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Original Articles

Dual inhibition of the PI3K and MAPK pathways enhances *nab*-paclitaxel/gemcitabine chemotherapy response in preclinical models of pancreatic cancer

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ABSTRACT

Standard chemotherapy for pancreatic ductal adenocarcinoma (PDAC), *nab*-paclitaxel (NPT) plus gemcitabine (Gem), has led to an average survival of 8.5 months. Presently, no therapeutics exist that effectively target the KRAS oncogene, activated in 95% of PDACs, but alternative strategies focus on inhibition of downstream effectors of KRAS signaling. Through combined inhibition of PI3K and MAPK signaling with MK-2206 (MK) and trametinib (Tra), enhancement of NPT + Gem response was evaluated. Median animal survival was significantly improved by the NPT + Gem combination (67% increase). Addition of MK-2206 or trametinib further increased median survival: NPT + Gem + MK (86%), NPT + Gem + Tra (105%), and NPT + Gem + MK + Tra (129%). In cell line-derived xenografts, the net tumor growth (in mm^3) compared to controls (878.5) was significantly reduced by NPT + Gem (191.2), NPT + Gem + MK (150.7), NPT + Gem + Tra (62.2) and NPT + Gem + MK + Tra (49.9) therapies. In patient-derived xenografts, the combination of MK-2206 and trametinib with chemotherapy had an additive response in reducing tumor growth. Effects of therapy on tumor cell proliferation and apoptosis corresponded with tumor growth inhibition. These findings suggest that the standard chemotherapy response of PDAC can be enhanced through dual targeting of PI3K and MAPK signaling, which could lead to improved PDAC therapy.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is currently the 3rd leading cause of cancer-related death in the USA and is expected to become the 2nd leading cause of cancer-death by 2030, surpassing colorectal cancer [1,2]. The prognosis of PDAC patients remains very poor with a 5-year overall survival rate of approximately 6%. Cardinal factors associated with poor prognosis are challenges to an early diagnosis, an aggressive progression of the disease and high resistance to traditional treatment approaches. Surgical resection gives the best chance for long-term survival of PDAC patients, however, only about 15–20% of patients qualify for resection because most patients have metastatic disease at the time of diagnosis [3]. In addition, the recurrence rate of PDAC is as high as 85% after curative resection [4,5]. Systemic chemotherapy is the main treatment for locally advanced unresectable or recurrent PDAC. The single agent gemcitabine (Gem) had been the standard treatment for advanced PDAC for more than two decades until FOLFIRINOX and *nab*-paclitaxel/gemcitabine became two superior first-line combination treatment options for PDAC [6–8]. FOLFIRINOX, a combination of 5-fluorouracil, leucovorin, irinotecan and oxaliplatin, increased the median survival of PDAC patients by 4.3 months (11.1 vs 6.8 months with Gem monotherapy). However, this regimen has a higher incidence of grade 3 to 4 adverse events, limiting its use to patients with good performance status [7]. A phase III trial (MPACT) demonstrated that the combination of gemcitabine with *nab*-paclitaxel (NPT) led to a 1.8-month improvement in patient survival compared to gemcitabine monotherapy [8]. Due to the still limited clinical efficacy of current standard chemotherapy for PDAC, novel

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Abbreviations

PDAC	Pancreatic ductal adenocarcinoma
NPT	*Nab*-paclitaxel
Gem	Gemcitabine
MK	MK-2206
Tra	Trametinib
IHC	Immunohistochemistry
PDX	Patient-derived xenografts

therapeutic approaches are urgently needed to create a meaningful impact on patient survival.

Genetic analysis exhibited *KRAS*, *p16/CDKN2A*, *TP53*, and *SMAD4* as the most frequently altered genes in PDAC. Among these, activating mutation in *KRAS* is the most frequently mutated oncogene in PDAC (> 95%) [9]. Thus, targeting this signaling pathway should have high therapeutic potential in PDAC. Unfortunately, the development of drugs that directly target KRAS remains elusive [10]. Therefore, alternative strategies focus on inhibition of major downstream effectors of KRAS signaling including the RAS/RAF/MEK/ERK (MAPK) and/or PI3K/AKT/mTOR signaling pathways.

In the RAS/RAF/MEK/ERK pathway, receptor tyrosine kinase (RTK) activation causes RAS phosphorylation that activates the RAF-MEK-ERK kinase signaling leading to several cellular processes involved in tumorigenesis including proliferation, transformation and survival [11]. Several small molecule inhibitors have been developed to target this pathway among which allosteric inhibitors of MEK have shown promising therapeutic efficacy [12]. Trametinib (Tra, Supplementary Fig. S1), a selective and reversible inhibitor of MEK1/2 kinase activity, has demonstrated antitumor efficacy in preclinical studies of several tumor types, with the largest effect in tumors harboring mutant *BRAF* or *RAS* [13]. Further, trametinib is an FDA-approved treatment for V600E-mutant metastatic melanoma patients [14,15]. In PDAC patient-derived xenografts, trametinib showed significant antitumor activity [16]. Recently, we demonstrated the potential of trametinib to enhance *nab*-paclitaxel-based chemotherapy in mouse models of PDAC [17]. In a phase II clinical study, trametinib combination with gemcitabine exhibited limited response in PDAC patients [18].

In the PI3K/AKT/mTOR pathway, RTK activation causes phosphorylation of PI3K which then phosphorylates PIP2 to PIP3, leading to AKT activation. The mammalian target of rapamycin (mTOR) is a downstream component of this pathway. The PI3K/AKT/mTOR pathway plays a central role in several cellular processes such as proliferation, survival and growth. Aberrant signaling of this pathway has been found in many cancers including PDAC and is mainly related to PTEN loss, PI3K amplification/mutation, AKT mutation or RTK activation [19]. MK-2206 is a novel, selective and allosteric inhibitor of AKT that has shown antitumor efficacy *in vitro* and *in vivo* (MK, Supplementary Fig. S1) [20]. MK-2206 is currently in phase II clinical trials for many solid tumors (clinicaltrials.gov).

Crosstalk exists between the RAS/RAF/MEK/ERK and/or PI3K/AKT/mTOR signaling pathways. Studies have shown activation of PI3K by RAS, and regulation of mTOR by ERK [21–23]. Also, alterations in both pathways have been found to occur together in many cancers including PDAC, indicating the requirement for the simultaneous inhibition of both the pathways for achieving maximum therapeutic benefit [24,25]. Some reports suggest that the RAS/RAF/MEK/ERK or PI3K/AKT/mTOR pathways can act as a compensatory mechanism with the inhibition of one pathway, further supporting the rationale of combined inhibition of these two pathways [26,27].

In this study, we explored the antitumor efficacy of simultaneous inhibition of both RAS/RAF/MEK/ERK and/or PI3K/AKT/mTOR signaling pathways in combination with *nab*-paclitaxel/gemcitabine chemotherapy in preclinical models of PDAC.

2. Materials and methods

2.1. Cell culture and reagents

Human PDAC cell lines (AsPC-1, BxPC-3, CFPAC, HPAF-II, Mia PaCa-2, SW-1990 and Panc-1) were purchased from the ATCC. A KPC mouse-derived cell line was generously provided by Dr. Reginald Hill (University of Southern California, CA). Pan02 cells were provided by Dr. Rolf Brekken (University of Texas Southwestern Medical Center, TX). Cells were cultured in DMEM or RPMI 1640 medium (Sigma Chemical Co. St. Louis, MO) containing 10% FBS and maintained at 37 °C in a humidified incubator with 5% CO_2 and 95% air. *Nab*-paclitaxel was obtained from Celgene Corporations (Summit, NJ). Gemcitabine and trametinib were purchased from LC labs (Woburn, MA). MK-2206 was purchased from Chemietek (Indianapolis, IN). The cell proliferation reagent WST-1 was purchased from Roche Diagnostic Corporation (Indianapolis, IN).

2.2. Cell viability assay

Cell viability was evaluated by the colorimetric WST-1 assay as previously described [28]. Briefly, PDAC cells (~4000 cells per well) were plated in a 96-well plate in regular cell growth medium containing 10% FBS. After 16 h, the medium was replaced with low serum medium containing 2% FBS and the cells were treated with 10 nM, 100 nM and 1 μM concentrations of *nab*-paclitaxel, MK-2206 and trametinib; or 100 nM, 1 μM and 10 μM concentrations of gemcitabine. After 72 h, WST-1 reagent (10 μl) was added in each well followed by additional incubation for 2 h. The absorbance was measured at 450 nm using a microplate reader.

2.3. Western blot analysis

Protein lysates were prepared by treating sub-confluent cells with *nab*-paclitaxel, gemcitabine, MK-2206 and trametinib (concentration of each drug 10 μM) and lysed after 16 h for Western blot analysis as previously described [28]. Protein lysates of subcutaneous tumors were prepared by snap freezing tumors in liquid N_2 and stored at −80 °C. These tumors were suspended in lysis buffer and homogenized using the Bullet Blender Homogenizer (Next Generation, Averill Park, NY), and extracts were sonicated on ice. Proteins in supernatants were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were incubated with the following antibodies: total ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), total AKT, phospho-AKT (Ser473), cleaved caspase-3, cleaved PARP-1 and GAPDH (Cell Signaling Technology, Beverly, MA). The membranes were then incubated with the corresponding HRP-conjugated secondary antibodies (Pierce Biotechnologies, Santa Cruz, CA). Protein bands were visualized using the enhanced chemiluminescence reagent (SignalFire, Cell Signaling) with an Image360 system and quantitated by densitometry.

2.4. Animal experiments

Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at the Indiana University School of Medicine (South Bend, IN). Female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (4–6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). NOD/SCID/IL2Rgnull (NSG) mice (6–8 weeks) were purchased from the Indiana University Simon Cancer Center In-Vivo Therapeutic Core.

Cell-derived subcutaneously xenograft study: AsPC-1 cells (7.5×10^5) were implanted subcutaneously into the right flank region of NOD/SCID mice. Two weeks after tumor cell injection, all mice had a measurable tumor. Mice were then randomized (n = 4 to 6 per group)

to receive PBS (control), *nab*-paclitaxel (5 mg/kg, twice a week), gemcitabine (50 mg/kg, twice a week), MK-2206 (50 mg/kg, 3 times a week) or trametinib (1 mg/kg, 5 times a week) via intraperitoneal injection for next 2 weeks. The tumor size was measured twice weekly, and tumor volume (V) was calculated using formula V = ½ (Length x Width^2). Net tumor growth was calculated by subtracting tumor volume on the first therapy day from that on the last day. Mice were euthanized after completion of treatment, tumors were dissected and processed for histological, immunohistochemical and Western blot analysis.

Patient-derived subcutaneous xenograft (PDX) study: A NSG mouse carrying pancreatic cancer patient-derived tumor was purchased from the Jackson Laboratory (Bar Harbor, ME). Tumor was sectioned into small pieces (1 cm × 1 cm) and subcutaneously implanted into the right flanks of NSG mice under anesthesia with isoflurane. Tumor growth was monitored twice weekly using a caliper. At about 1000 mm^3, tumors were extracted for serial transplantation into NSG mice and used for the experiment when tumor volume reached about 100–200 mm^3. PDX-bearing mice were randomized, grouped and treated for 2 weeks as mentioned previously. The tumor size was measured, and tumor volume was calculated.

Animal survival study: Female NOD/SCID mice (4–6 weeks of age) were injected intraperitoneally with AsPC-1 cells (7.5×10^5). Two weeks after tumor cell injection, mice were randomized (n = 6 to 8 per group) to receive therapy for two weeks as described in subcutaneous xenograft studies. Animals were euthanized when moribund according to predefined criteria [29,30]. Animal survival was evaluated from the first day of treatment until death.

2.5. Immunohistochemistry and immunofluorescence

Subcutaneous tumors were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Tumor sections (5 μm) were deparaffinized and rehydrated followed by heat-mediated antigen retrieval in citrate buffer. The tumor sections were then incubated with CAS blocking buffer for 20 min. Tumor cell proliferation was measured by overnight incubation with an anti-Ki67 antibody (Abcam, Cambridge, MA) at 4 °C and 40 min of incubation at room temperature with a Cy3 secondary antibody. Slides were mounted with a mounting medium containing DAPI (Invitrogen, Carlsbad, CA) and imaged using a fluorescence microscope. Proliferative activity was evaluated by calculating Ki67-positive cells from five different high-power fields (HPF) in a blinded manner. Tumor cell apoptosis was determined by TUNEL assay using "Apoptag Apoptosis Detection Kit" according to the manufacturer's (Millipore) instructions. Fluorescence microscopy was performed using IX81 Olympus microscope and images were captured with a Hamamatsu Orca digital camera (Hamamatsu Corporation, Bridgewater, NJ) with a DSU spinning confocal unit using cellSens Dimension software (Olympus, Center Valley, PA).

2.6. Statistical analysis

Statistical analysis for *in vivo* tumor growth studies was performed by one-way ANOVA for multiple group comparisons and Student's t-test for the individual group comparisons. Survival study statistics were performed using logrank group comparison (GraphPad Prism 6.0). P values less than 0.05 were considered statistically significant. *In vitro* cell proliferation data are expressed as the mean ± standard deviation. Statistical significance was analyzed by the two-tailed Student's t-test using GraphPad Prism 6.0 Software for individual group comparisons.

3. Results

3.1. MK-2206 and trametinib augment animal survival benefits of nab-paclitaxel/gemcitabine

In PDAC peritoneal dissemination xenografts with 2-week therapy schedule (Fig. 1A), animal survival was significantly increased by NPT + Gem chemotherapy (35 days, a 67% increase) compared with controls (21 days). Animal survival benefit of NPT + Gem was further improved by the addition of MK-2206 (39 days, an 86% increase over controls) or trametinib (43 days, a 105% increase). Importantly, the addition of both agents MK-226 and trametinib to NPT + Gem had an additive response in increasing animal survival (48 days, a 129% increase) (Fig. 1B). There was neither significant change in the body weight of mice during therapy period nor any discernible therapy-related toxicity in any therapy group (data not shown).

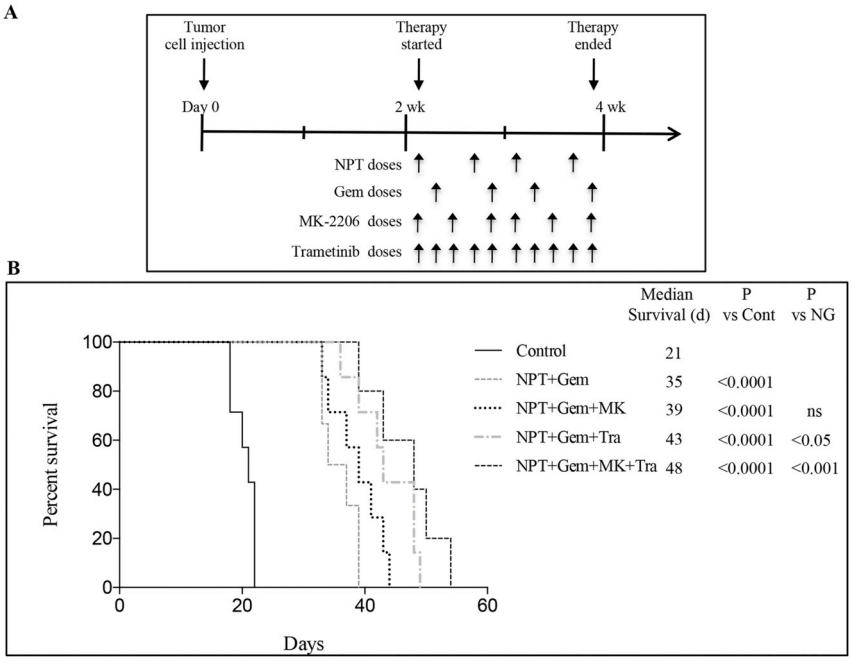

Fig. 1. Improvement in animal survival by addition of MK-2206 and trametinib to *nab*-paclitaxel/gemcitabine. **A.** Schematic representation of experimental procedure. AsPC-1 cells (0.75×10^6) were injected intraperitoneally in NOD/SCID mice and treatment was started after 2 weeks. **B.** Kaplan-Meier survival curve for mice injected with AsPC-1 cells and treated with *nab*-paclitaxel, gemcitabine, MK-2206 and trametinib for 2 weeks. The animal survival time was measured from the beginning of therapy. Statistical group differences in survival time were calculated using logrank testing.

3.2. MK-2206 and trametinib enhance tumor inhibition response of nab-paclitaxel/gemcitabine

In AsPC-1 PDAC cell-derived subcutaneous xenografts, 2-weeks after tumor cell injection, control mice (PBS treated) displayed rapid tumor growth for the next 16 days of therapy period. *Nab*-paclitaxel plus gemcitabine therapy inhibited tumor growth while the addition of MK-2206, trametinib or both agents together to chemotherapy regimen had a trend for additive effects (Fig. 2A). The net tumor growth after 16 days of therapy was 878.5 mm^3 in controls, 191.2 mm^3 after NPT + Gem, 150.7 mm^3 after NPT + Gem + MK, 62.2 mm^3 after NPT + Gem + Tra and 49.9 mm^3 after NPT + Gem + MK + Tra (Fig. 2B). At the completion of therapy, tumor weight was maximum in controls (0.76 g) that was decreased by NPT + Gem therapy (0.44 g). The addition of MK-2206 and trametinib to chemotherapy further decreased the tumor weight (Fig. 1C). In this experiment, there was no therapy-related change in the body weight of mice (Fig. 2D).

In PDAC patient-derived xenografts, *nab*-paclitaxel plus gemcitabine, MK-2206, trametinib or their combination delayed tumor growth. In this study, the addition of MK-2206 to chemotherapy had greater tumor inhibition effect than the addition of trametinib. Importantly, dual inhibition by MK-2206 and trametinib demonstrated additively enhanced response (Fig. 3A). The net increase in tumor size was 350 mm^3 in controls, 145.4 mm^3 after NPT + Gem, 20.4 mm^3 after NPT + Gem + MK, 122.1 mm^3 after NPT + Gem + Tra and −43.7 mm^3 (tumor regression) with NPT + Gem + MK + Tra (Fig. 3B). Mean tumor weight at the completion of therapy was again greatest in controls (0.4 g) but decreased by NPT + Gem (0.24 g) therapy, while the addition of MK-2206 or trametinib to chemotherapy exhibited an additive response in inhibiting tumor weight (Fig. 3C). Consistent with prior experiments, therapies appeared to be well tolerated as reflected by no significant weight change (Fig. 3D).

3.3. MK-2206 and trametinib with chemotherapy: tumor cell proliferation and apoptosis

Evaluation of subcutaneous tumors determined that *nab*-paclitaxel plus gemcitabine effectively reduced tumor cell proliferation (by 38.4%). Addition of MK-2206 and trametinib to chemotherapy led to further inhibition in tumor cell proliferation (range 51–57%). The combination of MK-2206 and trametinib with chemotherapy was most effective in decreasing tumor cell proliferation (67.8%) (Fig. 4A).

Tumor cell apoptosis detection revealed that compared with control samples (apoptotic index: 2.2), *nab*-paclitaxel plus gemcitabine prompted a small increase (3.7). The addition of MK-226 and trametinib, either alone or in combination, to chemotherapy resulted in a significant increase in tumor cell apoptosis (apoptotic index > 7.6) (Fig. 4B).

Further investigation of the tumor inhibitory mechanisms by Immunoblot analysis in subcutaneous tumor lysates exhibited that MK-2206 and trametinib decreased AKT and ERK phosphorylation, respectively. Additionally, both these agents demonstrated a concomitant increase in apoptosis-associated proteins cleaved caspase-3 and cleaved PARP-1 (Fig. 5).

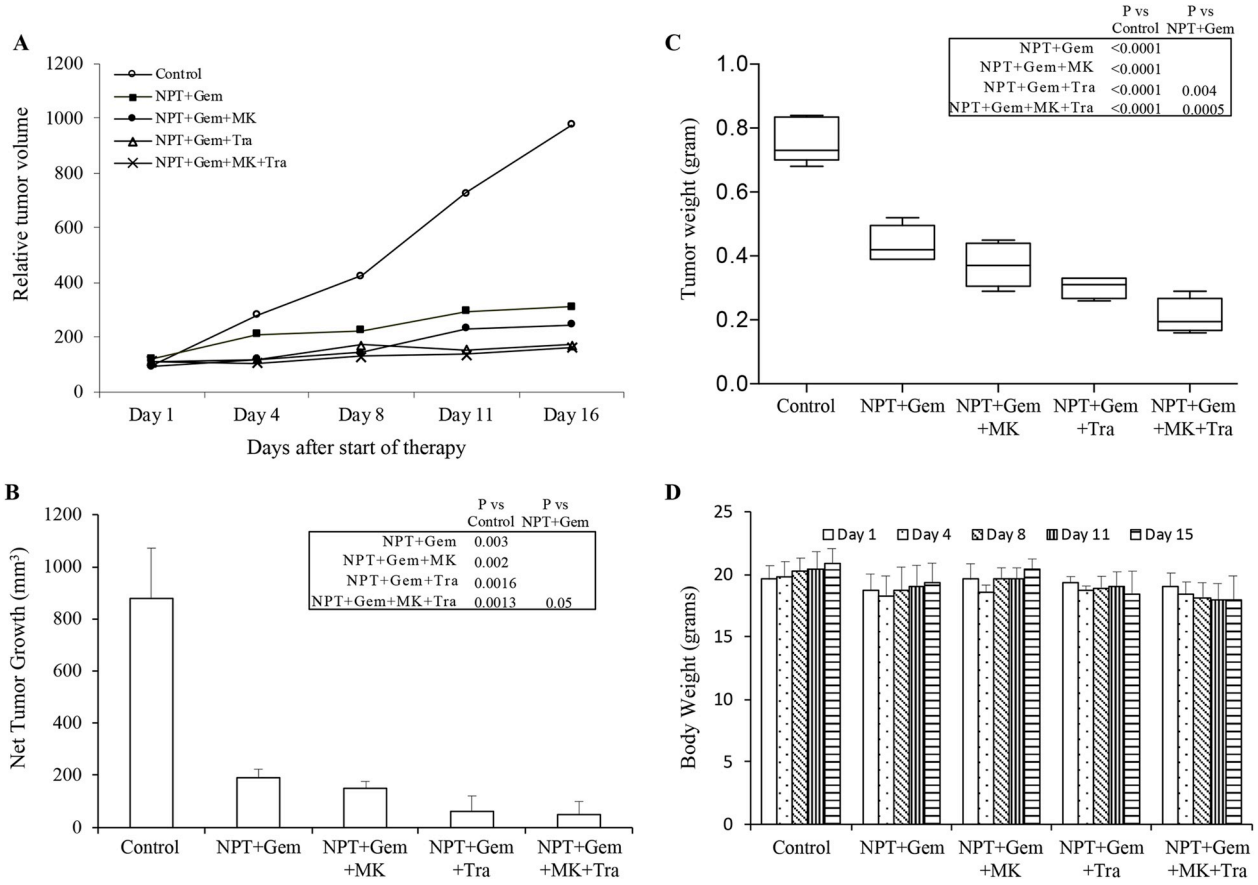

Fig. 2. Tumor growth inhibition by addition of MK-2206 and trametinib to *nab*-paclitaxel/gemcitabine in AsPC-1 cell-derived xenografts. Tumor-bearing nude mice were treated with *nab*-paclitaxel, gemcitabine, MK-2206 and trametinib for 2 weeks. **A.** Tumor size was measured twice a week using calipers and plotted. **B.** Net growth in tumor size was calculated by subtracting tumor volume on the first treatment day from that on the final day and plotted using bar graph. **C.** Mean tumor weight was calculated from final day tumor weights and presented as a bar chart. **D.** Mouse body weight was measured twice a week during the 2-week therapy period and presented as bar chart. Data are representative of mean values ± standard deviation from 5 mice per group.

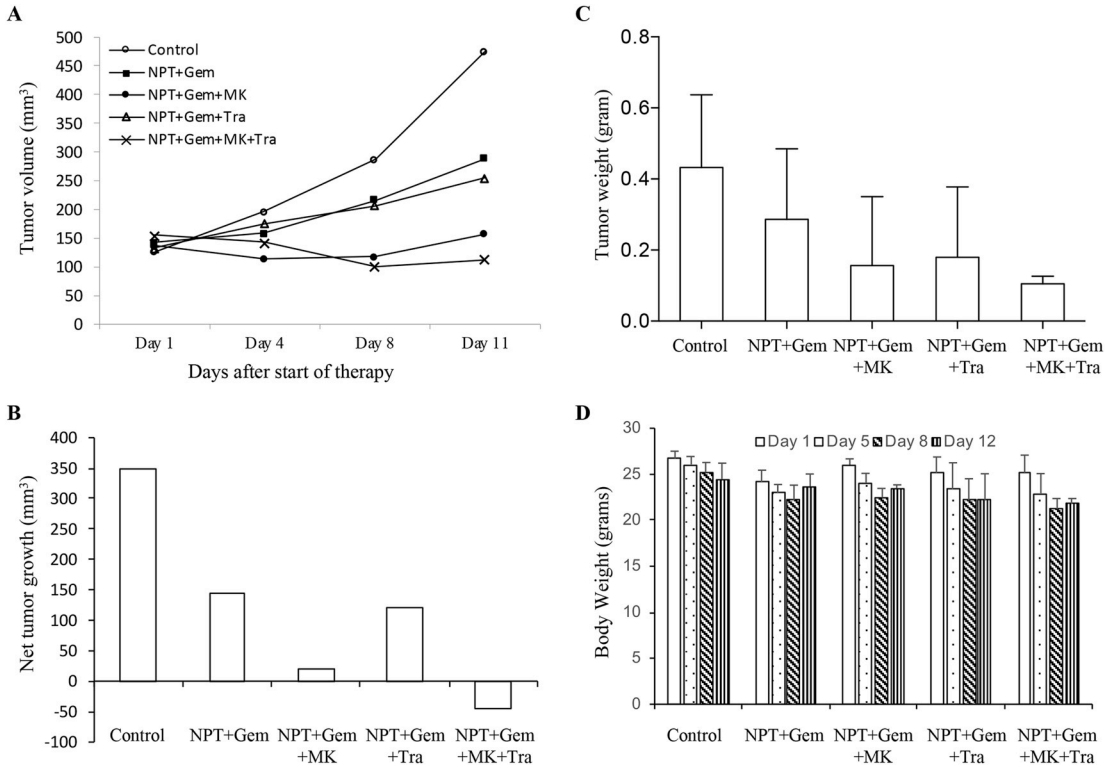

Fig. 3. Tumor growth inhibition by addition of MK-2206 and trametinib to *nab*-paclitaxel/gemcitabine in PDAC patient-derived xenografts. Tumor-bearing NSG mice were treated with *nab*-paclitaxel, gemcitabine, MK-2206 and trametinib for 2 weeks. **A.** Tumor size was measured twice a week using calipers and plotted. **B.** Net growth in tumor size was calculated by subtracting tumor volume on the first treatment day from that on the final day and plotted using bar graph. **C.** Mean tumor weight was calculated from final day tumor weights and presented as a bar chart. **D.** Mouse body weight was measured twice a week during the 2-week therapy period and presented as bar chart.

3.4. MK-2206 and trametinib with chemotherapy: In vitro cell viability and cellular targets

In vitro cell viability examination of *KRAS* mutant and wild-type PDAC epithelial cells indicated that *nab*-paclitaxel plus gemcitabine, MK-2206 and trametinib differentially suppressed cell proliferation. Among the *KRAS* mutant cell lines, AsPC-1 was least sensitive to these treatments while KPC mouse-derived cells were most sensitive (Fig. 6A). Percent inhibition in the cell proliferation in NPT + Gem, NPT + Gem + MK, NPT + Gem + Tra and NPT + Gem + MK + Tra was 36, 51, 44, 67 (AsPC-1, high dose levels); 58, 73, 70, 93 (Panc-1, high dose levels) and 75, 87, 99, 100 (KPC mouse-derived cells, medium dose levels) (Fig. 6A). Between the two *KRAS* wild-type cells tested, BxPC-3 cells were more sensitive to these treatments than Pan02 mouse PDAC cells (Fig. 6B). Percent inhibition in the cell proliferation was 92, 81, 88, 99 (BxPC-3, medium dose levels) and 26, 37, 39, 48 (Pan02, medium dose levels) (Fig. 6B).

Immunoblot analysis of AsPC-1 and Panc-1 cells determined that MK-2206 and trametinib blocked the expression of their respective cellular targets phospho-AKT and phospho-ERK, respectively. Importantly, reduction in the expression of phospho-AKT and phospho-ERK was concomitantly accompanied by increased levels of cleaved caspase-3 and cleaved PARP-1 proteins, to which the combination of MK-2206 and trametinib provided an additive effect (Fig. 7).

4. Discussion

Oncogenic KRAS activation is a nearly universal tumor-promoting pathway in PDAC, corroborating the rationale that blocking hyperactive KRAS signaling to reverse malignant transformation may be of central importance [9,10,31,32]. However, there are several barriers to targeting KRAS directly. Firstly, KRAS is a member of a large family of related proteins, which share very similar GDP/GTP-binding domains, making it extremely challenging to develop specific targeted drugs. Secondly, the KRAS protein lacks accessible binding pockets for high-affinity binding of small-molecule drugs. Thirdly and most importantly, KRAS has a very high affinity for GTP and GDP and there is a high concentration of GTP within the cell, thus rendering the creation of a competitive inhibitor immensely difficult [33]. Based on the challenges of direct KRAS targeting, the search has shifted towards downstream effectors, notable the MAPK and PI3K pathway [34].

Constitutive activated mutant *KRAS* leads to activation of several oncogenic pathways including those involving MAPK, PI3K-AKT, NF-kB, Wnt-β-catenin, Notch and TGF-β/Smad. Based on the facts that a large number of signaling pathways exist downstream to activating mutation in *KRAS* and these pathways are interconnected with each other at multiple levels, it is highly unlikely that single-agent therapy will produce a clinically meaningful therapeutic benefit to the *KRAS* mutant patient population. In a recent study, the suppression of PI3K pathway demonstrated a compensatory activation of the MAPK pathway, and inhibition of both the pathways was more effective [35]. Furthermore, in a mouse model of PDAC it was observed that MEK inhibition alone was cytostatic, but its antitumor response was significantly improved in combination with the PI3K inhibitor BKM120 or with erlotinib [36]. These studies indicate a critical importance for dual targeting of the PI3K-AKT and MAPK pathways for achieving the desired efficacy against *KRAS* mutant tumors such as PDAC. Our present study supports this rationale, as the combined administration of MK-2206 and trametinib was able to enhance standard cytotoxic therapy effects in various murine models of experimental PDAC.

The PDAC tumor microenvironment (TME) uniquely contains a dense desmoplastic stroma that often outnumbers the tumor cells [37]. This TME is highly heterogeneous consisting of tumor-associated fibroblasts, myofibroblasts, endothelial cells, immune cells and the

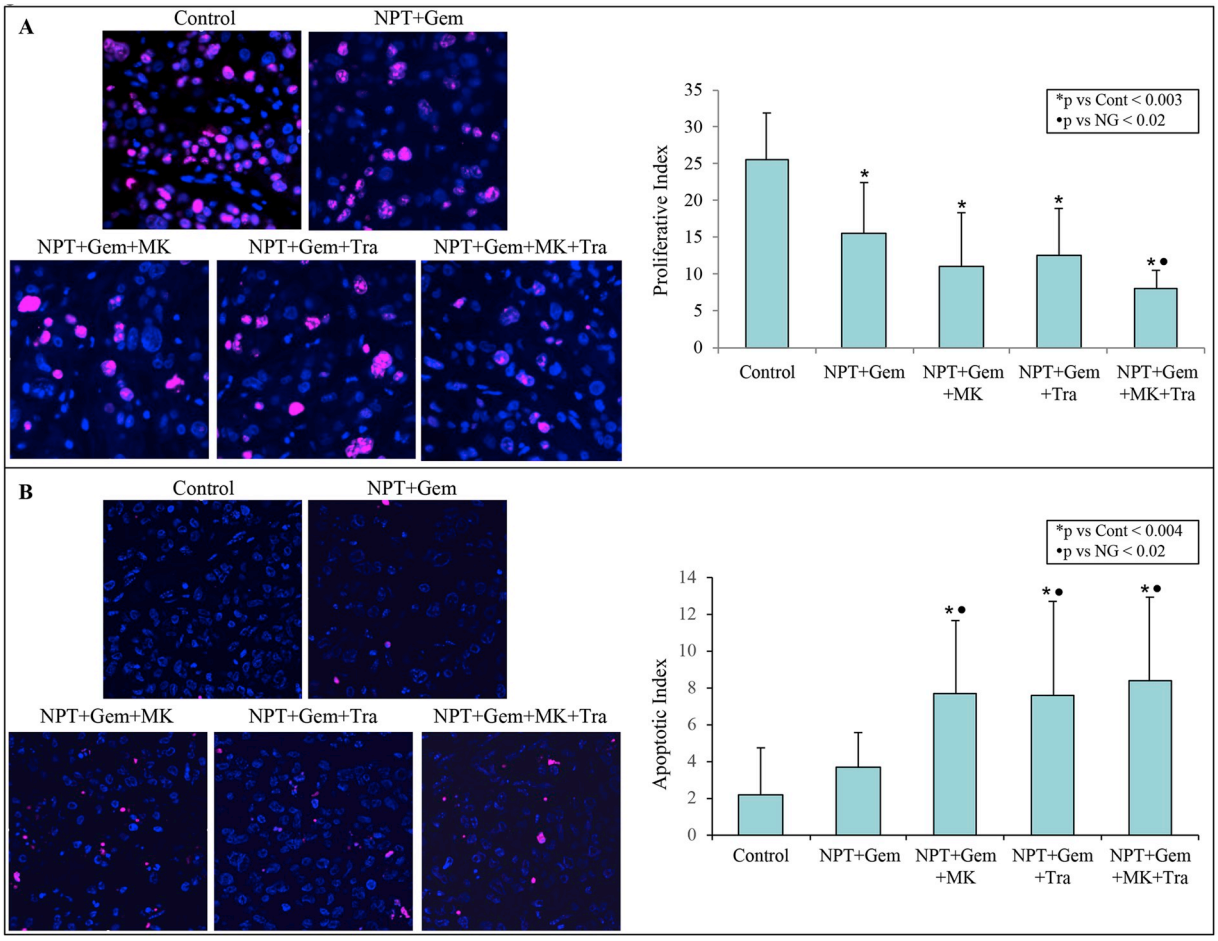

Fig. 4. Addition of MK-2206 and trametinib to *nab*-paclitaxel/gemcitabine: Effect on tumor cell proliferation and apoptosis. Nude mice bearing AsPC-1 PDAC cell-derived xenografts were treated with *nab*-paclitaxel, gemcitabine, MK-2206 and trametinib for 2 weeks. At the completion of treatment, tumors were dissected and processed for IHC analysis. **A.** Intratumoral proliferation was measured by immunostaining tumor sections for Ki67 nuclear antigen. Ki67-positive cells were counted in five different high-power fields. **B.** Intratumoral apoptosis was measured by staining tumor sections with TUNEL procedure. TUNEL-positive apoptotic cells were counted in five different high-power fields. For both immunostaining experiments, slides were photographed under a fluorescent microscope and the data are expressed as the mean ± standard deviation.

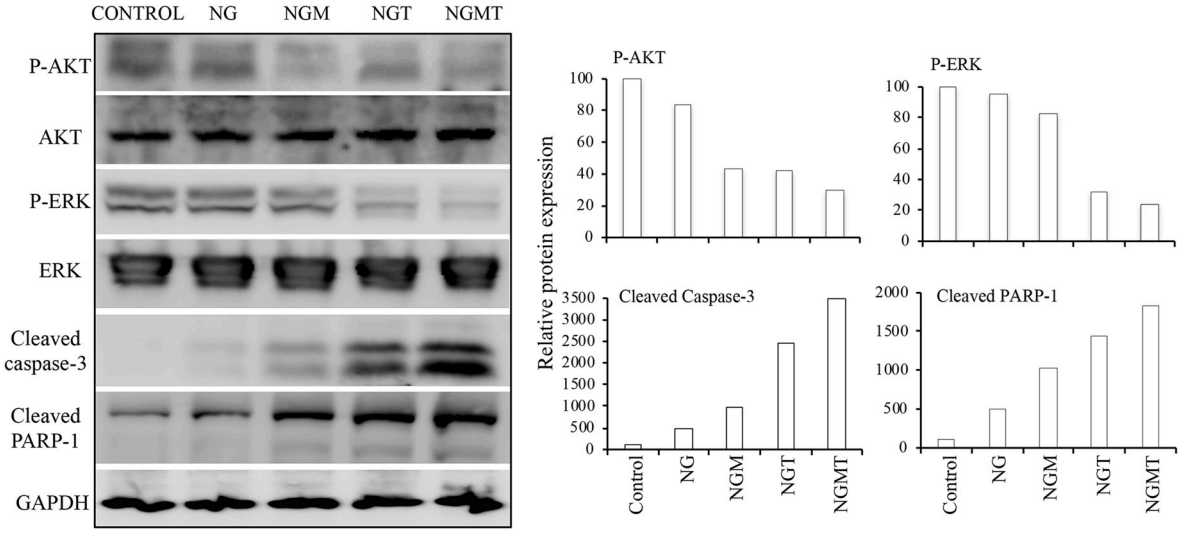

Fig. 5. Addition of MK-2206 and trametinib to *nab*-paclitaxel/gemcitabine: Effects on their molecular targets *in vivo*. Tumor lysates were prepared from tumor tissues obtained from AsPC-1 tumor-bearing mice after the completion of treatment and were analyzed by immunoblotting. Data are representative of pooled lysates obtained from tumor sections of at least 5 mice in each therapy group. The intensity of bands was quantitated by densitometry and is represented in the bar graph after normalizing values with corresponding total protein expression or GAPDH expression.

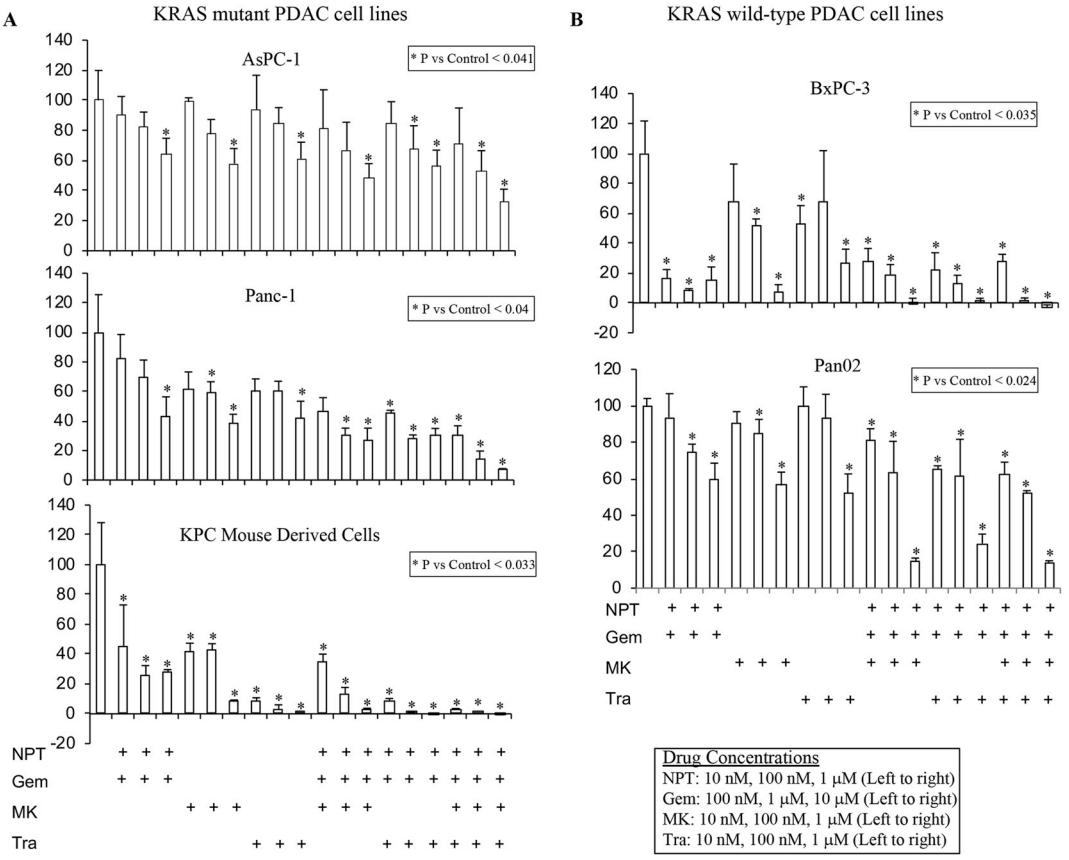

Fig. 6. Addition of MK-2206 and trametinib to *nab*-paclitaxel/gemcitabine: Effect on *in vitro* cell proliferation and cellular targets. A. KRAS mutant PDAC cells (AsPC-1, Panc-1, KPC mouse-derived cells) and B. KRAS wild-type PDAC cells (BxPC-3, Pan02), were plated on 96-well plates and treated with 10 nM, 100 nM and 1 μM concentrations of *nab*-paclitaxel, trametinib and MK-2206; or 100 nM, 1 μM and 10 μM concentrations of gemcitabine. After 72 h, the number of viable cells was calculated by WST-1 assay. Data are the mean ± SD of triplicate determinations.

extracellular matrix. The thick desmoplastic stroma provides a barrier for drugs to reach tumor cells and it has been associated with the intrinsic resistance to gemcitabine in PDAC [38]. The presence of dense stroma might be one of the reasons for the limited efficacy of trametinib and gemcitabine combination therapy in PDAC patients [18]. *Nab*-paclitaxel has been proposed to disrupt the PDAC stromal architecture causing increased perfusion and delivery of gemcitabine and as a result, leading to higher antitumor responses in a *nab*-paclitaxel and gemcitabine combination [39]. Additionally, we have previously demonstrated that *nab*-paclitaxel based chemotherapy has antitumor responses based on its antiproliferative, antistromal and proapoptotic effects [40]. These studies indicate that exploring the antitumor benefits of an AKT inhibitor MK-2206 and a MEK inhibitor trametinib with *nab*-paclitaxel/gemcitabine standard chemotherapy is a sensible therapeutic approach in PDAC. In this context it is interesting to explore whether MEK and AKT-directed therapy can mediate therapeutic benefits even in tumors without driving KRAS alterations, perhaps due to a partial dependence on these signaling components in tumor-related stromal progression. Our *in vitro* results do suggest that even in *KRAS* WT epithelial PDAC cells, dual targeting mediates such therapeutic benefit.

We detected a significant and differential expression of phospho-AKT, phospho-MEK and phospho-ERK (Supplemental Fig. S2) in all seven PDAC cells tested, confirming the prevalence of the effectors of the oncogenic KRAS pathway in PDAC cellular activation and progression. In our peritoneal dissemination and subcutaneous xenograft studies using aggressive *KRAS*-mutant cell lines, MK-2206 and trametinib both demonstrated an additive effect with *nab*-paclitaxel/gemcitabine chemotherapy, and the combination of MK-2206 plus trametinib with chemotherapy showed a maximum response. In these AsPC-1 cell-derived xenografts, trametinib appears to have a more pronounced effect than MK-2206 indicating more dependence on the MAPK pathway in these models. The effects of the addition of MK-2206 and trametinib was more obvious in the more clinically relevant peritoneal dissemination animal survival model compared with the subcutaneous model, which suggests the possibility of the local TME factors making AKT or MEK inhibition less relevant to PDAC growth in the subcutaneous setting. In contrast to AsPC-1 xenograft studies, subcutaneous PDX grafts were more sensitive to MK-2206 compared with trametinib, suggesting the prevalence of AKT signaling in this model. Target protein determination in tumor lysates showed that MK-2206 and trametinib significantly decreased phospho-AKT and phospho-ERK, respectively, and both these drugs increased the expression of apoptosis-associated proteins, suggesting that the MK-2206 and trametinib are sufficiently affecting their target within the local tumor setting. Importantly, the combination of MK-2206 and trametinib had an additive response in increasing the expression of apoptosis-related proteins, a downstream effect of inhibiting the PI3K-AKT or MAPK-MEK pathways.

In vitro proliferation studies of *KRAS* mutant and wild-type PDAC cells indicated that MK-2206 and trametinib both inhibited the proliferation, but the effect was not dependent on *KRAS* mutation status of these cells, as discussed earlier. These findings are not surprising as all these cells express phospho-AKT and phospho-ERK independent of their *KRAS* mutation status and that might be involved in their proliferation. The differential sensitivity of PDAC cells to AKT and MEK inhibition can be attributed to the *KRAS* mutational subtype, copy number, the presence of *PIK3CA* co-mutation and expression of growth factors and their

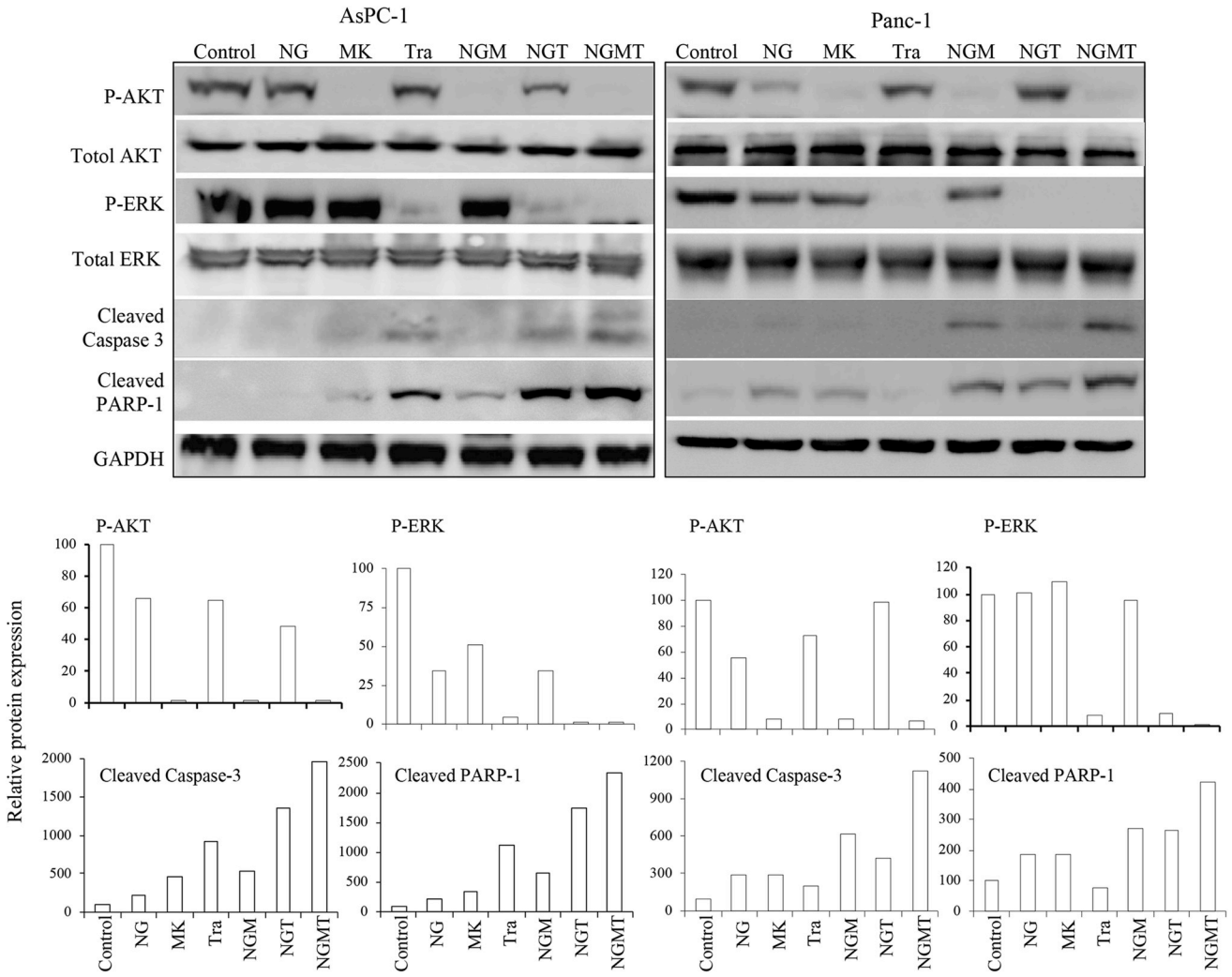

Fig. 7. Addition of MK-2206 and trametinib to *nab*-paclitaxel/gemcitabine: Effects on their molecular targets *in vitro*. A sub-confluent monolayer of AsPC-1 and Panc-1 cells was treated with 10 μM concentration of *nab*-paclitaxel, gemcitabine, MK-2206 or trametinib, for 16 h. Total cell extracts were analyzed by immunoblotting. The intensity of bands was quantitated by densitometry and is represented in the bar graph after normalizing values against corresponding total protein expression or GAPDH expression. Data are representative of two independent experiments with similar results.

receptors [41–44]. Among the *KRAS* mutant cell lines, least sensitivity of AsPC-1 cells to the treatments might be related its dependence on other cell growth signaling pathways such as JAK/STAT, TGF-β, Wnt/β-catenin, Notch and NF-kB, making these cells less sensitive to the PI3K/AKT and MAPK pathway inhibitors.

Considering the multifactorial effects of *nab*-paclitaxel/gemcitabine and inhibition of PI3K and MAPK pathways on PDAC progression, it is likely that specific molecular mechanisms regulate the potential of combination therapy in different compartments of TME and at different levels of tumor progression. Enhanced antitumor response of *nab*-paclitaxel/gemcitabine can be attributed to increased levels of drugs in tumors, better drug distribution and bioavailability, and higher retention of drugs used in combination. The exact mechanisms for the augmentation of chemotherapy by the dual inhibition of the PI3K-AKT or MAPK pathways remain ill-defined. Within the epithelial tumor cells, these can be associated with the direct inhibition of the tumor cell proliferation, survival, growth, and metastasis, and also by avoiding drug resistance due to blockade of compensatory mechanisms of these pathways.

This study exhibits the advantages of targeting the PI3K-AKT and MAPK pathways in PDAC that are consistent with other published reports to date. Our current results now also indicate that dual targeting of PI3K-AKT and MAPK pathways can have an additive effect in improving *nab*-paclitaxel/gemcitabine response in PDAC. Metabolic side effects are an important concern of the dual targeting of the PI3K-AKT and MAPK pathways with chemotherapy, therefore the antitumor benefits might have to be balanced with the potential for therapy-related toxicities. Although we have not observed any evidence of treatment-associated toxicity during a 2-week therapy period, toxicity associated with long-term therapy of MK-2206 and trametinib in combination with *nab*-paclitaxel/gemcitabine regimens remains to be elucidated. In conclusion, our study strongly supports the rationale of dual blocking of downstream targets of *KRAS*-mutation driven signaling and indicates the potential of MK-2206 and trametinib as targeting agents in combination with *nab*-paclitaxel/gemcitabine for clinical PDAC therapy.

Conflicts of interest

The authors indicate no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.05.037.

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