Anaplastic lymphoma kinase inhibitor NVP-TAE684 suppresses the proliferation of human pancreatic adenocarcinoma cells

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Abstract. Anaplastic lymphoma kinase (ALK) is known to be an important therapeutic target in various types of cancer. NVP-TAE684, a well-known inhibitor of ALK, was revealed to exert antitumor effects in several different malignancies. However, the molecular mechanisms responsible for these antitumor effects in cancer cells, including pancreatic adenocarcinoma cells, remain unknown. In the present study, NVP-TAE684 was investigated for its antitumor effects towards pancreatic adenocarcinoma cells. MTT assay, western blot analysis, flow cytometry, caspase-3/7 activity assay and Trypan blue exclusion assay were used and it was revealed that NVP-TAE684 suppressed the proliferation of seven human pancreatic adenocarcinoma cell lines (AsPC-1, Panc-1, MIA PaCa-2, Capan-1, CFPAC-1, Colo-357 and BxPC-3), and significantly increased G2/M arrest and apoptotic cell death. Furthermore, NVP-TAE684 inhibited the phosphorylation of ALK at Y1604, as well as that of downstream mediators such as AKT (S473) and ERK1/2 (Y202/T204). Notably, knocking down ALK with siRNAs also decreased proliferation and promoted G2/M arrest and apoptosis. Furthermore, inhibition of ALK with NVP-TAE684 or siRNA synergistically enhanced gemcitabine-induced cell death by inducing apoptosis. In conclusion, the findings of the present study indicated that NVP-TAE684 exerted its antitumor effects by inducing G2/M arrest and apoptosis via the inhibition of the ALK signaling pathway, and suggests its potential use as an antitumor agent against pancreatic adenocarcinoma.

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Introduction

Pancreatic adenocarcinoma, one of the most aggressive human malignancies, has the propensities of difficult diagnosis, early metastasis and resistance to treatment. Globally, a reported 458,918 new cases and 432,242 associated deaths were caused by pancreatic adenocarcinoma in 2018 (1). Due to a lack of effective treatments, the estimated five-year survival rate for patients with pancreatic adenocarcinoma has remained at <5%, which is the lowest among all types of cancers in general (1-3). To date, surgical resection is considered to be the most effective curative treatment, however, <20% of patients are eligible at the time of diagnosis (4). In addition, the majority of patients will eventually experience recurrence, and the five-year survival rate of patients who undergo complete surgical resection remains <25% (4). Other therapies for the treatment of pancreatic adenocarcinoma, including chemotherapy and radiotherapy, are important, but remain largely ineffective (5). Gemcitabine (2,2'-difluorodeoxycytidine, dFdC) is a standard, first-line compound that was approved for the treatment of metastatic and non-metastatic but locally advanced pancreatic adenocarcinoma in 1996 (6-8); however, resistance to this drug is a considerable limitation in disease treatment. Therefore, the investigation and application of novel targeted therapeutics that are less susceptible to intrinsic drug resistance, and with improved antitumor effects, are crucial to the successful treatment of pancreatic adenocarcinoma.

Anaplastic lymphoma kinase (ALK) belongs to one of the subfamilies of tyrosine kinases for the insulin receptor, and initiates a number of important cancer-associated signaling pathways. As such, ALK has been associated with the development of anaplastic large-cell lymphoma, non-small cell lung cancer, diffuse large B-cell lymphoma, inflammatory myofibroblastic tumors, neuroblastoma, anaplastic thyroid cancer, rhabdomyosarcoma and pancreatic adenocarcinoma (9-12).

ALK activation promotes a variety of functional properties such as cell survival, proliferation, differentiation and invasiveness through the modulation of the downstream signaling pathways of mediators including PI3K/AKT, MEK/ERK and STAT3 (9,12-14). Previous studies have revealed that the level of ALK phosphorylation is higher in pancreatic tumors than in normal pancreatic tissues; this was achieved by analyzing ALK expression in patient tissues, and inhibiting ALK activity using crizotinib (an inhibitor of c-MET/ALK), or ceritinib (a well-known ALK inhibitor with antitumor effects against pancreatic adenocarcinoma) (10,11). Combination treatment with ceritinib and gemcitabine also significantly inhibited the growth and/or survival of pancreatic adenocarcinoma (10). Moreover, NVP-TAE688, a well-known inhibitor of ALK, has been revealed to induce cell death in different types of cancer, including anaplastic large-cell lymphoma, non-small cell lung cancer, neuroblastoma and large B-cell lymphoma (15-20). In osteosarcoma, the inhibition of ALK by NVP-TAE684 sensitized cell apoptosis when used in combination with chemotherapeutic agents such as doxorubicin, paclitaxel, docetaxel or vincristine (21). Similar results were observed following the combined use of NVP-TAE684 and radiotherapy in patients with non-small cell lung cancer (22). Therefore, using NVP-TAE684 to inhibit ALK activity may be a clinically effective treatment option for a number of cancer types, including solid tumors. However, the antitumor effects of NVP-TAE684 in pancreatic adenocarcinoma have yet to be fully investigated.

Numerous cytotoxic and/or ALK-targeting agents inhibit tumor cell survival and proliferation by promoting cell cycle arrest at the G0/G1, S or G2/M phases (15,17,19,23-26). The G2/M phase is one of two major checkpoints for cell cycle regulation. Following DNA damage, cells are retained in G2/M and prevented from entering mitosis, which provides an opportunity for DNA repair and prevents the proliferation of damaged cells (27). Several studies have revealed that NVP-TAE684 and alectinib induced cell cycle arrest at the G0/G1 phase in anaplastic large-cell lymphoma, non-small cell lung cancer and neuroblastoma cells (15,17,19,25). In addition, crizotinib was revealed to induce G2/M arrest in ovarian cancer and non-small cell lung cancer cells (23,24). However, the molecular mechanisms of NVP-TAE684 in pancreatic adenocarcinoma cells have not been studied thus far.

In the present study, the antitumor effects of NVP-TAE684 were investigated using human pancreatic adenocarcinoma cells. The results indicated that NVP-TAE684 inhibited cellular proliferation and induced G2/M arrest and apoptotic cell death by targeting ALK in multiple human pancreatic adenocarcinoma cell lines.

Materials and methods

Cell culture and reagents. The human pancreatic adenocarcinoma AsPC-1, Capan-1 and Colo-357 (Tissue Culture and Biobanking Shared Resource; Georgetown University Lombardi Comprehensive Cancer Center) and BxPC-3 cell lines (ATCC) were maintained in RPMI-1640 media containing fetal bovine serum (FBS; 20% for AsPC-1; and 10% for Capan-1, Colo-357 and BxPC-3 cells), 100 U/ml penicillin, 100 μg/ml streptomycin and 1% sodium pyruvate at 37˚C in a humidified atmosphere containing 5% CO_2 as previously described (28,29). MIA PaCa-2 human pancreatic adenocarcinoma cells (ATCC) were maintained in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% FBS, 2.5% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2 as previously described (28,29). The Panc-1 and CFPAC-1 human pancreatic adenocarcinoma cell lines (ATCC) were maintained in DMEM supplemented 10% FBS, 10 U/ml penicillin and 10 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2 (28,29). The HPDE6-C7 immortal human pancreatic ductal epithelial cell line (provided by Dr Tsao, Montreal General Hospital and McGill University, Montreal, Canada) was cultured in keratinocyte serum-free medium supplemented with an epidermal growth factor, bovine pituitary extract and 1X antibiotic-antimycotic at 37˚C in a humidified atmosphere containing 5% CO_2 as previously described (30). Serum starvation was carried out by replacing the culture medium with fresh medium without FBS, and incubating for 24 h. The cell culture reagents were obtained from BioWhittaker (Lonza Group, Ltd.) and Invitrogen (Thermo Fisher Scientific, Inc.). NVP-TAE684 was purchased from Selleck Chemicals and dissolved in DMSO (Sigma-Aldrich; Merck KGaA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The human pancreatic adenocarcinoma cells (AsPC-1, Panc-1, MIA PaCa-2, Capan-1, CFPAC-1, Colo-357 and BxPC-3) were counted using the Luna™ Cell Counter (Logos Biosystems, Inc.) and seeded into 96-well flat-bottom plates at a density of $2x10^3$ cells/well. The cells were then exposed to NVP-TAE684 alone or in combination with gemcitabine at the indicated concentrations (0, 0.01, 0.1, 1 or 10 μM), and incubated at 37˚C for 72 h. After incubation, 10 μl MTT (1 mg/ml; Sigma-Aldrich; Merck KGaA) in PBS was added to each well and the cells were incubated at 37˚C for a further 4 h. Following centrifugation at 1000 x g for 2 min and removal of the medium, 150 μl DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 560 nm using an ELx808 Absorbance Microplate Reader (BioTek Instruments, Inc.) as previously described (28,29,31).

Western blot analysis. Human pancreatic adenocarcinoma cells were cultured to ~70% confluence and NVP-TAE684 was added at the indicated concentrations (0, 0.01, 0.1 or 1 μM). After exposure to NVP-TAE684, the cells were lysed in cell lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycophosphate, 10 mM NaF, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF and 1 mM DTT), and the protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). The proteins were separated by SDS-PAGE, transferred to PVDF membranes, blocked in 1X blocking buffer at room temperature for 1 h (Sigma-Aldrich; Merck KGaA) and probed with primary antibodies at 4˚C for overnight against the following: Phospho-ALK (Y1604) (dilution 1:250; product. no. 3341S), ALK (dilution 1:1,000; product. no. 3633S), phospho-AKT (S473) (dilution 1:500; product. no. 4060S), AKT (dilution

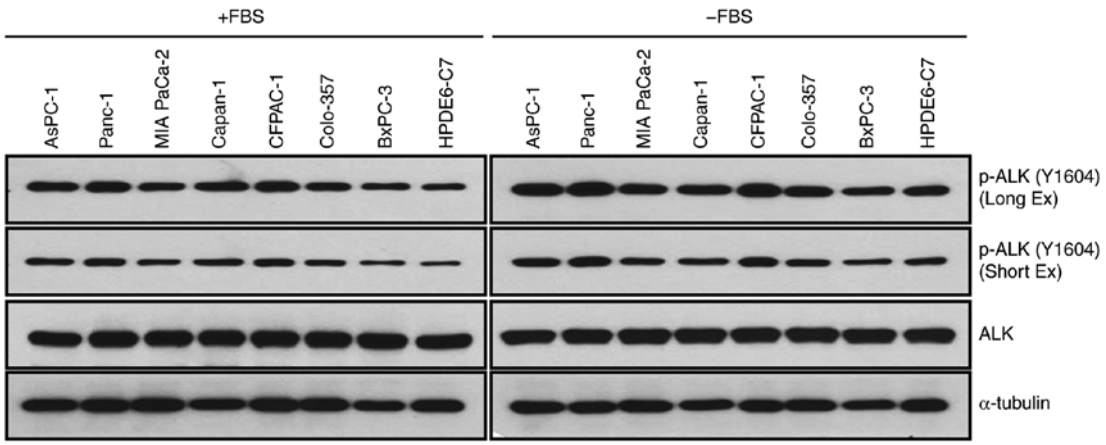

Figure 1. Phosphorylation levels of ALK in human pancreatic adenocarcinoma cells (AsPC-1, Panc-1, MIA-PaCa2, Capan-1, CFPAC-1, Colo-357 and BxPC-3) and immortal human pancreatic ductal epithelial cells (HPDE6-C7). Western blot analysis of cell extracts using the indicated antibodies. The levels of the phosphorylated at Y1604 and total form of ALK were determined. Anti-α-tubulin antibody was used for a loading and transfer control.

1:2,000; product. no. 4685S), phospho-ERK1/2 (Y202/T204) (dilution 1:1,000; product. no. 4370S), ERK1/2 (dilution 1:2,000; product. no. 9102S), phospho-STAT3 (Y705) (dilution 1:250; product. no. 9145S) (all Cell Signaling Technology, Inc.) and α-tubulin (dilution 1:2,000; product. no. T6074) (Sigma-Aldrich; Merck KGaA). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse (dilution 1:2,000; product. no. A9917) or anti-rabbit (dilution 1:2,000; product. no. 12-348) secondary antibodies (Sigma-Aldrich; Merck KGaA) at room temperature for 1 h and visualized using a chemiluminescence kit (Santa Cruz Biotechnology, Inc.) according to the manufacturer's protocol. The membranes were subsequently exposed to X-ray film (American X-ray and Medical Supply, Inc.) and developed as previously described (28,29,31).

Flow cytometry. Human pancreatic adenocarcinoma cells were treated with NVP-TAE684 and harvested by trypsinization. The cells were washed with PBS and fixed overnight in 70% ethanol at -20˚C. The cells were then incubated with 20 μg/ml propidium iodide (BD Biosciences) and 40 μg/ml RNase A (BD Biosciences) in 1X PBS, and analyzed using a FACSCalibur flow cytometer (BD Biosciences) as previously described (28,29,31).

Caspase-3/7 activity assay. Caspase-3/7 activity was determined using the Caspase-Glo® 3/7 Assay System (Promega Corporation) according to the manufacturer's protocol. MIA PaCa-2 and Colo-357 cells were treated with NVP-TAE684 alone or in combination with gemcitabine at the indicated concentrations (0, 0.1, 1 or 10 μM), and the caspase-3/7 activity of the cell lysates was determined. Luminescence was measured at 490 nm using a VICTOR X multilabel plate reader (PerkinElmer, Inc.) as previously described (32).

Small interfering RNA (siRNA) transfection. For the RNA interference experiments, 100 nM of each ALK siRNA (#1) (5'-AAUACUGACAGCCACAGGCAAUGUC-3'), ALK siRNA (#2) (5'-UUAGGUGGGACAGUACAGCUUCCCU-3') and the control siRNA (5'-GACGAGCGGCACGUGCACA-3') were purchased from Bioneer Corporation. MIA PaCa-2 cell transfection was conducted using Lipofectamine® 2000 at 37˚C for 6 h (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 48 h the transfected cells were processed for western blotting, cell proliferation, cell cycle analysis, and caspase-3/7 activity assay as previously described (28,29).

Trypan Blue exclusion assay. The cell monolayers were harvested by trypsinization, resuspended in complete medium, stained at room temperature for 3 min with trypan blue and counted. The number of viable cells was calculated using the Luna™ Cell Counter as previously described (28).

Statistical analysis. The two-tailed Student's t-test was used for two group comparisons and the one-way ANOVA with post hoc Tukey's honest significant difference (HSD) test was used to analyze the significance of the differences for more than two group comparisons. Data are expressed as the mean ± standard deviation (SD), and values of $P<0.05$ and $P<0.01$ were considered to indicate a statistically significant and highly statistically significant differences, respectively (33).

Results

ALK activity is induced in human pancreatic adenocarcinoma cells and immortal human pancreatic ductal epithelial cells. Firstly, the phosphorylation levels of ALK at Y1604 in human pancreatic adenocarcinoma cells (AsPC-1, Panc-1, MIA PaCa-2, Capan-1, CFPAC-1, Colo-357 and BxPC-3) and immortal human pancreatic duct epithelial cells (HPDE6-C7) was assessed, following culture in complete or serum-free medium (serum starvation) to identify whether serum affects the phosphorylation levels of ALK at Y1604. The western blot results revealed that all cells cultured in normal culture condition with the addition of serum possessed high levels of ALK phosphorylation at Y1604 (Fig. 1). Furthermore, it was observed that all cells cultured in serum-starved condition also possessed high levels of ALK phosphorylation at Y1604 (Fig. 1). These data indicated that the high phosphorylation levels of ALK are not influenced by the presence of serum. In

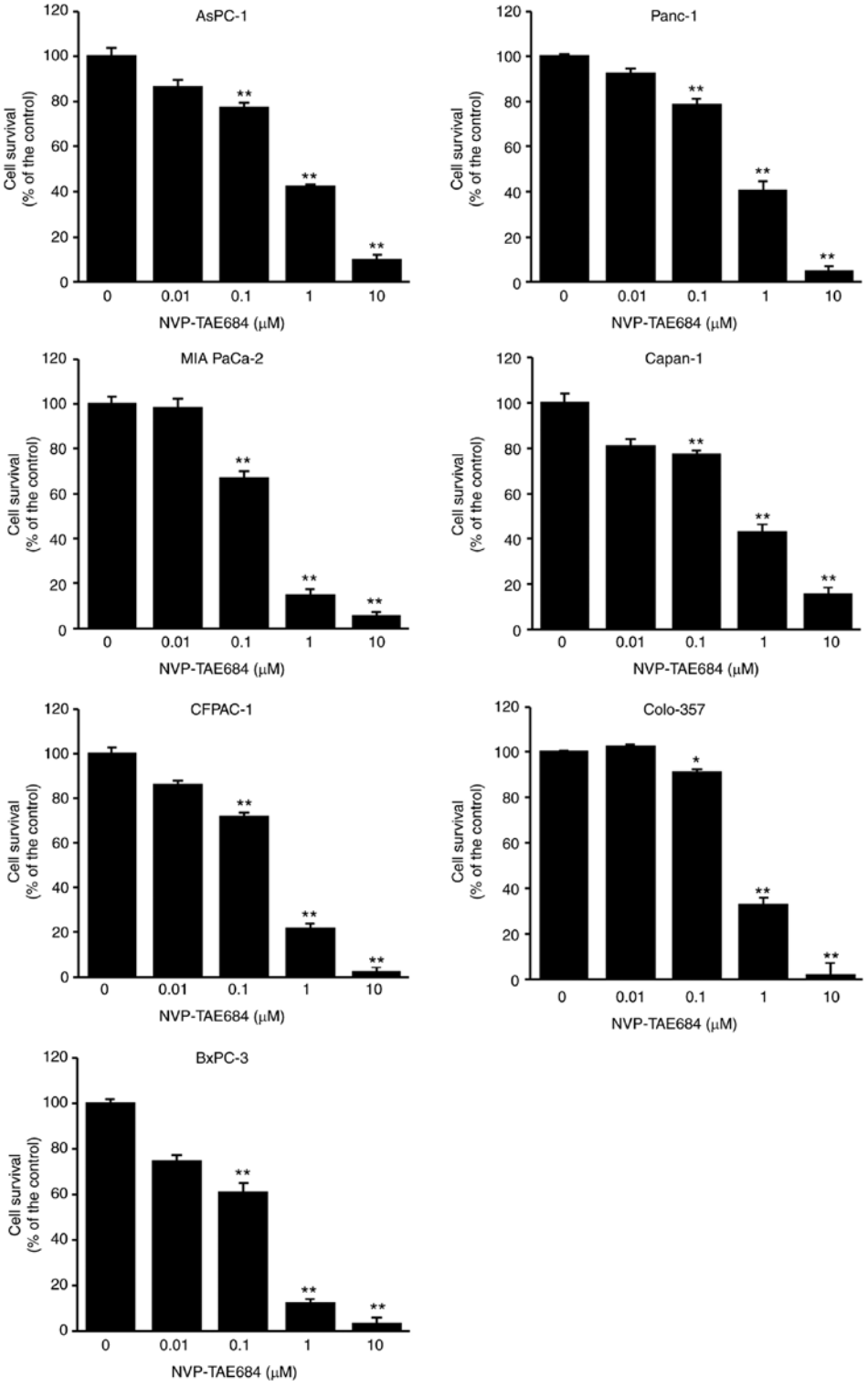

Figure 2. NVP-TAE684 inhibits cell survival. An MTT assay of AsPC-1, Panc-1, MIA PaCa-2, Capan-1, CFPAC-1, Colo-357 and BxPC-1 cells treated of NVP-TAE684 with indicated concentrations (0, 0.01, 0.1, 1 or 10 μM) for 72 h was used to determine cell survival. Representative data from three independent experiments performed in triplicate are presented as the mean ± SD. *P<0.05 and **P<0.01.

order to investigate the antitumor effects of NVP-TAE684 in pancreatic adenocarcinoma, all of the aforementioned human pancreatic adenocarcinoma cell lines were used for further experimentation.

NVP-TAE684 induces apoptotic cell death. NVP-TAE684 is a potent and selective inhibitor of ALK. In order to investigate the antitumor effects of NVP-TAE684 in pancreatic adenocarcinoma, AsPC-1, Panc-1, MIA PaCa-2, Capan-1, CFPAC-1, Colo-357 and BxPC-3 cells were treated with various concentrations of NVP-TAE684 (0, 0.01, 0.1, 1 and/or 10 μM) for 72 h, and cell viability was assessed using an MTT assay. NVP-TAE684 significantly reduced the number of viable cells in all cell lines in a dose-dependent manner (Fig. 2).

Table I. Antitumor effects of NVP-TAE684 in human pancreatic adenocarcinoma cells.

Human pancreatic adenocarcinoma cells	IC_{50} (μM)						
	AsPC-1	Panc-1	MIA PaCa-2	Capan-1	CFPAC-1	Colo-357	BxPC-3
NVP-TAE684	0.85±0.005	0.81±0.01	0.29±0.002	0.86±0.012	0.44±0.007	0.66±0.009	0.25±0.006

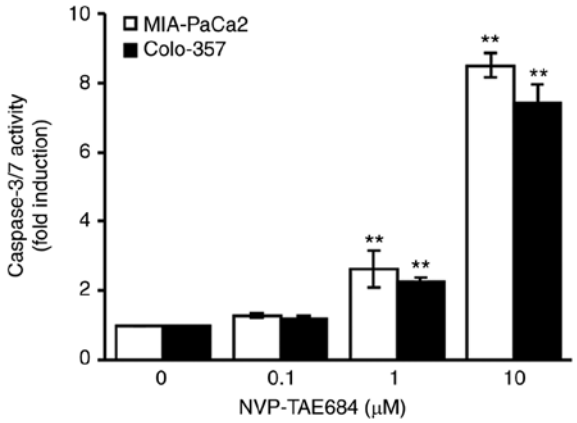

Figure 3. NVP-TAE684 induces apoptotic cell death. MIA PaCa-2 and Colo-357 cells were treated with the indicated concentrations of NVP-TAE684 for 24 h. A caspase-3/7 activity assay was used to determine apoptotic cell death. Representative data from three independent experiments performed in triplicate are presented as the mean ± SD. $^{**}P<0.01$.

In addition, the IC_{50} of NVP-TAE684 was determined to be 0.85±0.005, 0.81±0.01, 0.29±0.002, 0.86±0.012, 0.44±0.007, 0.66±0.009 and 0.25±0.006 in AsPC-1, Panc-1, MIA PaCa-2, Capan-1, CFPAC-1, Colo-357 and BxPC-3 cells, respectively (Table I). Furthermore, MIA PaCa-2, BxPC-3 and CFPAC-1 cells were highly sensitive to NVP-TAE684, while Colo-357, AsPC-1, Panc-1 and Capan-1 cells appeared to be less sensitive (Fig. 2).

To further investigate the mechanisms by which NVP-TAE684 promotes apoptosis and identify the molecular mechanisms of sensitivity and/or resistance of pancreatic adenocarcinoma cells to NVP-TAE684, MIA PaCa-2 cells (which were found to be highly sensitive to NVP-TAE684) and Colo-357 cells (which were less sensitive to NVP-TAE684) were treated with various concentrations of NVP-TAE684 (0, 0.1, 1 and/or 10 μM) for 24 h. Apoptosis was detected using caspase-3/7 activity analysis, and a significant increase in caspase-3/7 activity was observed in both cell lines cells following treatment with 1 and 10 μM NVP-TAE684 (Fig. 3). Collectively, these data indicated that NVP-TAE684 significantly reduced proliferation and induced apoptosis in human pancreatic adenocarcinoma cells.

NVP-TAE684 induces G2/M and sub-G1 arrest. Next, the NVP-TAE684-induced inhibitory effects on ALK on cell cycle progression were investigated. MIA PaCa-2 and Colo-357 cells were treated for 24 h with the indicated concentrations of NVP-TAE684, and their cell cycle profiles were flow cytometrically assessed. NVP-TAE684 significantly promoted cell cycle arrest at the G2/M phase [from 17.5 to 74.7% in MIA PaCa-2 cells (1 μM), and from 14.1 to 73.2% in Colo-357 cells (10 μM)] and significantly decreased the number of cells in the G0/G1 phase (from 49.6 to 22.5% in MIA PaCa-2 cells, and from 56.8 to 22.4% in Colo-357 cells) and S phase (from 32.9 to 2.8% in MIA PaCa-2 cells, and from 29.1 to 4.4% in Colo-357) (Fig. 4A). An increase in the sub-G1 population was also observed following the administration of NVP-TAE684 (at various concentrations), though this was more apparent in MIA PaCa-2 than Colo-357 cells (Fig. 4B). Collectively, these data indicated that NVP-TAE684 significantly promoted cell cycle arrest at the G2/M phase in human pancreatic adenocarcinoma cells.

NVP-TAE684 decreases ALK activity. In order to identify whether the decrease in cell proliferation, and the increase in apoptosis and G2/M arrest were associated with the inhibition of ALK phosphorylation, MIA PaCa-2 and Colo-357 cells were treated with NVP-TAE684 (0, 0.01, 0.1 and/or 1 μM) for 8 h. NVP-TAE684 markedly reduced the levels of ALK phosphorylation at Y1604 in both cell lines (Fig. 5). Furthermore, the phosphorylation levels of downstream mediators of the ALK signaling pathway, such as AKT, ERK1/2 and STAT3, were also determined following NVP-TAE684 treatment. Under these conditions, NVP-TAE684 also markedly reduced the phosphorylation levels of AKT (at S473) and ERK1/2 (at Y202/T204), and to a lesser degree, STAT3 (at Y705) in both cell lines (Fig. 5). Collectively, these findings demonstrated that the antitumor effects of NVP-TAE684 in human pancreatic adenocarcinoma cells were closely associated with the inhibition of ALK phosphorylation and mostly through significant reduction of AKT and ERK1/2 phosphorylation.

ALK-knockdown decreases cellular proliferation and induces apoptotic death and G2/M arrest. Since the NVP-TAE684-induced inhibition of ALK inhibited cellular proliferation, and induced apoptotic cell death and G2/M arrest, MIA PaCa-2 cells were transfected with either ALK siRNA (#1 or #2) or the control siRNA to compare the effects of NVP-TAE684 and ALK-knockdown, and to confirm the antitumor effects of NVP-TAE684. At a concentration of 0.1 μM ALK siRNA (#1 or #2), ALK-knockdown decreased the levels of total and phosphorylated ALK in MIA PaCa-2 cells (Fig. 6A). Under these conditions, the phosphorylation levels of AKT (S473) and ERK1/2 (Y202/T204) but not STAT3 (Y705) were also decreased in these cells (Fig. 6A). In addition, knocking down ALK decreased cell survival (Fig. 6B) and induced apoptotic cell death, as indicated by the induction of caspase-3/7 activity (Fig. 6C) and the accumulation of cells in the sub-G_1 phase (from 8.4 in the control group to 25.2% in the ALK siRNA (#1), and 17.1% in the ALK siRNA (#2)

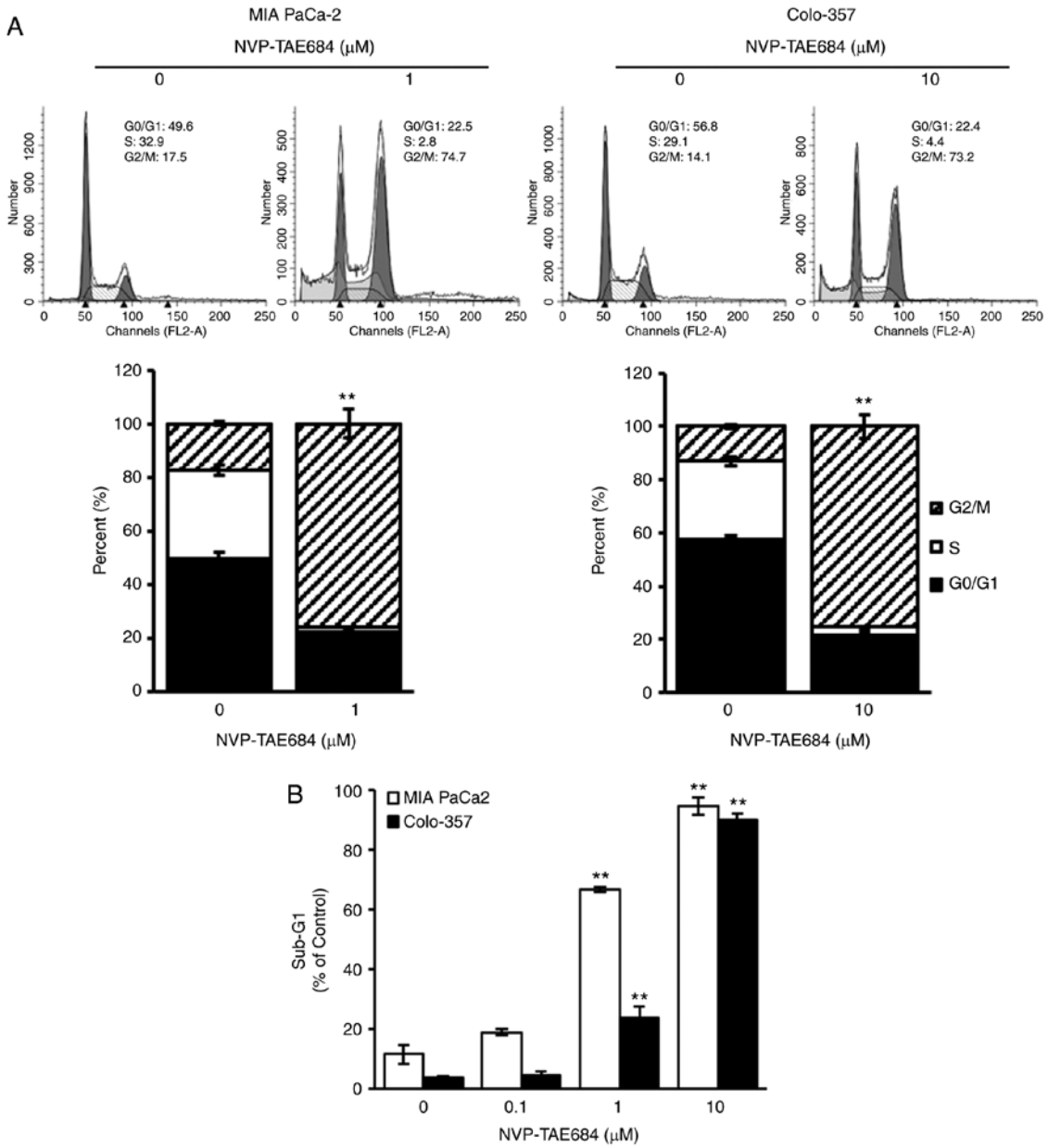

Figure 4. NVP-TAE684 induces G2/M and Sub-G1 phase cell cycle arrest. (A and B) Analysis by FACS of MIA PaCa-2 and Colo-357 cells treated with the indicated concentrations of NVP-TAE684 for 24 h was used to determine cell cycle arrest. (B) The sub-G1 population from FACS analysis was analyzed. Representative data are presented as the mean ± SD from three independent experiments. **P<0.01.

groups (Fig. 6E). Furthermore, ALK-knockdown promoted cell cycle arrest at the G2/M phase from 14.5 in the control group to 47.7% in the ALK siRNA (#1), and 31.9% in the ALK siRNA (#2) groups. The cell population in the G0/G1 phase was also decreased from 39.6% in the control group to 24.6 and 24.3% in the ALK siRNA (#1) and (#2) groups, respectively, and the proportion of cells in the S phase was reduced from 45.9% in the control group to 24.6 and 43.8% in the ALK siRNA (#1) and (#2) groups, respectively (Fig. 6D). Collectively, targeting ALK with either NVP-TAE684 or ALK siRNA reduced survival, induced apoptosis and promoted G2/M arrest in human pancreatic adenocarcinoma cells.

Synergistic cytotoxic effects of NVP-TAE684 and gemcitabine. In order to investigate the potential beneficial effects of NVP-TAE684 and gemcitabine combination therapy, MIA PaCa-2, Colo-357, AsPC-1 and BxPC-3 cells were treated with NVP-TAE684 and gemcitabine for 72 h at the indicated concentrations. The combination of NVP-TAE684 and gemcitabine synergistically inhibited cellular proliferation (Fig. 7A). To further investigate the synergism between NVP-TAE684 and gemcitabine, both compounds were used to assess the induction of apoptotic cell death in MIA PaCa-2 and Colo-357 cells. The cells were treated with either NVP-TAE684 or gemcitabine alone, or a combination of both drugs, at the indicated concentrations for 24 h, and a caspase-3/7 assay was performed to evaluate apoptosis. Compared with cells treated with either drug alone, the combination of NVP-TAE684 and gemcitabine synergistically increased apoptosis in both cell lines by significantly inducing caspase-3/7 activity (Fig. 7B). Furthermore, in order to confirm whether inhibiting ALK enhanced sensitivity to gemcitabine, MIA PaCa-2 cells

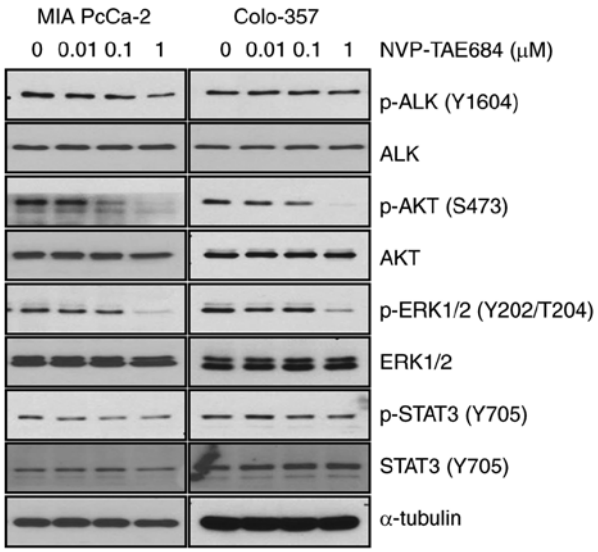

Figure 5. NVP-TAE684 inhibits downstream proteins of the ALK signaling pathway. Western blot analysis of MIA PaCa-2 and Colo-357 cells treated of NVP-TAE684 with various concentrations (0, 0.01, 0.1 or 1 μM) for 8 h were used to determine the ALK signaling pathway with indicated antibodies. Anti-α-tubulin antibody was used for loading and transfer control.

pretreated with either ALK siRNA (#1 or #2) or the control siRNA were incubated with the indicated concentrations of gemcitabine for 72 h. The results revealed that a combination of ALK siRNA and gemcitabine more effectively reduced proliferation than a combination of the control-siRNA and gemcitabine (Fig. 7C). Collectively, these results indicated that targeting ALK with NVP-TAE684 or ALK siRNA enhanced gemcitabine-induced cell death in human pancreatic adenocarcinoma cells by inducing apoptosis.

Discussion

In the present study, the mechanisms underlying the antitumor effects of NVP-TAE684, a potent and selective inhibitor of ALK, were investigated in human pancreatic adenocarcinoma cells. The results indicated that the levels of ALK phosphorylation were increased in human pancreatic adenocarcinoma cell lines (AsPC-1, Panc-1, MIA PaCa-2, Capan-1, CFPAC-1, Colo-357 and BxPC-3) and immortal human pancreatic duct epithelial cells (HPDE6-C7), and that NVP-TAE684 inhibited cell survival in all of the pancreatic adenocarcinoma cell lines investigated. Furthermore, NVP-TAE687 significantly induced G2/M phase cell cycle arrest and apoptotic cell death, and decreased the phosphorylation of ALK and downstream members of the ALK signaling pathway. To further confirm the effects of NVP-TAE687, ALK siRNA-knockdown also reduced cell survival and induced G2/M arrest and apoptotic cell death; additionally, the inhibition of ALK with NVP-TAE684 or siRNA enhanced gemcitabine-induced apoptosis. To the best of our knowledge, the present study is the first to report that NVP-TAE684-induced ALK inhibition reduced cell viability and induced apoptosis and G2/M phase cell cycle arrest in human pancreatic adenocarcinoma cells.

A number of small molecular kinase inhibitors have been developed to target ALK and its downstream signaling-pathway proteins, the effects of which have been confirmed in various cancer types, including anaplastic large-cell lymphoma, non-small cell lung cancer, neuroblastoma, large B-cell lymphoma and pancreatic adenocarcinoma (10,11,15-20). Notably, using NVP-TAE684 to retard ALK activity exerted significant antitumor effects in anaplastic large-cell lymphoma, non-small cell lung cancer, neuroblastoma and large B-cell lymphoma (15-20). In addition, NVP-TAE684-associated ALK inhibition decreased cell survival and induced apoptosis in osteosarcoma, which was enhanced by combination treatment with chemotherapeutic drugs such as doxorubicin, paclitaxel, docetaxel and vincristine (21), and with radiotherapy in non-small cell lung cancer (22). Furthermore, targeting ALK activity with crizotinib or ceritinib resulted in significant antitumor effects against pancreatic adenocarcinoma (10,11), and inhibiting ALK with ceritinib significantly enhanced the sensitivity of pancreatic adenocarcinoma cells to gemcitabine (10). However, there are currently no studies focused on the antitumor effects and molecular mechanisms of NVP-TAE684 in pancreatic adenocarcinoma, or comparisons with effective ALK inhibitors such as NVP-TAE 684, crizotinib or ceritinib in the treatment of other types of cancer as previously reported (10,11,15-20). In the present study, seven human pancreatic adenocarcinoma cell lines with relatively high ALK phosphorylation levels at Y1604 were revealed to be sensitive to NVP-TAE684 treatment. Among them, MIA PaCa-2, BxPC-3 and CFPAC-1 cells exhibited high sensitivity to NVP-TAE684, and Colo-357, AsPC-1, Panc-1 and Capan-1 cells were relatively less sensitive to NVP-TAE684. Notably, NVP-TAE684-induced ALK inhibition also significantly enhanced the antitumor effects of gemcitabine in pancreatic adenocarcinoma cells. Therefore, these developments indicated the possible clinical significance of targeted therapy with well-known ALK inhibitors such as NVP-TAE684, ceritinib and/or crizotinib for the effective treatment of various cancers with high ALK activity, including pancreatic adenocarcinoma.

The G2/M checkpoint is an important regulatory point of the cell cycle (27), and cell cycle arrest at this phase indicates that damaged DNA is difficult to repair (34,35). Previously, ALK inhibition using crizotinib was revealed to promote G2/M arrest in A2780 and SKOV3 ovarian cancer cells (23) and non-small cell lung cancer cells including HCC78, SPC-A1 and PC-9 (24,36). On the other hand, inhibition of ALK with NVP-TAE684 induced G1 arrest in H2228 and HCC78 non-small cell lung cancer cells (17,36), Karpas-299 anaplastic large-cell lymphoma cells (15) and LM1 diffuse large B-cell lymphoma cells (19). In the present study, NVP-TAE684 induced cell cycle arrest at the G2/M phase in MIA PaCa-2 and Colo-357 pancreatic adenocarcinoma cells. However, the molecular mechanisms of NVP-TAE684-associated cell cycle arrest could not be demonstrated, which may be due to the increased downregulation of CD30 and off-target inhibition of aurora kinase (15,36,37).

It is well known that ALK activates various downstream signaling pathways including those of AKT, ERK1/2 and STAT3 which regulate cellular proliferation, survival, division and invasion (9,12-14). Therefore, to determine the ability of NVP-TAE684 to target downstream signaling pathway proteins, western blot analysis was performed using pancreatic adenocarcinoma cells treated with various concentrations of NVP-TAE684. Among the proteins of these downstream

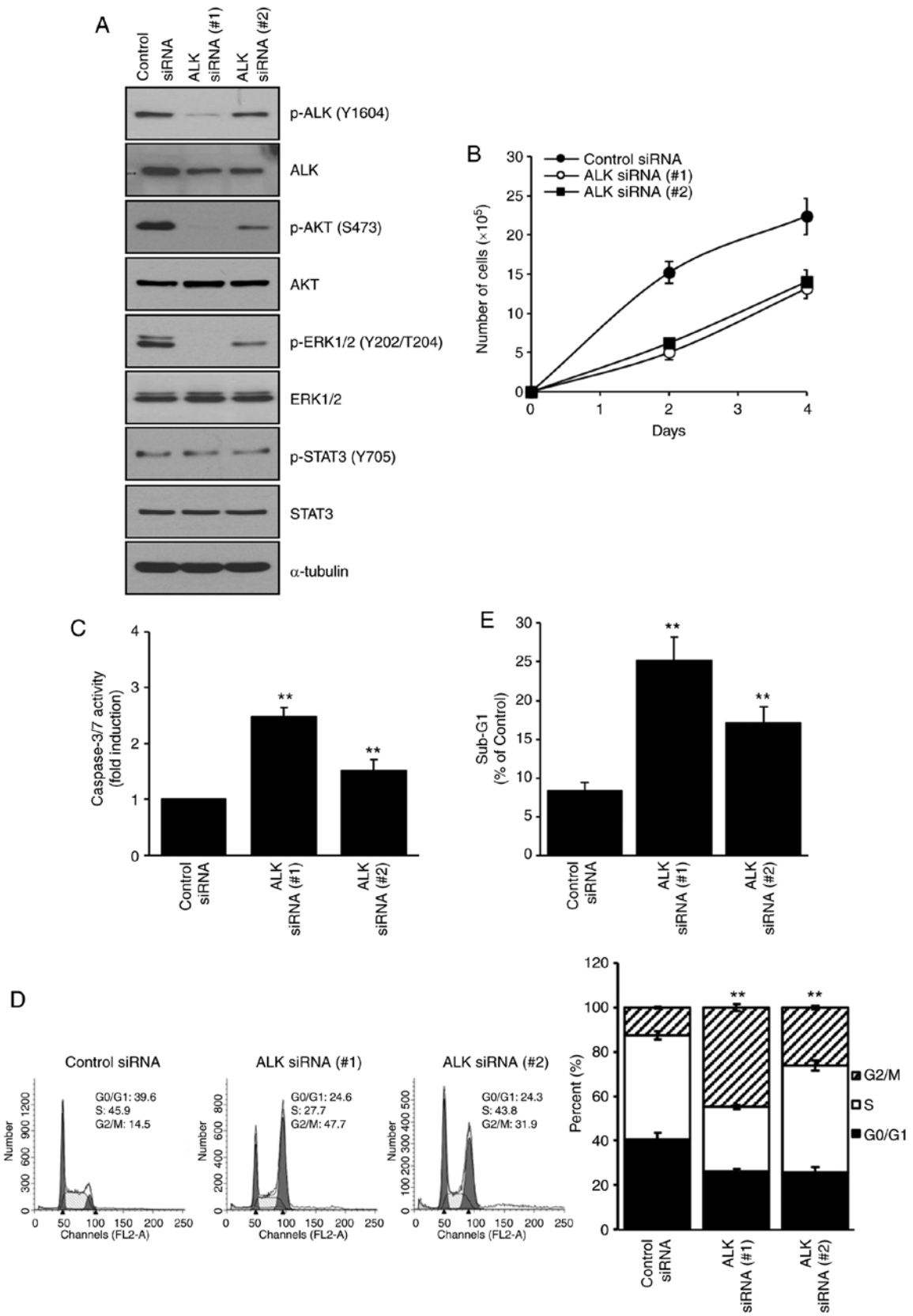

Figure 6. Knockdown of ALK inhibits cell survival and induces apoptotic cell death. MIA PaCa-2 cells were transfected with either ALK siRNA (#1 and #2) or Control siRNA for 48 h as described in Materials and methods. (A) Western blot analysis was performed with the indicated antibodies. Anti-α-tubulin antibody was used for loading and transfer control. (B) Results of the trypan blue exclusion assay of MIA PaCa-2 cells which were transfected with either ALK siRNA (#1 and #2) or Control siRNA for 2 and/or 4 days. Representative data from three independent experiments performed in triplicate are shown as mean ± SD. (C) MIA PaCa-2 cells were transfected with either ALK siRNA (#1 and #2) or Control siRNA for 48 h as described in (A) and caspase-3/7 activity analysis was used to measure apoptotic cell death. Representative data from three independent experiments performed in triplicate are presented as the mean ± SD. **P<0.01. (D and E) MIA PaCa-2 cells were transfected with either ALK siRNA (#1 and #2) or Control siRNA for 48 h as described in A and FACS analysis was used to determine cell cycle arrest. (E) The sub-G1 population from FACS analysis was analyzed. Representative data are presented as the mean ± SD from three independent experiments. **P<0.01.

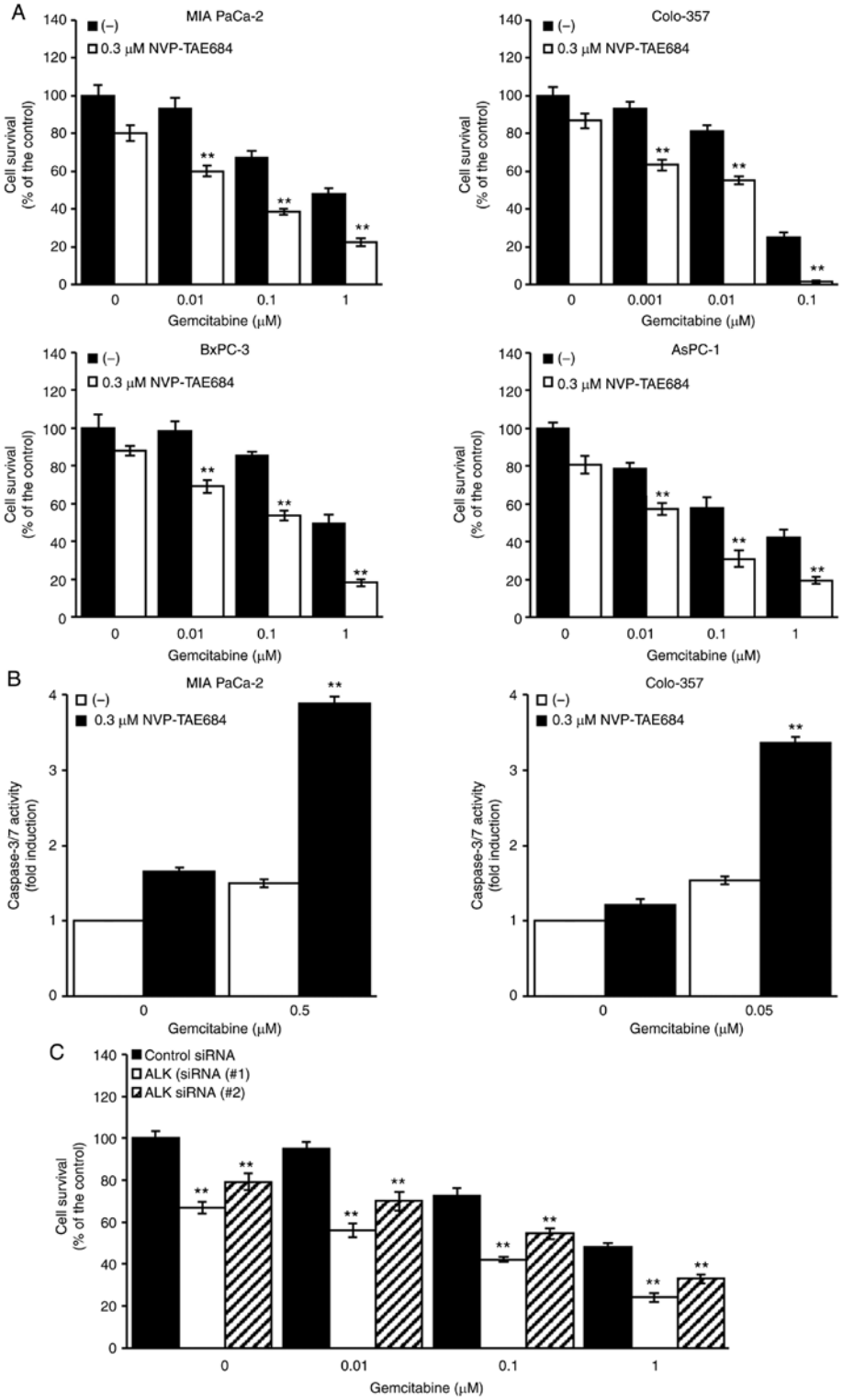

Figure 7. Synergistic antitumor effects by combination of NVP-TAE684 and gemcitabine. (A) An MTT assay of MIA PaCa-2, Colo-357, BxPC-3 and AsPC-1 cells co-treated of NVP-TAE684 and gemcitabine with indicated concentrations for 72 h was used to determine cell survival. Representative data from three independent experiments performed in triplicate are presented as the mean ± SD. **P<0.01. (B) MIA PaCa-2 and Colo-357 cells were treated with either NVP-TAE684, gemcitabine alone or in combination of both drugs with indicated concentrations for 48 h and then caspase-3/7 activity was measured to determine apoptotic cell death. Representative data from three independent experiments performed in triplicate are presented as the mean ± SD. **P<0.01. (C) An MTT assay of MIA PaCa-2 cells transfected with either ALK siRNA (#1, #2) or Control siRNA for 48 h as described in Fig. 6A and further treated with gemcitabine with indicated concentrations for 72 h was used to determine cell survival. Representative data from three independent experiments performed in triplicate are presented as the mean ± SD. **P<0.01.

signaling pathways, NVP-TAE684 effectively inhibited the phosphorylation of AKT (S473) and ERK1/2 (Y202/T204), and to a lesser degree, STAT3 (Y705) in pancreatic adenocarcinoma cells, indicating that NVP-TAE684-induced ALK inhibition effectively reduced the activities of these signaling pathways. Overall, it was hypothesized that the antitumor effects of

NVP-TAE684 were closely associated with the inhibition of ALK signaling, which ultimately resulted in a reduction in cell survival and the induction of apoptotic cell death and G2/M arrest in pancreatic adenocarcinoma cells.

In conclusion, the findings of the present study demonstrated that inhibiting ALK activity with NVP-TAE684 reduced cell survival and induced apoptotic cell death and G2/M arrest in pancreatic adenocarcinoma cells. However, there are still some limitations to our research. First, the effect(s) of NVP-TAE684 on cell survival of normal cell lines was not investigated; second, western blot images of cleaved caspase-3 expression are required to examine the induction of apoptotic cell death by NVP-TAE684; third, animal model study/patient-derived xenograft experiments need to be performed to better investigate the important role of NVP-TAE684 in pancreatic adenocarcinoma cells; last the molecular mechanisms of the antitumor effects of NVP-TAE684, alone or in combination with other chemotherapeutic drugs such as gemcitabine, require further investigation, NVP-TAE684 may be a novel compound for the treatment of patients with pancreatic adenocarcinoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HQD, VTT, THD and YSS conceived and designed the study. HQD, VTT, HTN, PTN, HTHT, TNHB, VPLD, TTD and KSY conducted the experiments and performed the statistical analysis. HQD, VTT and YSS wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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