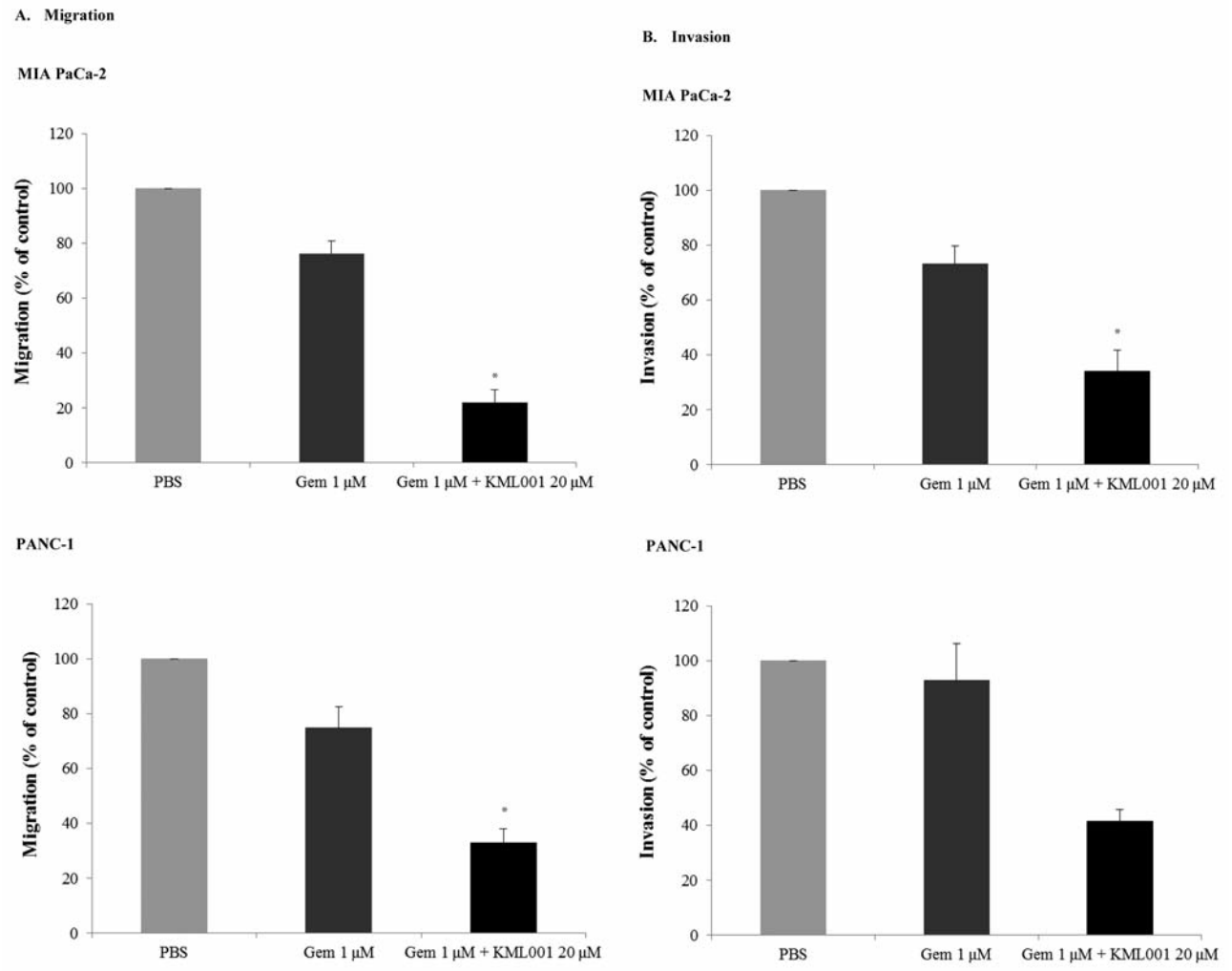

Figure 2. *KML001 enhances the anti-migrative and anti-invasive effects of gemcitabine on pancreatic cancer cells. MIA PaCa-2 and PANC-1 cells were treated with gemcitabine (1 μM) alone and in combination with KML001 (20 μM) for 48 h. Cells were placed in serum-free culture medium and plated into the upper compartment of either a migration or invasion chamber. After 24 h, cells in the upper chamber were removed and cells that had migrated (A) or invaded (B) into the lower surface of the membrane were fixed and stained with Wright-Giemsa. The relative-fold change calculated for migration and invasion of cells treated with gemcitabine alone or in combination with KML001 was normalized to that of the phosphate-buffered saline (PBS)-treated cells and expressed as a percentage of the control, which was assumed to be 100%. Cell migration was significantly reduced in both cell lines. Cell invasion was significantly reduced in MIA PaCa-2 cells. The experiment was performed in triplicate. PBS, Control treated with phosphate-buffered saline; Gem, gemcitabine; KML001, sodium meta-arsenite. Data are the mean±SEM of three independent experiments. *$p<0.005$.*

Results

KML001 enhances the antiproliferative effects of gemcitabine against MIA PaCa-2 cells. Figure 1 shows the effects of treatment with 1 μM gemcitabine alone and in combination with 20 μM KML001 on MIA PaCa-2 and PANC-1 cell growth. In MIA PaCa-2 cells, cell proliferation was significantly inhibited following combination treatment (12.1% inhibition after 24 h and 26.3% inhibition after 48 h) compared to gemcitabine treatment-alone ($p<0.001$) (Figure 1A). In PANC-1 cells, however, cell proliferation paradoxically increased after combination treatment compared with gemcitabine treatment alone ($p=0.006$; Figure 1B).

KML001 enhances the anti-migrative and anti-invasive effects of gemcitabine on pancreatic cancer cells. Combination treatment of 1 μM gemcitabine and 20 μM KML001 significantly reduced cell migration by 70.9% and 56.0% in MIA PaCa-2 and PANC-1 cells, respectively ($p=0.024$ in both cases) compared to treatment with gemcitabine alone (Figure 2A). Compared to treatment with gemcitabine alone, combination treatment significantly

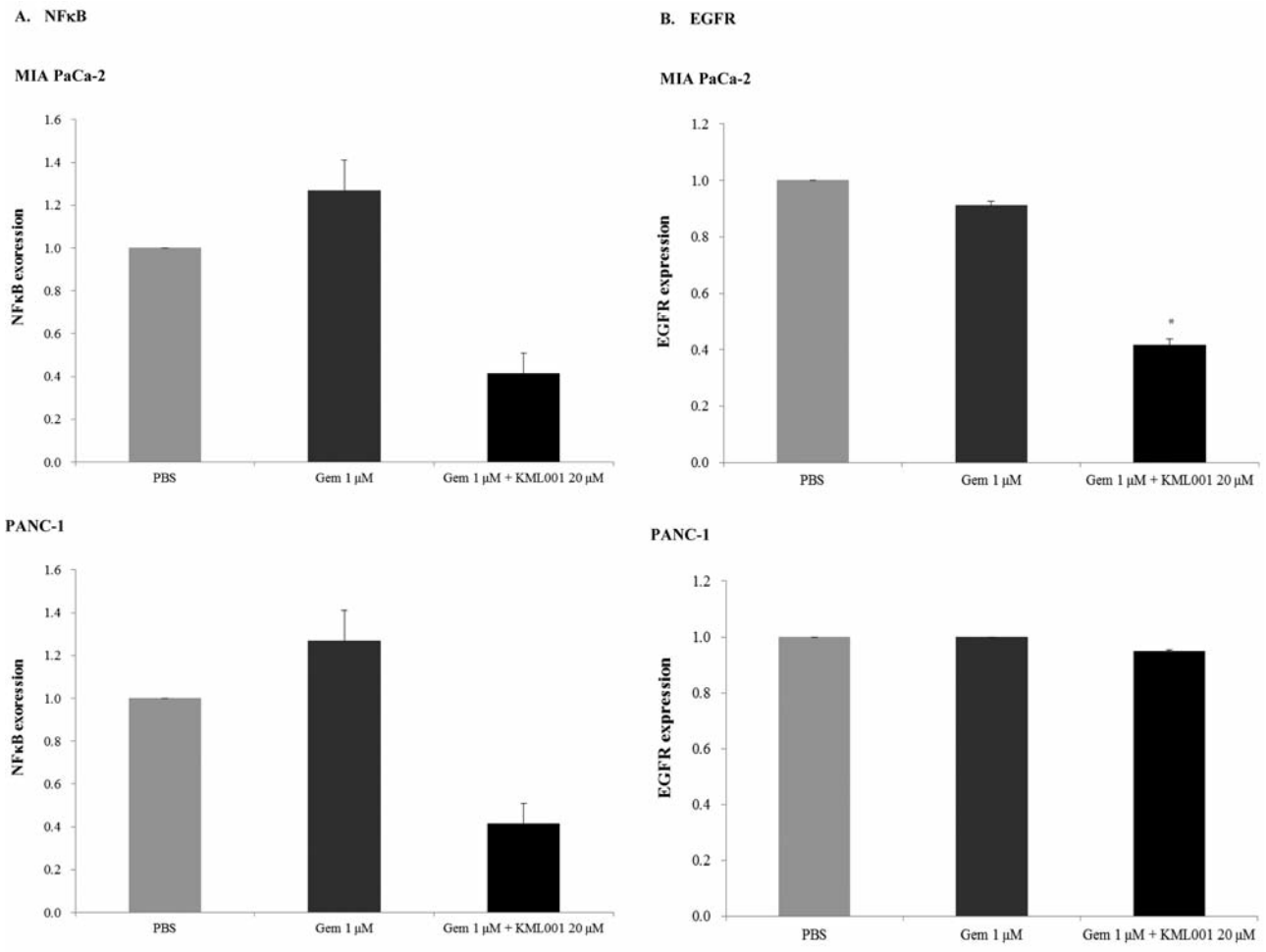

Figure 3. *continued*

reduced cell invasion by MIA PaCa-2 cells by 53.5% (p=0.024), but did not significantly affect cell invasion by PANC-1 cells (decrease of 55.2%, p=0.055) (Figure 2B).

KML001 enhances the anticancer activities of gemcitabine by suppression of MMP2 and EGFR. Constitutive NF-κB activity plays a key role in pancreatic tumorigenesis through activation of its downstream target genes and confers resistance to gemcitabine in pancreatic cancer cells (11, 12). We examined whether the effect of KML001 on the anticancer activity of gemcitabine is associated with the inhibition of NF-κB p65 expression. ELISA results showed that compared to gemcitabine treatment-alone, combination treatment reduced NF-κB p65 expression by 83.1% in MIA PaCa-2 cells and by 67.4% in PANC-2 cells, but the decreases were not statistically significant (p=0.055 in both cases) (Figure 3A).

Activation of the EGFR signaling pathway in cancer cells has been linked with increased cell proliferation, angiogenesis, metastasis, and lower apoptosis (13). Combination treatment significantly reduced EGFR expression by 54.3% (p=0.024) in MIA PaCa-2 cells compared to gemcitabine treatment-alone; a 6% decrease in EGFR expression observed in PANC-1 cells was not statistically significant (p=0.091) (Figure 3B).

MMP2 plays a key role during invasion and metastasis of malignant cells (14). We explored the influence of KML001 on the anti-migrative and anti-invasive effects of gemcitabine in association with MMP2 expression and found that compared to gemcitabine treatment-alone, combination treatment significantly reduced MMP2 expression by 43.4% in PANC-1 cells (p=0.024). A 5.7% decrease seen in MIA PaCa-2 cells was not statistically significant (p=0.156) (Figure 3C).

Next we examined VEGFC expression, which is associated with lymphangiogenesis, lymph node metastasis, and angiogenesis (15-19). In MIA PaCa-2 cells, combination treatment did not significantly affect VEGFC expression (decrease of 11.2%; p=0.055) compared with gemcitabine

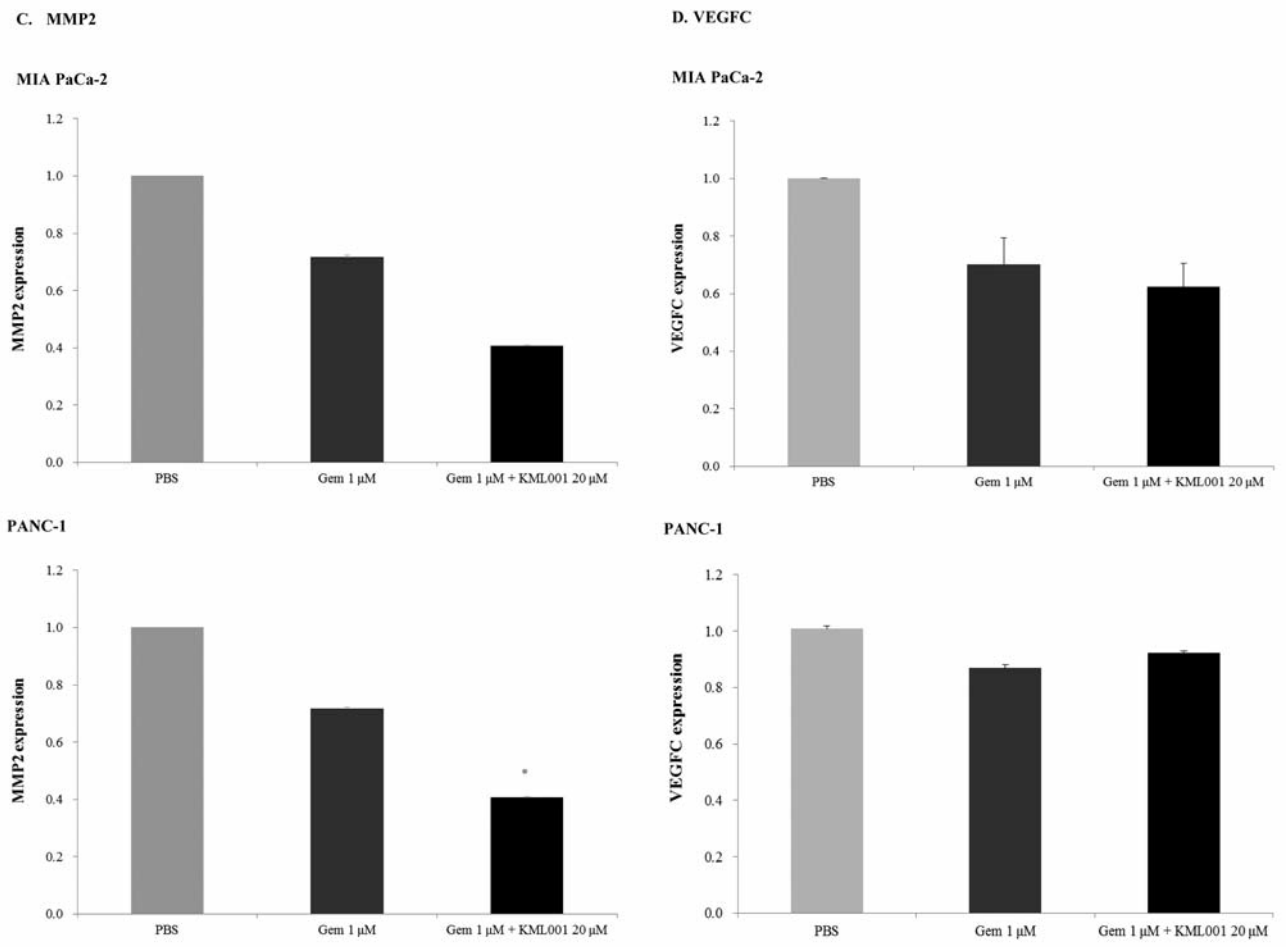

Figure 3. *KML001 enhances the anticancer activities of gemcitabine by partial suppression of nuclear factor-kappa B (NFκB), matrix metalloproteinase (MMP2), and epidermal growth factor receptor (EGFR). A: Nuclear extracts were prepared from MIA PaCa-2 and PANC-1 cells treated with PBS (control), gemcitabine alone, or a combination of gemcitabine and KML001 and subjected to analysis for NFκB p65 expression as measured by enzyme-linked immunosorbent assay (ELISA). The culture media of control and combination-treated MIA PaCa-2 and PANC-1 cells were harvested for the detection of EGFR (B), MMP2 (C), and vascular endothelial growth factor-C (VEGFC) (D) using ELISA. Combination treatment did not significantly affect NFκB p65 expression compared to gemcitabine treatment alone, but did significantly reduce EGFR expression in MIA PaCa-2 cells and MMP2 in PANC-1 cells. The experiment was performed in triplicate. PBS, Control treated with phosphate-buffered saline; Gem, gemcitabine; KML001, sodium meta-arsenite. Data are the mean±SEM of three independent experiments. *p<0.005.*

treatment alone. Interestingly, in PANC-1 cell line, VEGFC expression was increased following combination treatment compared with gemcitabine treatment-alone (Figure 3D).

Discussion

Our previous research revealed that KML001 inhibited cell proliferation, migration, and invasion of pancreatic cancer cells. In the present study, for the first time, we showed that KML001 enhanced the antiproliferative, anti-migrative, and anti-invasive effects of gemcitabine on pancreatic cancer cells. Suppression of EGFR and MMP2 by KML001 may contribute to potentiation of the antitumor activities of gemcitabine.

Several studies have reported that KML001 accelerates apoptosis of cancer cells in cases of leukemia, prostate cancer, and melanoma (6, 20, 21). The anticancer mechanism of KML001 in prostate cancer involves DNA damage at the telomeres of cancer cell chromosomes (6), but its biological mechanism of antitumor activity in pancreatic cancer remains uncertain. Previous results of our group have shown that KML001 inhibits proliferation and invasion of pancreatic cancer cells partly through down-regulation of NF-κB activation (8), and the current study builds upon our previous work.

Gemcitabine is currently the standard therapeutic agent available for treating advanced pancreatic cancer; however, monotherapy with gemcitabine has resulted in only modest

improvement in overall survival because of systemic toxicity and chemoresistance. Therefore, gemcitabine-based combination therapy has been investigated to overcome chemoresistance (3, 22). The current study demonstrates that KML001 enhances the antiproliferative, anti-migrative, and anti-invasive activities of gemcitabine against pancreatic cancer cells.

It has been demonstrated that NF-κB p65 was constitutively activated in approximately 67% of pancreatic cancer cells (11), and the results of many investigations indicate that NF-κB is associated with chemoresistance of pancreatic cancer (12, 22, 23). NF-κB inhibition has been shown to increase the sensitivity of cancer cells to the apoptotic action of chemotherapeutic agents (23). Although the decreases in NF-κB p65 expression following combination treatment revealed by our ELISA results were not statistically significant, further study with larger sample sizes may reveal a statistically significant effect.

In the present study, combination treatment significantly reduced EGFR expression in MIA PaCa-2 cells compared to gemcitabine treatment-alone. EGFR is known to be overexpressed in pancreatic cancer (24). Unregulated EGFR activation can promote a range of oncogenic activities, such as proliferation, migration, stromal invasion, tumor neo-vascularization, and resistance to pro-apoptotic signals in transformed cells (13, 24). We also found that combination treatment significantly reduced MMP2 expression in PANC-1 cells compared to gemcitabine treatment-alone; MMP2 is known to play a critical role in tumor cell invasion and metastasis (25). These results suggest that KML001 potentiated the anticancer activities of gemcitabine partly through suppression of EGFR and MMP2.

In conclusion, KML001 enhanced the antiproliferative, anti-migrative, and anti-invasive effects of gemcitabine in pancreatic cancer cells. Down-regulation of EGFR and MMP2 expression may play a role in the potentiating effect of KML001 on anticancer activities of gemcitabine. Combination treatment of gemcitabine and KML001 could be a new therapeutic regimen for the treatment of pancreatic cancer. Further studies are required to confirm the antitumor activity and molecular biological mechanisms of KML001.

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