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## Nintedanib, a triple angiokinase inhibitor, enhances cytotoxic therapy response in pancreatic cancer

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### Abstract

Angiogenesis remains a sensible target for pancreatic ductal adenocarcinoma (PDAC) therapy. VEGF, PDGF, FGF and their receptors are expressed at high levels and correlate with poor prognosis in human PDAC. Nintedanib is a triple angiokinase inhibitor that targets VEGFR1/2/3, FGFR1/2/3 and PDGFR $\alpha/\beta$  signaling. We investigated the antitumor activity of nintedanib alone or in combination with the cytotoxic agent gemcitabine in experimental PDAC. Nintedanib inhibited proliferation of cells from multiple lineages found in PDAC, with gemcitabine enhancing inhibitory effects. Nintedanib blocked PI3K/MAPK activity and induced apoptosis *in vitro* and *in vivo*. In a heterotopic model, net local tumor growth compared to controls (100%) was  $60.8 \pm 10.5\%$  in the gemcitabine group,  $-2.1 \pm 9.9\%$  after nintedanib therapy and  $-12.4 \pm 16\%$  after gemcitabine plus nintedanib therapy. Effects of therapy on intratumoral proliferation, microvessel density and apoptosis corresponded with tumor growth inhibition data. In a PDAC survival model, median animal survival after gemcitabine, nintedanib and gemcitabine plus nintedanib was 25, 31 and 38 days, respectively, compared to 16 days in controls. The strong antitumor activity of nintedanib in experimental PDAC supports the potential of nintedanib-controlled mechanisms as targets for improved clinical PDAC therapy.

### Keywords

Pancreatic cancer; Nintedanib; Angiogenesis; Chemotherapy; Combination therapy

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## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the United States, and is expected to become the second-leading cause of cancer death by 2030 [1]. Despite recent advancements in multimodality strategies and availability of novel and more effective antineoplastic combination treatments, the 5-year survival rate for PDAC overall remains less than 6% [2]. Late-stage diagnosis, early and aggressive invasion with metastatic disease and resistance to conventional cytotoxic therapy are the major factors for dismal outcomes in the majority of patients. Much attention has been placed on systemic treatment options for pancreatic cancer as either definitive or perioperative therapy. Single agent gemcitabine (Gem), a deoxycytidine nucleoside analog, has been the standard of care for advanced PDAC since 1997 after producing a response rate of 5% and a median survival of 5.7 months in a pivotal randomized trial [3]. Greater efficacy against advanced pancreatic cancer has recently been demonstrated when nab-paclitaxel was added to gemcitabine [4]. The more intense cytotoxic regimen FOLFIRINOX (oxaliplatin/irinotecan/5-FU/leucovorin) showed better responses than gemcitabine but it also resulted in increased toxicity [5]. Despite these recent trial-based moderate improvements, there is still an urgent requirement for novel therapeutic strategies to improve overall survival of patients with pancreatic cancer.

Angiogenesis is an essential process for tumor growth and metastasis, and remains a target process with potential for cancer therapy including PDAC. Pathological angiogenesis as encountered in tumor growth represents a highly regulated yet disturbed balance between proangiogenic and antiangiogenic mechanisms [6]. Among several positive regulators of angiogenesis, growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) can induce the division of cultured endothelial cells thus indicating a direct action on these cells [6]. VEGF, PDGF, FGF and their receptors are expressed at high levels and correlate with poor prognosis in pancreatic cancer [7,8], providing a strong rationale for exploiting the therapeutic potential of multitarget antiangiogenic agent combinations with an established cytotoxic regimen. Single-target antiangiogenic agents including bevacizumab, a monoclonal antibody against VEGF-A [9,10], the cyclooxygenase inhibitor celecoxib [11] and the PDGF signaling inhibitor imatinib [12] have been studied in combination therapy in PDAC with limited success [13]. A second-class of antiangiogenic agents, small molecules with multi-tyrosine kinase inhibitor (TKI) activity including sunitinib and sorafenib that target several pro-angiogenic receptor tyrosine kinase axes showed promising antitumor response but this did not translate into higher survival rates in PDAC. Most broad-spectrum antiangiogenic TKIs such as sorafenib, sunitinib, pazopanib and vandetanib have complex efficacy data and in many cases the use of these drugs is associated with a significant increase in the incidence and risk of side effects [14–16]. Lower specificity of most of these TKIs toward their therapeutic targets, particularly at the FGF-FGFR axis, is a major factor for risk of increased toxicity [17]. Nintedanib (formally known as BIBF 1120) is a novel, specific and potent triple angiokinase inhibitor of VEGFR1/2/3, FGFR1/2/3 and PDGFR $\alpha/\beta$ ; it also targets other kinases such as RET, FLT-3 and Src in the low nanomolar range. Nintedanib has very high specificity toward its therapeutic targets with significantly lower

IC<sub>50</sub>: VEGFR1/2/3 (IC<sub>50</sub> 13–34 nM); FGFR1/2/3 (IC<sub>50</sub> 37–108 nM); and PDGFR $\alpha/\beta$  (IC<sub>50</sub> 59–65 nM). Due to its unique targeting profile with high specificity, nintedanib has the potential to effectively prevent tumor growth and metastasis while minimizing toxicity and resistance development across a broad range of cancers. In preclinical animal studies, nintedanib showed antitumor activity in several tumor types [18]. In some clinical studies, nintedanib combination with chemotherapy showed promising antitumor activity where other antiangiogenic agents failed to show a response suggesting nintedanib might be superior [19,20]. Thus, we aimed to determine the therapeutic efficacy of the multi-targeting antiendothelial agent nintedanib in combination with the standard chemotherapy agent gemcitabine in experimental pancreatic cancer.

## Materials and methods

### Cell culture and reagents

The human pancreatic cancer cell lines AsPC-1, BxPC-3, Panc-1, MIA PaCa-2, the human umbilical vein endothelial cells (HUVEC), and the human fibroblast cell line WI-38 were all purchased from the American Type Culture Collection (ATCC, Rockville, MD). Pancreatic cancer associated stromal cells were kindly provided by Dr. Melissa Fishel at the Indiana University Pancreatic Cancer Signature Center, Indianapolis. The murine pancreatic cancer cell line PanO2 was obtained from the National Cancer Institute (Bethesda, MD). Cells were initially grown and multiple aliquots were cryopreserved. All the cell lines were used within 6 months after culture start. AsPC-1 and BxPC-3 cells were grown in RPMI 1640 medium; Panc-1, MIA PaCa-2, PanO2, WI-38 and pancreatic cancer stromal cells were grown in DMEM (Sigma Chemical Co. St. Louis, MO), both media were supplemented with 10% FBS. HUVECs were grown in EndoGRO-LS medium containing endothelial cell growth supplements (Millipore Corp., Billerica, MA). Gemcitabine was purchased from Eli Lilly Corporation (Indianapolis, IN). Nintedanib was purchased from LC Laboratories (Woburn, MA). The cell proliferation reagent WST-1 was purchased from Roche Diagnostic Corporation (Indianapolis, IN).

### Cell viability assay

Cell viability assays were performed in 96-well plates using the colorimetric WST-1 reagent as previously described [21]. Briefly, cells were plated in a 96-well plate and treated with nintedanib and gemcitabine. After a 72-hour incubation of cells with nintedanib and gemcitabine, 10  $\mu$ l WST-1 reagent was added in each well, and absorbance at 450 nm was measured after 2 hours using a microplate reader. Drug sensitivity graphs were plotted using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA).

### *In vitro* scratch (wound-healing) assay

The *in vitro* scratch assays were performed to measure cell migration in 12-well plates. Monolayers of PanO2 cells or HUVECs in low-serum media were scratched once per well with a 200  $\mu$ l pipette tip to create an artificial *in vitro* cell-free wound. Cells were washed twice to remove non-adherent cells and then treated with 10  $\mu$ M nintedanib. The scratch closure (wound healing) was measured as a percentage of original scratch area after 24-hour incubation of cells with nintedanib and gemcitabine.

### Western blot analysis

Cells were plated in T25 flasks and sub-confluent monolayers were treated with nintedanib and gemcitabine and lysed after 16 hours. Tumor tissue lysates were prepared as previously described [22]. Briefly, tumor tissues were immediately snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . These tumor tissues were suspended in lysis buffer and homogenized using the Bullet Blender Homogenizer (Next Generation, Averill Park, NY), and extracts were sonicated. Proteins in supernatants were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Membranes were incubated overnight at  $4^{\circ}\text{C}$  with the following antibodies: total AKT, phospho-AKT (Ser473), total ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), cleaved caspase-3 (all from Cell Signaling Technology, Beverly, MA),  $\alpha$ -tubulin and GAPDH (both from Sigma). The membranes were then incubated with the corresponding HRP-conjugated secondary antibodies (Pierce Biotechnologies, Santa Cruz, CA) for 1-2 hours. Specific bands were detected using the enhanced chemiluminescence reagent (ECL, Bio-Rad) on autoradiographic film and quantitated by densitometry.

### Tumor implantation and *in vivo* tumor growth experiment

Animal experiments were performed according to the guidelines and approved Institutional Animal Care and Use Committee protocols of the University of Texas Southwestern Medical Center (Dallas, TX) (Animal Protocol Number 2012-0081) and the Indiana University School of Medicine (South Bend, IN) (Animal Protocol Number 16-023). Female athymic nu/nu mice (aged 4–6 weeks) were used to establish a subcutaneous xenograft model as previously described [23]. Mice were injected with AsPC-1 cells ( $0.75 \times 10^6$ ), randomly grouped and intraperitoneal therapy started after two weeks with PBS (control), nintedanib (25 mg/kg, 5 $\times$  a week) and gemcitabine (50 mg/kg, 2 $\times$  a week). The tumor size was measured twice weekly and tumor volume (V) was calculated by using the formula  $[V = \frac{1}{2} (L \times (W)^2)]$ , where L= length and W = width. After completion of the 2 week therapy, the animals were euthanized, tumors were removed, weighed, dissected and processed for histological or immunohistochemical analysis.

### Immunohistochemical analysis

Tumor tissues fixed in 4% paraformaldehyde were embedded in paraffin. Intratumoral proliferative activity was measured by using Ki67 nuclear antigen staining as per manufacturer's protocol (Abcam, Cambridge, MA). Briefly, tissue sections (5  $\mu\text{m}$ ) were deparaffinized and rehydrated followed by heat-mediated antigen retrieval using citrate buffer. The tissue sections were incubated with CAS blocking buffer followed by 1-hour incubation with anti-Ki67 antibody (1:200) and 40 minutes incubation with Cy3 (1:200) secondary antibody. Slides were mounted with DAPI containing mounting solution (Invitrogen, Carlsbad, CA). Proliferative activity was evaluated by calculating Ki67-positive cells from five different high-power fields (HPF) in a blinded manner. Intratumoral apoptosis was analyzed by staining tissue sections with "Apoptag Apoptosis Detection Kit" according to the manufacturer's (Millipore) instructions. For evaluating intratumoral microvessel density (MVD), paraffin-embedded tissues were sectioned (5  $\mu\text{m}$ ), deparaffinized and rehydrated followed by heat-mediated antigen retrieval using citrate buffer. The tissue sections were incubated for 20 minutes in CAS blocking buffer followed

by overnight incubation at 4 °C with anti-endomucin clone V.5C7 (1:100 dilution in blocking solution) antibody (Millipore; MAB2624). Subsequently, the tissue sections were incubated in 1:200 Cy3-labeled secondary antibody at room temperature for 40 minutes. Tissues were then washed and mounted with DAPI containing mounting solution. Endomucin positive vessels were calculated within a microscopic HPF in a blinded manner. Fluorescence microscopy was used to detect fluorescent signals 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 Slidebook software (Intelligent Imaging Innovations, Philadelphia, PA).

### Animal survival analysis

Animal survival studies were performed using 6- to 8-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice [24]. The mice were intraperitoneally injected with AsPC-1 ( $0.75 \times 10^6$ ) cells. Two weeks later the animals were randomly grouped (n = 6–8 per group) and treated intraperitoneally with PBS (control), nintedanib (25 mg/kg, 5× a week) and gemcitabine (50 mg/kg, 2× a week) for a duration of two weeks. Mice were euthanized when moribund according to predefined criteria including rapid weight loss or gain (>15%), tumor size, lethargy, inability to remain upright and lack of strength. Animal survival was evaluated from the first day of treatment until death.

### Statistical analysis

*In vitro* cell proliferation data are expressed as mean  $\pm$  standard deviation. Statistical significance was analyzed by the two-tailed Student's t-test using GraphPad Prism 6.0 Software (GraphPad Software, San Diego, CA) for individual group comparison. Statistical analysis for *in vivo* tumor growth studies was performed by oneway ANOVA for multiple group comparison and Student's t-test for the individual group comparison. Survival study statistics were evaluated using logrank group comparison (GraphPad Prism 6.0). Values of p < 0.05 were considered to represent statistically significant group differences.

## Results

### Nintedanib inhibits PDAC related cell proliferation and migration, and enhances gemcitabine response

Analysis of human PDAC cells AsPC-1, BxPC-3, MIA PaCa-2, Panc-1, murine PDAC cells PanO2, PDAC associated stromal cells, HUVECs endothelial cells and WI-38 fibroblasts revealed that nintedanib dose-dependently inhibited proliferation of all these PDAC related cells. Importantly, combination of nintedanib with gemcitabine had enhancing inhibitory effects. At 1  $\mu$ M concentration, percent inhibition in cell proliferation by gemcitabine, nintedanib and gemcitabine plus nintedanib was 54%, 56%, 74% for AsPC-1, 46%, 45%, 69% in Panc-1, 90%, 62%, 94% for PanO2, 23%, 38%, 48% in PDAC stromal cells, 92%, 72%, 99% for HUVECs and 87%, 45%, 99% in WI-38 cells (Fig. 1A and B). Nintedanib and gemcitabine also inhibited proliferation of the other PDAC cell lines BXPC-3 and MIA PaCa-2, and combination of nintedanib and gemcitabine had additive effects (data not shown). The effect of nintedanib on cell migration was evaluated by an *in vitro* scratch (wound healing) assay. Nintedanib treatment caused a 75% inhibition in wound healing in

PanO2 cells ( $p = 0.0002$ ) and a 83% inhibition in HUVECs as compared with controls ( $p = 0.022$ ) (Supplemental Fig. S1).

### **Nintedanib blocks PI3K/MAPK activity and induces apoptosis in PDAC associated stromal cells**

Activated signaling of VEGF/FGF/PDGF pathways, the main inducers of tumor angiogenesis and growth, has been shown to converge on induced expression and signaling of PI3K/AKT and MAPK/ERK pathways [25]. This well-defined effect of nintedanib on targeting VEGF/FGF/PDGF signaling was investigated by examining phospho-AKT, phospho-ERK and apoptosis-related cleaved caspase-3 protein expression in PDAC relevant cells. Immunoblot analysis revealed that nintedanib, either alone or in combination with gemcitabine, caused a decrease in the levels of phospho-AKT but no significant effect on phospho-ERK in AsPC-1 cells; decrease in phospho-AKT, phospho-ERK and increase in apoptosis-related protein cleaved caspase-3 in PDAC stromal cells and HUVECs; decrease in phospho-AKT, no change in phospho-ERK and increase in cleaved caspase-3 for WI-38 cells (Fig. 2). In other PDAC cells, nintedanib decreased p-AKT levels in all three cells tested (BxPC-3, MIA PaCa-2 and Panc-1), while decreasing p-ERK levels in BxPC-3 and MIA PaCa-2 cells but not in Panc-1 cells (Supplemental Fig. S2).

### **Nintedanib inhibits local tumor growth and enhances gemcitabine antitumor response**

In AsPC-1 subcutaneous xenografts, treatment of tumor-bearing mice with nintedanib (25 mg/kg, 5× a week for 2 weeks), either alone or in combination with gemcitabine (50 mg/kg, 2× a week for 2 weeks), resulted in statistically significant net tumor growth inhibition (Fig. 3A and B). In this study, compared to controls ( $100 \pm 29$ ), the percent net local tumor growth was  $60.8 \pm 10.5$  ( $p = 0.02$ ) in the gemcitabine group,  $-2.1 \pm 9.9$  ( $p = 0.0001$ ) after nintedanib therapy and  $-12.4 \pm 16$  ( $p = 0.0001$ ) after gemcitabine plus nintedanib therapy, respectively (Fig. 3A and B). Furthermore, the net tumor growth inhibition in nintedanib monotherapy and gemcitabine plus nintedanib groups was statistically different compared to gemcitabine alone. Therapy with gemcitabine, nintedanib and the combination resulted in decreased mean tumor weight compared to control treated animals. Mean tumor weight in different treatment groups was as follows: control  $0.32 \pm 0.05$  g, gemcitabine  $0.23 \pm 0.06$  g ( $p = 0.028$  vs. control), nintedanib  $0.14 \pm 0.04$  g ( $p = 0.0003$  vs. control), and gemcitabine plus nintedanib  $0.12 \pm 0.04$  g ( $p = 0.0002$  vs. control) (Fig. 3C).

No significant change in mouse body weight was observed in the nintedanib, gemcitabine, or gemcitabine plus nintedanib therapy groups, and there was no other discernible treatment-related toxicity (Supplemental Fig. S3A).

### **Nintedanib downregulates signaling proteins, induces intratumoral apoptosis, inhibits proliferation and reduces microvessel density**

The effects of nintedanib on the downstream signaling proteins PI3K/AKT and MAPK/ERK were also examined by Western blot analysis of protein lysates from AsPC-1 tumor xenografts. A significant decrease in the expression of phospho-AKT and phospho-ERK was observed in the nintedanib treated groups (Fig. 3D). Evaluation of intratumoral apoptosis by measuring levels of cleaved caspase-3 protein revealed that gemcitabine and

nintedanib both induced cleavage of caspase-3 and that the combination of these agents had additive effects on this apoptosis-related protein (Fig. 3D). Intratumoral apoptosis was further confirmed by immunohistochemical analysis of tumor tissues. TUNEL assay revealed that the gemcitabine monotherapy group displayed a 3-fold increase ( $p = 0.002$ ), while nintedanib monotherapy caused a 7-fold increase ( $p < 0.003$ ) in apoptotic index as compared with the control group. The combination treatment of gemcitabine and nintedanib generated a 9-fold increase in the apoptotic index compared to controls ( $p < 0.003$ ). In addition, induction in intratumoral apoptosis in the nintedanib-monotherapy and combination therapy groups was significantly higher than that for the gemcitabine monotherapy group ( $p < 0.0002$ ) (Fig. 4A).

Additional mechanisms of *in vivo* antitumor activity of nintedanib were examined by determining intratumoral proliferation in tumor tissues. The tumors of nintedanib treated mice presented a decreased tumor cell proliferation rate. A 79% decrease ( $p = 0.0008$ ) in intratumoral proliferative index was observed in the nintedanib-treated group as compared with controls. Gemcitabine monotherapy caused a 49% decrease in intratumoral proliferation as compared with controls ( $p = 0.019$ ). The combination treatment group of gemcitabine and nintedanib caused a 86% decrease in proliferative activity as compared with controls ( $p = 0.00001$ ). The proliferative index in the nintedanib-monotherapy and combination therapy groups was also significantly reduced as compared with the gemcitabine-monotherapy group ( $p < 0.03$ ) (Fig. 4B).

The effect of nintedanib and gemcitabine treatment on tumor vasculature was measured through endomucin staining of tumor tissue sections. Nintedanib and gemcitabine both reduced intratumoral microvessel counts. Microvessel density in nintedanib monotherapy and gemcitabine plus nintedanib combination therapy groups was significantly lower than in the gemcitabine monotherapy group (Fig. 4C). Furthermore, the combination treatment group gemcitabine plus nintedanib showed some additive effects on decreasing microvessel counts, but these were not significantly lower than in the nintedanib alone group. Mean microvessel counts per HPF were  $24 \pm 11$  (control),  $12.7 \pm 4.6$  (Gemcitabine),  $5.1 \pm 2.4$  (nintedanib) and  $3.2 \pm 1.4$  (gemcitabine plus nintedanib) (Fig. 4C).

### **Nintedanib prolongs animal survival and enhances gemcitabine survival benefit**

The effect of nintedanib and gemcitabine on animal survival was evaluated in an intraperitoneal murine xenograft model [26]. After 2-weeks of therapy, the median animal survival was 16 days in the control group and 25 days after single agent gemcitabine (a 56% increase compared with controls,  $p = 0.036$ ). The median animal survival was significantly improved by single agent nintedanib (31 days, a 94% increase over controls,  $p = 0.0004$ ; and a 24% increase compared with single agent gemcitabine,  $p = 0.042$ ) and the combination of gemcitabine plus nintedanib (38 days, a 138% increase compared with controls,  $p = 0.001$ ). Median survival in the combination therapy group was also significantly greater than that in the monotherapy groups (52% increase over gemcitabine,  $p = 0.001$ ; and 23% increase compared with nintedanib,  $p = 0.01$ ) (Fig. 5). There was no obvious treatment-related toxicity in this study and no significant change in mouse body weight during the 2-week

therapy with nintedanib (25 mg/kg, 5×/week) and gemcitabine (50 mg/kg, 2×/week) (Supplemental Fig. S3B).

In a separate experiment with higher doses of nintedanib (50 mg/kg) and gemcitabine (100 mg/kg), there was some drug related toxicity as observed by a decrease in mouse body weight. In this experiment, mice were only treated for one week; the resulting animal survival in the control group was 18 days, after gemcitabine 25 days and after nintedanib 34 days. However, animal survival in the combination therapy group (35 days) was not significantly longer than that in the nintedanib monotherapy group (Supplemental Fig. S4).

## Discussion

In PDAC, high expression of several growth factors and their receptors is a major promoter of angiogenesis and metastasis [27]. Therefore, antiangiogenic therapy is a sensible and promising therapeutic avenue due to its potential for synergistic or additive effects with other antitumor agents. However, given the complexity of several aberrant angiogenic pathways and regulators, it is unlikely that any single agent will be effective for all PDAC patients. Therefore, targeting multiple pathways that are most commonly expressed in the progression of pancreatic cancer will be a critical step in developing novel therapeutic strategies to achieve a meaningful clinical benefit.

Several antiangiogenic agents have been evaluated in pancreatic cancer, while these agents showed significant preclinical effects, only a modest effect was observed in clinical studies, probably due to induction of tumor escape mechanisms [28,29]. Two major challenges in the success of antiangiogenic therapy are development of resistance in the primary tumor and induction of metastasis [30]. Sunitinib, a multitarget angiogenic inhibitor, has been shown to inhibit local tumor growth but it also increased VEGF expression and metastatic burden, resulting in no survival benefit [31,32]. This has been corroborated in our murine PDAC models, in which we also observed that single agent sunitinib effectively blocked local tumor growth but was unable to create any significant survival benefit [33,34]. The single-target anti-VEGF agent bevacizumab has not shown any significant clinical activity in pancreatic cancer in combination with gemcitabine [15]. However, our preclinical studies demonstrated the benefits of combining polymechanistic, multi-targeting antiangiogenic agents beyond bevacizumab with cytotoxic therapy [33,34]. In the present study, we evaluated the efficacy of nintedanib, a triple angiokinase inhibitor that acts by blocking not only VEGFR, but also FGFR and PDGFR, which are involved in the development of resistance to anti-VEGF therapy and induction of metastasis.

The dense desmoplastic stroma surrounding malignant epithelial cells that is composed of several cellular and acellular components including fibroblasts, stellate cells and endothelial cells creates a complex tumor microenvironment that plays a major role in PDAC development, invasion, metastasis and resistance to chemotherapy [35]. Targeting fibroblasts and endothelial cells for solid tumor treatment has been shown to be a potentially effective strategy [36,37]. We observed that nintedanib inhibited proliferation of pancreatic tumor stromal cells and representative fibroblast WI-38 cells. Pancreatic stromal cells were less sensitive to nintedanib as compared with WI-38 cells. As expected, nintedanib had a





therefore used this cell line in murine xenograft studies of experimental therapeutic evaluation of nintedanib as previously reported [26]. The AsPC-1 intraperitoneal xenograft model for animal survival is highly reproducible, well characterized and closely resembles the metastatic progression pattern of the clinical disease. We observed that in our murine PDAC models, the single-agent nintedanib not only inhibited local tumor growth but, unlike many other antiangiogenic therapies, also significantly enhanced animal survival. Enhancement in animal survival by single agent nintedanib, in contrast to other antiangiogenic agents used in our model [23,33,34,44], emphasize either the advantages of its triple angiokinase spectrum through preventing resistance and further tumor progression through the FGF/PDGF pathways [45], or the benefits of an even broader target range relevant for inhibition of epithelial–mesenchymal pathways [39]. In survival studies, combination of nintedanib with gemcitabine showed maximum survival benefit at optimized, i.e. reduced doses. Although previous clinical studies of nintedanib in combination with chemotherapy suggest a favorable toxicity profile and long-term efficacy [19,20], in our study with higher nintedanib doses the reduction in animal body weight indicated that nintedanib doses need to be selected more cautiously. Importantly, all nintedanib induced antitumor effects and *in vivo* marker responses showed additive effects in combination with gemcitabine. Gemcitabine itself has antiproliferative and proapoptotic effects on tumor cells as well as endothelial cells and fibroblasts. The exact mechanisms for the enhancement in antitumor activity of gemcitabine by nintedanib addition remains unclear; however, some possible mechanisms include normalization of tumor microvessels, increased gemcitabine delivery into the tumor microenvironment due to reduced interstitial pressure, reduced desmoplastic stromal density or direct augmentation of gemcitabine antitumor effects [34,46,47].

In summary, the results of the present study demonstrate that the triple angiokinase inhibitor nintedanib has strong antitumor activity as a single agent in two murine PDAC xenograft models. Furthermore, nintedanib combination with the cytotoxic agent gemcitabine caused significant additive antitumor effects. These findings suggest that the multifactorial nature of pancreatic cancer progression may be more effectively approached through combinations of cytotoxic agents with antitumor agents that specifically target several redundant or parallel molecular pathways. This multitargeting profile, the *in vivo* benefit and the potential for a low toxicity profile provide a strong rationale for clinical evaluation of nintedanib in combination with effective cytotoxic agents.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

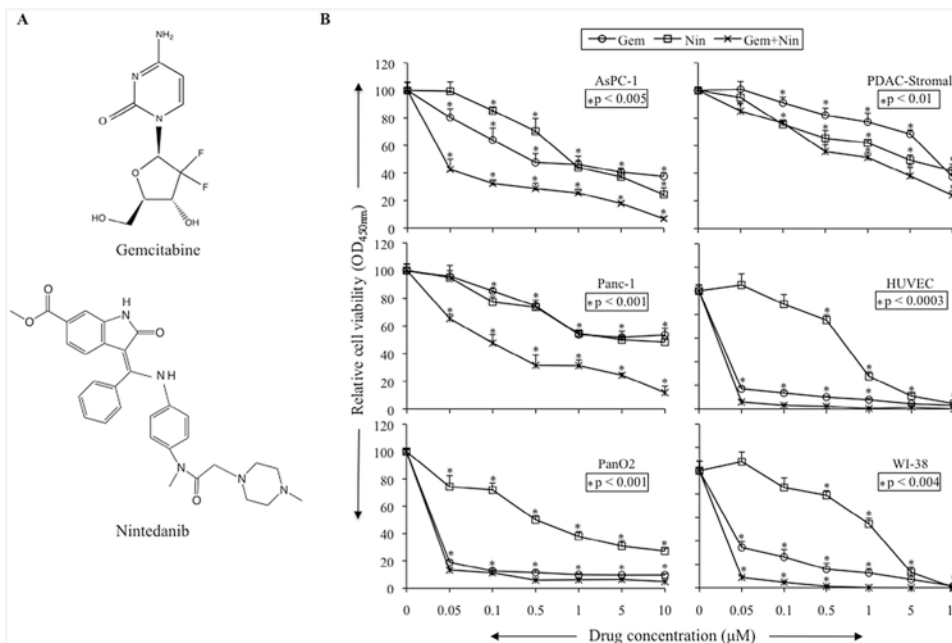
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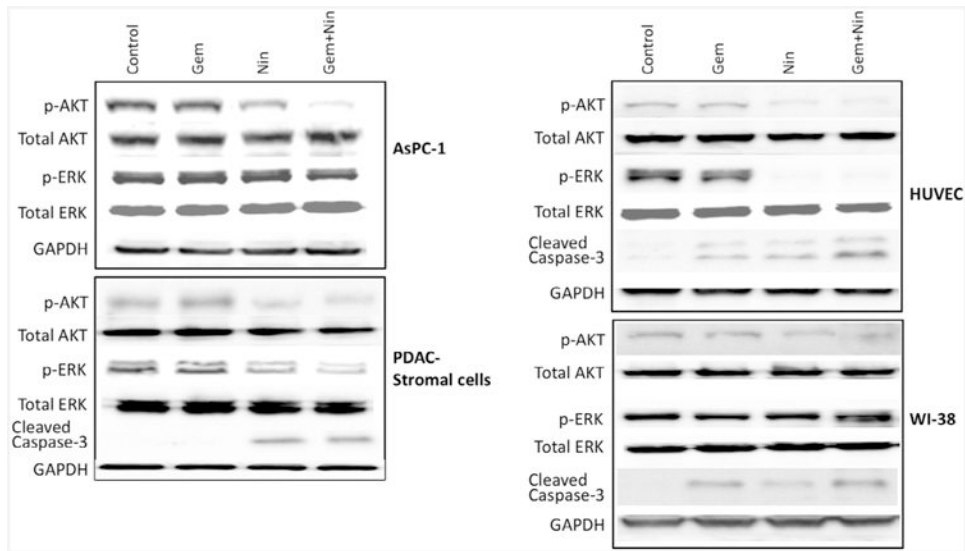
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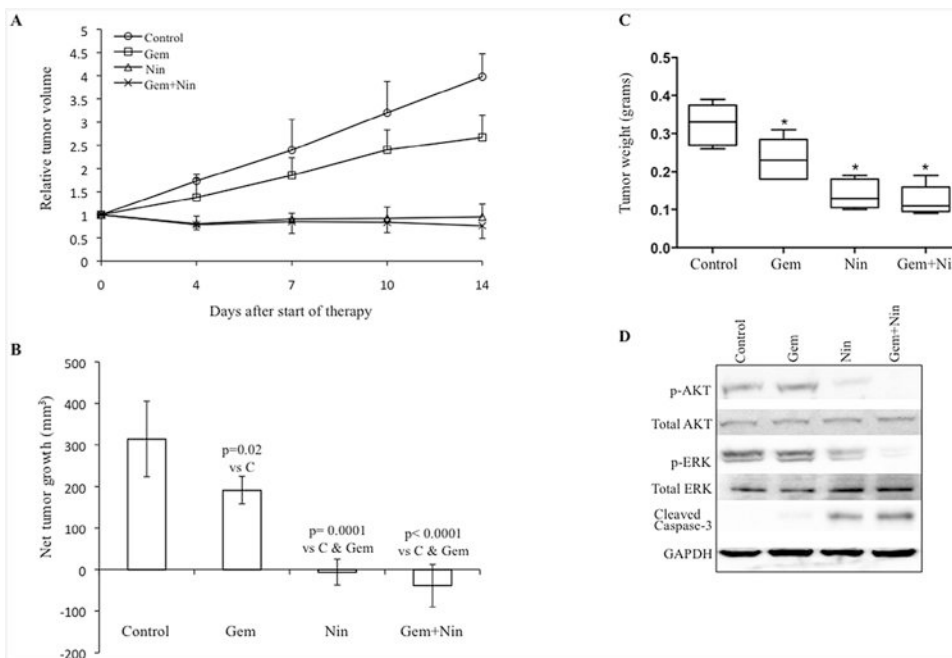
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**Fig. 1.** Nintedanib inhibits *in vitro* cell proliferation of PDAC related cell types and had additive effects in combination with gemcitabine. (A) Chemical structures of gemcitabine and nintedanib. (B) Cells were plated on 96-well plates and treated with 50 nM, 100 nM, 500 nM, 1 μM, 5 μM and 10 μM concentrations of gemcitabine and nintedanib. After 72 hours, 10 μl WST-1 reagent was added in each well and incubated for 2 additional hours. The absorbance at 450 nm was measured using a microplate reader. The resulting number of viable cells was calculated by measuring absorbance of color produced in each well. Data are the mean ± standard deviation of triplicate determinations.

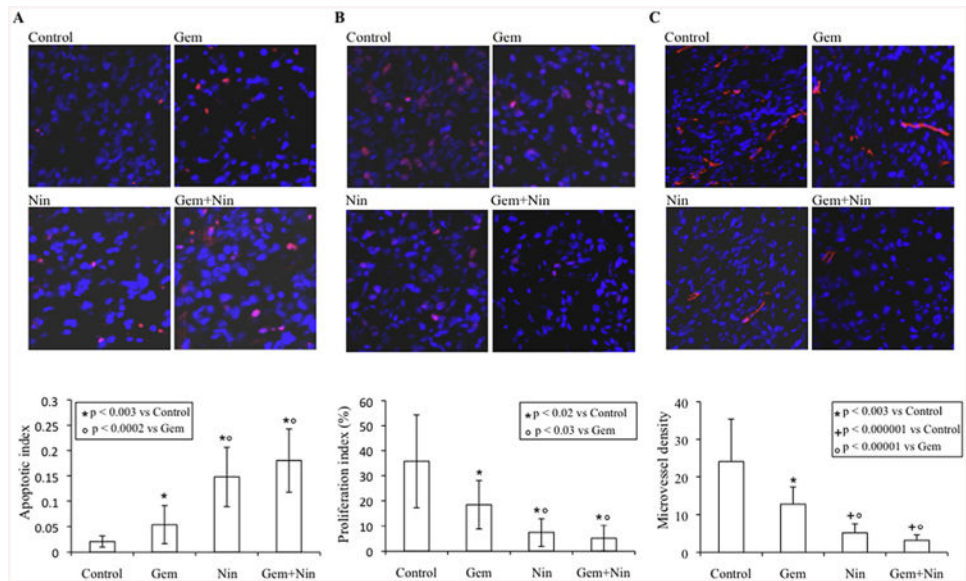


**Fig. 2.** Nintedanib effect on PI3K/MAPK activity and apoptosis in four different PDAC related cells. A sub-confluent monolayer of cells was treated with nintedanib (10  $\mu$ M) and gemcitabine (10  $\mu$ M), either alone or in combination for 16 hours. Total protein lysates were prepared and analyzed by immunoblotting for p-AKT, total AKT, p-ERK, total ERK, cleaved caspase-3 and GAPDH (loading control) proteins. Data are representative of two independent experiments with similar results.

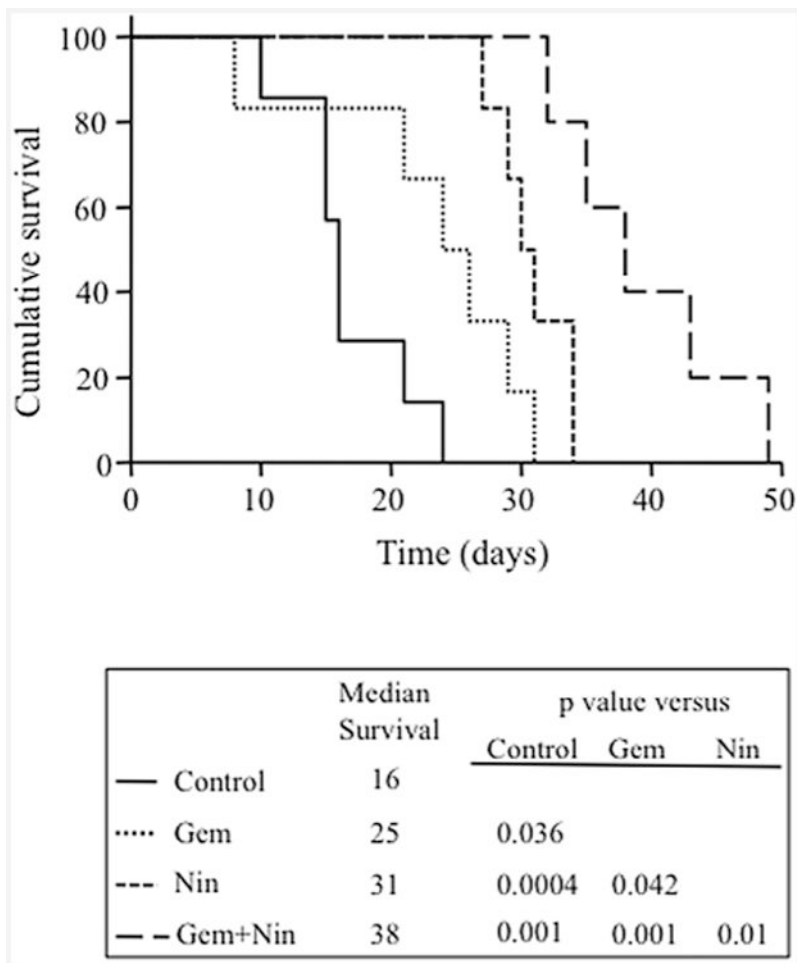


**Fig. 3.** Nintedanib and gemcitabine inhibit growth of established local PDAC tumor. Nude mice were subcutaneously injected with AsPC-1 cells and treated with nintedanib and gemcitabine for 2 weeks. Tumor growth was measured twice a week using calipers. (A) Relative tumor volume is calculated by dividing the tumor volume on any day by the tumor volume at the start of therapy (day 0). (B) Net tumor growth was calculated by subtracting tumor volume on the first treatment day from that on the final day. Data are representative of mean values  $\pm$  standard deviation from 6 to 8 mice per group. (C) Mean tumor weight was calculated from final day tumor weights in each group and is presented as a box plot. Box height denotes interquartile range; horizontal line within the box denotes median; and whiskers represent minimum and maximum values. Symbols \* represent significant difference ( $p < 0.03$ ) versus controls. (D) Nintedanib blocks PI3K/MAPK signaling proteins and induces apoptosis-related proteins. Tumor lysates were prepared from tumor tissue samples obtained from AsPC-1 tumor bearing mice and were analyzed by immunoblotting. The intensity of bands was quantitated by densitometry and is represented in the bar graph after normalizing values with  $\alpha$ -tubulin expression. Data are representative of three independent experiments with similar results.





**Fig. 4.** Effects of nintedanib and gemcitabine therapy on intratumoral apoptosis, proliferation and microvessel density. Nude mice were subcutaneously injected with AsPC-1 cells ( $0.75 \times 10^6$ ) and treated with nintedanib and gemcitabine, either alone or in combination, for 2 weeks. (A) Intratumoral apoptosis was measured by staining the tumor tissue section with TUNEL procedure. (B) Intratumoral proliferation was measured by immunostaining with Ki67 nuclear antigen. The proliferation index was calculated by dividing Ki67 positive cells from the total number of cells per HPF. (C) Microvessel density was evaluated by staining tumor tissue sections with endomucin antibody. For all three immunostaining experiments, slides were photographed under a fluorescent microscope and counting was performed in five different high power fields. The data are expressed as the mean  $\pm$  standard deviation.



**Fig. 5.** Effects of nintedanib and gemcitabine therapy on animal survival. AsPC-1 cells ( $0.75 \times 10^6$ ) were injected intraperitoneally into NOD/SCID mice and treatment started after 2 weeks with nintedanib (25 mg/kg, 5 times a week) and gemcitabine (50 mg/kg, 2 times a week), either alone or in combination, for 2 weeks. The graph represents the animal survival time from the beginning of therapy. Statistical group differences in survival time were calculated using logrank testing (GraphPad Prism 6.0).