Enhancement of Nab-paclitaxel Antitumor Activity through Addition of Multitargeting Antiangiogenic Agents in Experimental Pancreatic Cancer

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Abstract

Nab-paclitaxel (NPT) has recently shown efficacy in pancreatic ductal adenocarcinoma (PDAC). Targeting tumor angiogenesis is a sensible combination therapeutic strategy for cancer including PDAC. We tested the hypothesis that nab-paclitaxel response in PDAC can be enhanced by the mechanistically different antiangiogenic agents, bevacizumab (Bev) or sunitinib (Su), despite its inherently increased tumor penetration and drug delivery. Compared to controls (19 days) median animal survival was increased after NPT therapy (32 days, a 68% increase, p<0.0008); other regimens with enhanced survival were NPT+Bev (38 days, a 100% increase, p<0.0004), NPT+Su (37 days, a 95% increase, p<0.0004) and NPT+Bev+Su (49 days, a 158% increase, p<0.0001) but not Bev, Su or Bev+Su therapy. Relative to controls (100±22.8), percent net local tumor growth was 28.2±23.4 with NPT, 55.6±18 (Bev), 38.8±30.2 (Su), 11±7.2 (Bev+Su), 32.8±29.2 (NPT+Bev), 6.6±10.4 (NPT+Su) and 13.8±12.5 (NPT+Bev+Su). Therapeutic effects on intratumoral proliferation, apoptosis, microvessel density and stromal density corresponded with tumor growth inhibition data. In AsPC-1 PDAC cells, nab-paclitaxel IC$_{50}$ was reduced >6-fold by addition of sunitinib (IC$_{25}$) but not by bevacizumab. In HUVEC endothelial cells, nab-paclitaxel IC$_{50}$ (82 nM) was decreased to 41 nM by bevacizumab and to 63 nM by sunitinib. In fibroblast WI-38 cells, nab-paclitaxel IC$_{50}$ (7.2 μM) was decreased to 7.8 nM by sunitinib, but not by bevacizumab. These findings suggest that the effects of one of the most active cytotoxic agents
against PDAC, nab-paclitaxel, can be enhanced with antiangiogenic agents, which clinically could relate to greater responses and improved antitumor results.
Introduction

Pancreatic ductal adenocarcinoma (PDAC) has the worst survival rate of all solid tumors. Despite recent advancements in surgical procedures and availability of novel and more effective antineoplastic combination strategies, the 5-year survival rate for PDAC overall remains less than 5% (1). Due to the lack of ability for early detection of pancreatic cancer, most patients present with metastatic or locally advanced disease, and are therefore not able to benefit from primary tumor resection. Therefore, much attention has been focused towards systemic treatment options for PDAC for possible definitive or perioperative therapy benefits.

Single agent gemcitabine (Gem) has been a standard of care in advanced PDAC since 1997 after producing a response rate of 5% and a median survival of 5.7 months in a pivotal randomized trial (2). Currently, several clinical studies are exploring gemcitabine-based combinations, either in conjunction with other cytotoxic agents or targeting biologics, to develop more effective and less toxic regimens for PDAC patient.

Taxanes such as docetaxel and paclitaxel are mitotic inhibitors that showed promising antitumor activities in solid tumors (3, 4). These taxanes had limited clinical activity against PADC (5, 6). Nano-particle albumin (nab) bound paclitaxel (NPT) is a water-soluble, cremophor-free, albumin-bound 130-nm particle formulation of paclitaxel. Nab-paclitaxel is approved for the treatment of metastatic breast cancer based on its superior activity compared to solvent-based paclitaxel (7). Nab-paclitaxel initially was developed to avoid toxicity on April 3, 2017. © 2014 American Association for Cancer Research. mct.aacrjournals.org Downloaded from
associated with solvent cremophor required to solubilize paclitaxel. Preclinical and clinical data have demonstrated superior efficacy and safety of nab-paclitaxel over solvent-based paclitaxel (7, 8). A recent phase III clinical trial in patients with metastatic adenocarcinoma of the pancreas showed a median survival of 8.5 months in patients treated with nab-paclitaxel plus gemcitabine compared to 6.7 months in the gemcitabine alone group (9). Recent experiments in our lab demonstrated superior antitumor activity of nab-paclitaxel compared with docetaxel or gemcitabine, providing a strong rationale for evaluating nab-paclitaxel as first line chemotherapy or as a backbone of combination regimens with potential biologic targeting or antiangiogenic agents in patients with pancreatic cancer (10).

Angiogenesis, an essential process for tumor growth and metastasis, is a well-established target for cancer therapy including PDAC. Antiangiogenic agents including bevacizumab (Bev), a monoclonal antibody against vascular endothelial growth factor (VEGF) (11, 12), the cyclooxygenase inhibitor celecoxib (13), the epidermal growth factor receptor inhibitor erlotinib (14) and various receptor tyrosine kinase (RTK) inhibitors (15) have been studied in combination therapy in PDAC models. Sunitinib is a multikinase inhibitor of several tumorigenic RTKs, including VEGF receptors (type 1 and 2), platelet-derived growth factor (PDGF) receptors (α and β), c-KIT, FLT3 and RET (16, 17). Sunitinib is currently approved for the treatment of advanced renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumors (18). VEGF receptor and PDGF receptors are overexpressed and have been correlated with poor prognosis in
human PDAC (19, 20). Sunitinib has been shown to have antitumor efficacy in experimental PDAC (21, 22). These findings suggest that the multifactorial nature of pancreatic cancer may be more effectively approached through treatment of multiple molecular targets, and thus provide a strong rationale for studying the therapeutic potential of antiangiogenic agents in combination with an effective cytotoxic agent. The present study evaluated combination treatment benefits of nab-paclitaxel with the multitargeting combination of the antiangiogenic agents bevacizumab and sunitinib for potentially enhanced PDAC clinical applications.

**Materials and Methods:**

**Materials**

Nab-paclitaxel was obtained from Abraxis BioSciences (Los Angeles, CA), bevacizumab was purchased from Genentech (South San Francisco, CA) and sunitinib was purchased from LC Laboratories, Inc. (Woburn, MA). The cell proliferation reagent WST-1 was purchased from Roche Diagnostic Corporation (Indianapolis, IN).

**Cell culture**

The human pancreatic cancer cell lines AsPC-1 and Panc-1, 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). Cells were initially grown and multiple aliquots were cryopreserved. All the cell lines were used within 6 months after resuscitation. AsPC-1 cells were grown in RPMI 1640 medium; Panc-1 and WI-38 cells were grown in DMEM (Sigma
Chemical Co. St. Louis, MO), both supplemented with 10% FBS. HUVECs were grown in EndoGRO-LS medium containing endothelial cell growth supplements (Millipore Corp., Billerica, MA).

**Cell viability assay**

Assays were performed in 96-well plates using the colorimetric WST-1 reagent as previously described (23). Briefly, cells were plated in a 96-well plate and treated with nab-paclitaxel, bevacizumab and sunitinib. After a 72-hour incubation, 10 μl WST-1 reagent was added in each well, and absorbance at 450 nm was measured after 2 hours using a microplate reader. Drug sensitivity curves and IC_{50} values were calculated using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA).

**Western blot analysis**

Sub-confluent monolayers of cells were treated with nab-paclitaxel (10 μM), bevacizumab (1 mg/ml) or sunitinib (10 μM) and lysed after 16 hours. Tumor tissue lysates were prepared as previously described (24). Briefly, tumor tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C. These samples were crushed in liquid nitrogen using a sterilized mortar, re-suspended in lysis buffer, 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°C with the following antibodies: phospho-stathmin (Ser38), cleaved poly (ADP-ribose) polymerase-1 (PARP-1) (Cell Signaling Technology, Beverly, MA), α-tubulin and GAPDH (both from Sigma). The membranes were then incubated with the corresponding HRP-
conjugated secondary antibodies (Pierce Biotechnologies, Santa Cruz, CA). Enhanced chemiluminescence reagent (ECL, Perkin Elmer Life Sciences, Boston, MA) was used to detect specific bands, which were then quantitated by densitometry.

**Animal survival analysis**

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). Animal survival studies were performed using 6- to 8-week-old female nonobese diabetic / severe combined immunodeficient (NOD/SCID) mice (25). The mice were intraperitoneally injected with AsPC-1 (0.75x10^6) cells. Two weeks later the animals were randomly grouped (n=6 to 8 per group) and treated intraperitoneally with PBS (control), nab-paclitaxel (10 mg/kg, twice weekly), bevacizumab (10 μg per mouse, twice weekly) or sunitinib (20 mg/kg, 5 times weekly) for 2 weeks. Nab-paclitaxel, bevacizumab and sunitinib doses were selected based on previous studies in the literature (26-28). Mice were euthanized when turning 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.

**Tumor implantation and in vivo tumor growth experiment**
Female athymic nu/nu mice (aged 4-6 weeks) were used to establish subcutaneous xenograft model as previously described (29). Mice were injected with AsPC-1 cells (0.75 x 10^6) or Panc-1 cells (10 x 10^6), randomly grouped (n=6 to 8 per group) and intraperitoneal therapy started after two weeks with PBS (control), nab-paclitaxel (10 mg/kg, twice weekly), bevacizumab (10 μg per mouse, twice weekly) and sunitinib (20 mg/kg, 5 times weekly). 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 treatment, 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 μm) 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 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. Intratumoral microvessel
density (MVD) was evaluated by incubating tissue sections with PECAM-1 (1:100) antibody (BD Pharmingen, Bedford, MA) overnight at 4°C followed by 40 minutes incubation with Cy3 secondary antibody. Slides were mounted with DAPI containing mounting solution. PECAM-1 positive vessels were calculated within a microscopic HPF in a blinded manner. Intratumoral stromal density was evaluated by incubating tissue sections with α-SMA or collagen 1 (1:200) antibody overnight at 4°C followed by 40 minutes incubation with Cy3 conjugated secondary antibody. Slides were mounted with DAPI containing mounting solution. 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).

**Statistical analysis**

In vitro cell proliferation data are expressed as mean ± standard deviation. Statistical significance was analyzed by the two-tailed Student’s t-test using GraphPad Prism 4 Software (GraphPad Software, San Diego, CA) for individual group comparison. Statistical analysis for in vivo tumor growth studies was performed by one-way 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 4). Values of p<0.05 were considered to represent statistically significant group differences.
Results:

Effects of nab-paclitaxel, bevacizumab and sunitinib on animal survival

Animal survival benefit was evaluated using an intraperitoneal PDAC murine xenograft model in NOD/SCID mice after two weeks of therapy. The median animal survival was 19 days in the control group and was significantly improved by single agent nab-paclitaxel (32 days, a 68% increase compared with control, p=0.0008), modestly improved by sunitinib (24 days, p=0.045) but without any improvement with bevacizumab therapy (21 days, p=0.18). Median survival after the combination of bevacizumab with sunitinib (22 days, p=0.157) was also not better than the control group. Combination of nab-paclitaxel with bevacizumab enhanced animal survival to 38 days (a 100% increase compared with controls, p=0.0004 versus control, p=0.0005 versus bevacizumab, p=0.03 versus nab-paclitaxel) while the combination of nab-paclitaxel with sunitinib extended animal survival to 37 days (a 95% increase compared with controls, p=0.0004 versus control, p=0.008 versus sunitinib, p=0.17 versus nab-paclitaxel). Importantly, the combination of nab-paclitaxel with both antiangiogenic agents bevacizumab and sunitinib demonstrated the greatest observed survival benefit with a median survival of 49 days (a 158% increase compared with controls, p=0.0001 versus control, p< 0.001 versus monotherapy groups) (Figure 1). No significant change in mouse body weight was observed during 2-week therapy in all groups indicating that there was no significant drug related toxicity even in the triple combination therapy group (Supplemental Figure S1A).

Nab-paclitaxel, bevacizumab and sunitinib therapy effects on local tumor growth
Therapy effects on local tumor growth were evaluated in AsPC-1 subcutaneous tumor xenografts in athymic nu/nu mice. Treatment of AsPC-1 tumor bearing mice with all three agents, either alone or in combination, caused an inhibition in local tumor growth. Evaluation of tumor volume during a treatment period of 3 weeks revealed that the combination therapy groups Bev+Su, NPT+Su and NPT+Bev+Su were more effective in inhibiting local tumor growth (Figure 2A). However, some inhibition of local tumor growth in vivo was observed after all treatments: compared to controls (100±22.8), percent net local tumor growth was 28.2±23.4 with NPT, 55.6±18 (Bev), 38.8±30.2 (Su), 11±7.2 (Bev+Su), 32.8±29.2 (NPT+Bev), 6.6±10.4 (NPT+Su) and 13.8±12.5 (NPT+Bev+Su) (Figure 2B). There was no significant change in mouse body weight during the experiment (Supplemental Figure S1B). The tumor weight measurement at completion of therapy was very comparable to the final day tumor volume data (Supplemental Figure S2). We also evaluated NPT, Bev and Su treatment effects on local tumor growth in Panc-1 subcutaneous xenografts in athymic nu/nu mice and observed a similar pattern as seen in AsPC-1 xenografts with all three agents inhibiting tumor growth. Compared to the percent net local tumor growth in controls (100±46), the most effective groups showed a net gain of only 4.3±24% (Bev+Su), or a net reduction of -11.2±23% (NPT+Su) and -12.4±28% (NPT+Bev+Su) (Supplemental Figure S3). Overall, group differences were highly statistically significant as determined by one-way ANOVA (p<0.0001).

Intratumoral mechanisms of antitumor effects
The mechanisms of antitumor activities of nab-paclitaxel, bevacizumab and sunitinib, either alone or in combination, were investigated by immunohistological and immunoblot analyses of tumor tissues obtained from AsPC-1 subcutaneous xenografts of athymic nude mice after 3 weeks of therapy. Ki67 staining to analyze intratumoral proliferative activity demonstrated that nab-paclitaxel and sunitinib monotherapy were more effective in inhibiting intratumoral cell proliferation than bevacizumab alone. Compared with single agent treatments, the combination treatment groups NPT+Su (p<0.0005) and NPT+Bev+Su (p<0.0001) were more effective, but the differences were not statistically significant compared to the Bev+Su or NPT+Bev groups. Compared to controls (100%), the intratumoral proliferative index, measured by calculating Ki67 positive cells over total number of cells per HPF, was decreased in the NPT (39±18%), Bev (69±6%), Su (36±11%), Bev+Su (34±9%), NPT+Bev (36±8%), NPT+Su (17±10%) and NPT+Bev+Su (13±6%) therapy groups (Figure 3A).

The mechanism of cell proliferation inhibition was further investigated by analyzing the expression of phospho-stathmin and α-tubulin, proteins regulating microtubule dynamics. Immunoblot analysis of tumor lysates from the subcutaneous xenografts revealed that nab-paclitaxel and sunitinib increased the expression of phospho-stathmin, an inactive form of stathmin, but no change in tubulin expression. Bevacizumab also caused a small increase in phospho-stathmin expression but no change in tubulin expression (Figure 3B, Supplemental Figure S4).
TUNEL staining was performed to analyze the effects of nab-paclitaxel, bevacizumab and sunitinib on intratumoral apoptosis. Apoptotic index calculated by TUNEL-positive cells over total cells per HPF revealed that sunitinib was more effective in inducing apoptosis followed by nab-paclitaxel and bevacizumab monotherapy. Combinations of nab-paclitaxel with bevacizumab or sunitinib were also effective but not significantly better than sunitinib alone (Figure 4). Mean apoptotic index in different treatment groups was as follows: 0.02±0.01 (control), 0.07±0.02 (NPT), 0.04±0.01 (Bev), 0.14±0.05 (Su), 0.18±0.03 (Bev+Su), 0.11±0.05 (NPT+Bev), 0.19±0.04 (NPT+Su) and 0.21±0.05 (NPT+Bev+Su) (Figure 4).

The effects of nab-paclitaxel and antiangiogenic agents bevacizumab and sunitinib, on tumor vasculature was assessed by PECAM-1 staining of tumor tissue sections. All three agents caused a reduction in microvessel density, sunitinib being most effective. The combination treatment group NPT+Bev+Su showed some additive effects on decreasing microvessel counts, but these were not significantly lower than after sunitinib alone (Figure 5). Mean microvessel counts per HPF were as follows: 21±4 (control), 12.9±2 (NPT), 14.4±3.2 (Bev), 7±1 (Su), 9.3±1.5 (Bev+Su), 10.9±1.7 (NPT+Bev), 6.5±2.1 (NPT+Su) and 3.8±1.7 (NPT+Bev+Su) (Figure 5). Microvessel density in the setting of nab-paclitaxel therapy was only significantly reduced by the addition of sunitinib, but not after bevacizumab.

The effects of nab-paclitaxel and antiangiogenic agents on tumor stroma were examined by fluorescent IHC staining of stromal marker proteins collagen-1
and α-SMA. Relative to control, collagen-1 pixel intensity was decreased in treatment groups by (in percent) 66.6±14 (NPT), 31.2±10.2 (Bev), 54.1±6.2 (Su), 70.6±8.7 (Bev+Su), 71.9±6.3 (NPT+Bev), 81.1±1.7 (NPT+Su) and 83.2±2.6 (NPT+Bev+Su) (Supplemental Figure S5). Pixel intensity of α-SMA expression was also significantly decreased by NPT (68%, p<0.0003) but less affected by Bev and Su; combination treatment groups were not significantly different from the NPT monotherapy group (Supplemental Figure S6).

Effects on in vitro cell proliferation and related protein expression

To delineate the antitumor mechanism of the addition of bevacizumab and sunitinib to nab-paclitaxel on various cellular lineage components present within the tumor microenvironment, in vitro cell viability assays were performed using WST-1 reagent in the representative PDAC AsPC-1 cells, HUVEC endothelial cells and WI-38 fibroblast cells. In AsPC-1, nab-paclitaxel IC₅₀ 5.1 μM was reduced more than 6-fold by addition of an IC₂₅ dose of sunitinib, but no significant effect was observed by bevacizumab addition at an IC₂₅ dose (Figure 6A). In HUVEC, the nab-paclitaxel IC₅₀ of 82 nM was decreased to 41 nM by bevacizumab and to 63 nM by sunitinib. In WI-38 cells, the nab-paclitaxel IC₅₀ of 7.2 μM was decreased to 7.8 nM by sunitinib, while no significant decrease was observed after bevacizumab addition (Figure 6A). The underlying mechanisms were further evaluated by analyzing the effects of these treatments on the expression of phospho-stathmin, α-tubulin, and the apoptosis marker protein cleaved PARP-1. Nab-paclitaxel treatment caused a significant increase in phospho-stathmin and cleaved PARP-1 in all three cell-types. Nab-paclitaxel
decreased α-tubulin expression in AsPC-1 cells but not in HUVEC or WI-38 cells. Sunitinib treatment caused an increase in cleaved PARP-1 protein but no change in phospho-stathmin or α-tubulin expression. Bevacizumab treatment had no effect on phospho-stathmin, α-tubulin, or cleaved PARP-1 protein expressions in all three cell-types (Figure 6B).

Discussion:

Late-stage diagnosis, early and aggressive local invasion and metastatic progression, and lack of effective therapeutic options are the major contributors in the generally dismal prognosis of pancreatic cancer patients. The modest effectiveness of gemcitabine, the dominant chemotherapeutic agent used in PDAC, raises the need for novel therapeutic strategies to be explored and the underlying mechanisms of resistance to systemic treatments to be elucidated.

PDAC is characterized by the formation of a dense stroma that not only plays an important role in cancer development, progression, invasion and metastasis but also provides a mechanical barrier for the optimal delivery of chemotherapy (30). Nab-paclitaxel has recently 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, as observed in the recent clinical PDAC trial (31). Recent studies in our lab have shown that nab-paclitaxel is the most effective single cytotoxic agent when compared with gemcitabine or docetaxel in experimental PDAC (10). Additionally, in a breast cancer model, nab-paclitaxel
has also been proposed to improve primary tumor oxygenation by inhibiting the formation of novel microvessel and by disrupting established microvessel thus improving the antitumor response of radiation therapy and targeted therapy (32, 33). Therefore, the evaluation of combination treatment benefits of nab-paclitaxel with antiangiogenic agents seems logical and plausible in advanced and metastatic cancers. Since angiogenesis is critical for primary and metastatic PDAC progression, antiangiogenic treatment is a conceptually promising therapeutic strategy based on its potential for synergistic interaction with other antitumor agents, limited toxicity and enhanced antitumor effects (34). However, resistance to angiogenic inhibitors in the primary tumor and induction of metastasis are two possibly major challenges for antiangiogenic therapy (35). In renal cell cancer, sunitinib has been shown to decrease local tumor growth but to also increase VEGF expression and metastatic burden at the same time, resulting in no survival benefit (36, 37). Also, in vivo antitumor activities of sunitinib have been shown to depend not only upon its antiangiogenic activities, but also upon its direct impact on tumor cells and stromal components (16, 17, 38). The scope of the present study was to evaluate the treatment benefits and underlying mechanism of nab-paclitaxel combinations with bevacizumab or sunitinib in experimental PDAC.

Animal survival studies in an intraperitoneal murine xenograft model, which itself is well representative of the progression pattern of PDAC (39), revealed that nab-paclitaxel significantly improved animal survival that was further extended by the single target antiangiogenic agent bevacizumab or the
multitargeting antiangiogenic agent sunitinib to similar extent. Importantly, the maximum survival benefit was achieved when nab-paclitaxel was combined with both bevacizumab and sunitinib. Advantages in combining nab-paclitaxel with bevacizumab and sunitinib might be explained by previous studies showing that sunitinib inhibits signaling pathways involved in bevacizumab resistance (36). These finding clearly support the importance of blocking multiple pathways by using mechanistically different antiangiogenic agents combined with effective chemotherapy regimen for PDAC treatment. Previous studies in our lab have shown that in experimental PDAC, gemcitabine response can be improved by addition of one or more antiangiogenic agents (22, 29, 39-41). The current study thus extends this general notion for comparable combinations with the antimitotic agent nab-paclitaxel, giving support to a rationale for using polymechanistic antiangiogenic therapy independent from the underlying cytotoxic agent's mechanism. In addition, this strategy appears to function even in the setting of enhanced intratumoral delivery, a characteristic for nab-paclitaxel over other unbound taxanes.

Subcutaneous murine xenograft studies revealed that nab-paclitaxel inhibited local tumor growth, but in contrast to the survival experiment, single agent bevacizumab and sunitinib were also effective. This difference in effectiveness of antiangiogenic agents in two different models can be attributed to the fact that the subcutaneous model lacks metastatic progression and is generally more easily affected by antiangiogenic agents (42).
Ki67 nuclear antigen staining is a prognostic marker in metastatic pancreatic cancer (43). Decreased Ki67 staining by nab-paclitaxel and sunitinib, either alone or in combination groups, indicate its role as biomarker for enhanced combination therapy efficacy. High resistance of PDAC to conventional therapies is attributed to its resistance to apoptosis (44). The greater apoptosis-inducing activity of sunitinib compared with nab-paclitaxel or bevacizumab indicates that the contribution made by sunitinib in combination therapy are likely in part apoptotic pathway driven; this would explain the propensity of sunitinib to enhance NPT effects more than those of Bev. High PECAM-1 staining, a well-established marker of microvessel density / angiogenesis, in control tumor tissues confirmed an abnormal hypervascularity of PDAC. A decrease in microvessel density indicating antiangiogenic effects were observed by all therapies via nab-paclitaxel, bevacizumab and sunitinib; this effect was more pronounced in combination therapy groups. Furthermore, nab-paclitaxel decreased the tumor stromal density markers, collagen 1 and $\alpha$-SMA, that corroborate with previously published report that nab-paclitaxel disrupts stromal architecture (31). Nab-paclitaxel containing combination therapy groups were also effective in decreasing collagen 1 and $\alpha$-SMA content. Overall, all these tissue-based analyses showed differential effects of nab-paclitaxel, bevacizumab and sunitinib but in general correlate with in vivo combination therapy effects, and could therefore be considered as clinical biomarkers of treatment efficacy for these combinations. Of course, all markers are mere surrogates for the actual events taken place within the tumor tissue during treatment, but the aggregate
impression is that there is a considerable activity by NPT against stromal (vascular and fibroblastic) targets within the PDAC microenvironment that can still be enhanced through the antiangiogenic agents used.

During further evaluation of the mechanism of action of nab-paclitaxel combination with bevacizumab and sunitinib, in vitro cell viability studies revealed that nab-paclitaxel inhibited proliferation of the PDAC cell line AsPC-1. This cell line is very aggressive and highly resistant to gemcitabine which we consider useful for an experimental therapeutic approach to PDAC (39). As expected, the addition of bevacizumab had no meaningful effect on AsPC-1 proliferation, while sunitinib addition effectively decreased the nab-paclitaxel IC50, supporting the notion that sunitinib has indeed direct antiepithelial effects in addition to its antiangiogenic properties (22). Targeting endothelial cells and fibroblast for solid tumor treatment have been shown to be a potentially effective strategy (45, 46). Furthermore, nab-paclitaxel inhibited representative EC and fibroblast proliferation, suggesting that its effect is not limited to epithelial cells but may well extend to tumor vasculature and stromal architecture. In HUVECs, nab-paclitaxel effects were further improved by bevacizumab and sunitinib, but in WI-38 fibroblasts nab-paclitaxel effects were only enhanced by sunitinib and not by bevacizumab. Stathmin, an important microtubule dynamics regulatory protein, is overexpressed in several cancers (47). Antimicrotubule activities of taxanes have shown to be correlated with phosphorylation and thus inactivation of stathmin, decrease in tubulin and increase in apoptosis (48). In the present study, nab-paclitaxel induced phospho-stathmin and cleaved PARP-1 in all three cell-types.
Nab-paclitaxel decreased $\alpha$-tubulin only in AsPC-1 but not in HUVECs or WI-38 cells, indicating that this is a cell line specific phenomenon, and not an essential component for an antiproliferative effect of nab-paclitaxel. Also, as represented in figure 3B, there was no significant change in $\alpha$-tubulin expression in tumor lysates probably due to the presence of multiple nonepithelial cell types within the samples derived from tumor tissues. In all three cell types, sunitinib increased cleaved PARP-1 expression while bevacizumab had no effect, again supporting aspects of the multitargeting nature of sunitinib. The in vitro expression pattern of cleaved PARP-1 by nab-paclitaxel, bevacizumab and sunitinib in all three cell types was very comparable to in vivo apoptosis data indicating that cleaved PARP-1 is a good marker for therapeutic activity in this setting.

In summary, the present study demonstrates that although nab-paclitaxel already has strong antitumor activity as a single agent, this is significantly enhanced by combination with the antiangiogenic agents bevacizumab and sunitinib. Although the exact mechanism for the enhancement of nab-paclitaxel activity by bevacizumab or sunitinib remains unclear, it could be attributed to a decrease in angiogenesis, reduction of desmoplastic stroma formation, increased delivery of nab-paclitaxel to tumor, and greater efficacy in growth inhibition of multiple cells types within the tumor microenvironment (49). These results strongly support a rationale for combining polymechanistic, multi-targeting antiangiogenic agents with effective systemic therapy such as nab-paclitaxel for clinical PDAC treatment.
References:

Figure legends:

Figure 1: Animal survival evaluation after treatment with nab-paclitaxel, bevacizumab or sunitinib. AsPC-1 tumor bearing NOD/SCID mice were treated for 2 weeks with nab-paclitaxel (10 mg/kg, twice weekly), bevacizumab (10 μg per mouse, twice weekly) or sunitinib (20 mg/kg, 5 times weekly), either alone or in combination. The curve represents animal survival time from the start of therapy. Statistical group differences in survival time were calculated using logrank testing (GraphPad Prism 4).

Figure 2: Nab-paclitaxel, bevacizumab and sunitinib effects on growth of established local PDAC tumors. Nude mice were subcutaneously injected with AsPC-1 cells and therapy was started after 2 weeks. (A) Relative tumor growth is calculated by dividing the tumor volume at any time by the tumor volume at the start of treatment. (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 ± standard deviation from 6-8 mice per group. Symbols * represent significant difference (p<0.05) versus controls.

Figure 3: Intratumoral proliferative activity analysis after nab-paclitaxel, bevacizumab or sunitinib therapy. AsPC-1 tumor bearing athymic nude mice were treated for 3-weeks, tumors were dissected and processed for immunohistochemical analysis. (A) Tumor tissue sections were stained with Ki67 antibody and slides were photographed under a fluorescent microscope. Ki67-
positive cells were counted in five different high power fields. (B) Expression of phospho-stathmin and α-tubulin proteins in tumor lysates. The intensity of bands was quantitated by densitometry. Data are representative of three independent experiments with similar results.

Figure 4: Intratumoral apoptotic activity measurement after nab-paclitaxel, bevacizumab or sunitinib therapy. AsPC-1 tumor bearing athymic nude mice were treated for 3-weeks, tumors were dissected and processed for immunohistochemical analysis. Apoptotic activity was measured by staining tumor tissue sections with TUNEL procedure and photographed under a fluorescent microscope. TUNEL-positive apoptotic cells were counted in five different high power fields. The data are expressed as the mean ± standard deviation.

Figure 5: Tumor vasculature analysis after nab-paclitaxel, bevacizumab or sunitinib therapy. AsPC-1 tumor bearing athymic nude mice were treated for 3-weeks, tumors were dissected and processed for immunohistochemical analysis. Tumor vascular was analyzed by staining tumor tissue sections with PECAM-1 antibody and photographed under a fluorescent microscope. PECAM-positive microvessels were counted in five different high power fields. The data are expressed as the mean ± standard deviation.
Figure 6: Nab-paclitaxel, bevacizumab and sunitinib effects on in vitro cell proliferation and related protein expression. (A) Cells were seeded into 96-well plates and treated with different concentrations of nab-paclitaxel with or without IC$_{25}$ dose of bevacizumab or sunitinib. After 72 hours incubation, WST-1 reagent (10 μl) was added and absorbance was measured at 450 nm that is proportional to the viable cells in each well. Data are the mean ± SD of triplicate determinations. Drug sensitivity curves and IC$_{50}$ values were calculated using GraphPad Prism 4.0 software. (B) Antiproliferative effects of nab-paclitaxel, bevacizumab and sunitinib correlates with increased phospho-stathmin and cleaved PARP-1 expression. Sub-confluent cell monolayers were treated with nab-paclitaxel (10 μM), bevacizumab (1 mg/ml) or sunitinib (10 μM), either alone or in combination for 16 hours. Total cell extracts were analyzed by immunoblotting for p-stathmin, cleaved PARP-1, α-tubulin, GAPDH and actin proteins. Data are representative of two independent experiments with similar results.
Figure 2

A

Relative Tumor Growth

Days after start of therapy

B

Net Tumor Growth (mm³)

Control  NPT  Bev  Su  Bev+Su  NPT+Bev  NPT+Su  NPT+Bev+Su

*
Figure 4

The figure shows immunofluorescent images comparing different treatments across four groups: Control, NPT, Bev, and Su. There are additional subgroups: Bev+Su, NPT+Bev, NPT+Su, and NPT+Bev+Su.

A graph is also presented below the images, illustrating the apoptotic index for each group. The graph includes error bars indicating variability. The legend in the figure indicates that *p < 0.001 vs Control and **p < 0.05 vs NPT.
Figure 5

The images show immunofluorescent staining for microvessels in different treatment groups: Control, NPT, Bev, Su, Bev+Su, NPT+Bev, NPT+Su, and NPT+BEV+Su. The bar graph below the images illustrates the microvessel density per high power field (HPF) for each group. The symbols * and ** indicate statistical significance: * p < 0.04 vs Control and ** p < 0.02 vs NPT.
Molecular Cancer Therapeutics

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