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

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Abstract

Nanoparticle albumin–bound paclitaxel (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 NPT 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 with 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 þ Bev (38 days, a 100% increase, \( P = 0.0004 \)), NPT þ Su (37 days, a 95% increase, \( P = 0.0004 \)), and NPT þ Bev þ Su (49 days, a 158% increase, \( P = 0.0001 \)) but not bevacizumab, sunitinib, or Bev þ Su therapy. Relative to controls (100 ± 22.8), percentage net local tumor growth was 28.2 ± 23.4 with NPT, 55.6 ± 18 (Bev), 38.8 ± 30.2 (Su), 11 ± 7.2 (Bev þ Su), 32.8 ± 29.2 (NPT þ Bev), 6.6 ± 10.4 (NPT þ Su), and 13.8 ± 12.5 (NPT þ Bev þ Su). Therapeutic effects on intratumoral proliferation, apoptosis, microvessel density, and stromal density corresponded with tumor growth inhibition data. In AsPC-1 PDAC cells, NPT IC50 was reduced >6-fold by the addition of sunitinib (IC50) but not by bevacizumab. In human umbilical vein endothelial cells (HUVEC), NPT IC50 (82 nmol/L) was decreased to 41 nmol/L by bevacizumab and to 63 nmol/L by sunitinib. In fibroblast WI-38 cells, NPT IC50 (7.2 μmol/L) was decreased to 7.8 nmol/L by sunitinib, but not by bevacizumab. These findings suggest that the effects of one of the most active cytotoxic agents against PDAC, NPT, can be enhanced with antiangiogenic agents, which clinically could relate to greater responses and improved antitumor results. Mol Cancer Ther; 13(5); 1032–43. ©2014 AACR.

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). Because of 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 toward systemic treatment options for PDAC for possible definitive or perioperative therapy benefits.

Single agent gemcitabine 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 patients with PDAC.

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 PDAC (5, 6). Nanoparticle albumin–bound paclitaxel (nab-paclitaxel, NPT) is a water-soluble, cremophor-free, albumin-bound 130-nm particle formulation of paclitaxel. NPT is approved for the treatment of metastatic breast cancer based on its superior activity compared with solvent-based paclitaxel (7). NPT initially was developed to avoid toxicity associated with the solvent cremophor required to solubilize paclitaxel. Preclinical and clinical data have demonstrated superior efficacy...
and safety of NPT 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 NPT plus gemcitabine compared with 6.7 months in the gemcitabine-alone group (9). Recent experiments in our laboratory demonstrated superior antitumor activity of NPT compared with docetaxel or gemcitabine, providing a strong rationale for evaluating NPT 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 VEGF (11, 12), the COX inhibitor celecoxib (13), the EGF 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 NPT with the multitargeting combination of the antiangiogenic agents bevacizumab and sunitinib for potentially enhanced PDAC clinical applications.

Materials and Methods

Materials

NPT was obtained from Abraxis BioScience, bevacizumab was purchased from Genentech, and sunitinib was purchased from LC Laboratories, Inc. The cell proliferation reagent WST-1 was purchased from Roche Diagnostics Corporation.

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). 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 Dulbecco’s Modified Eagle Medium (DMEM; Sigma), both supplemented with 10% FBS. HUVECs were grown in EndoGRO-LS medium containing endothelial cell growth supplements (Millipore Corporation).

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 NPT, 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 IC₅₀ values were calculated using GraphPad Prism 6.0 software (GraphPad Software).

Western blot analysis

Subconfluent monolayers of cells were treated with NPT (10 μmol/L), bevacizumab (1 mg/mL), or sunitinib (10 μmol/L) 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, resuspended in lysis buffer, and extracts were sonicated. Proteins in supernatants were separated by SDS-PAGE and transferred to PVDF (polyvinylidene difluoride) membranes (Bio-Rad). Membranes were incubated overnight at 4 °C with the following antibodies: phospho-stathmin (Ser38), cleaved PARP-1 (Cell Signaling Technology), α-tubulin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; both from Sigma). The membranes were then incubated with the corresponding horseradish peroxidase (HRP)–conjugated secondary antibodies (Pierce Biotechnology). Enhanced chemiluminescence reagent (ECL; PerkinElmer) 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 (0.75 × 10⁶) cells. Two weeks later, the animals were randomly grouped (n = 6 to 8 per group) and treated intraperitoneally with PBS (control), NPT (10 mg/kg, twice weekly), bevacizumab (10 μg per mouse, twice weekly), or sunitinib (20 mg/kg, 5-times weekly) for 2 weeks. NPT, bevacizumab, and sunitinib doses were selected on the basis of previous studies in the literature (26–28). 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
Tumor implantation and in vivo tumor growth experiment

Female athymic nu/nu mice (ages 4–6 weeks) were used to establish a subcutaneous xenograft model as previously described (29). Mice were injected with AsPC-1 cells (0.75 × 10^6) or Panc-1 cells (10 × 10^6), randomly grouped (n = 6 to 8 per group), and intraperitoneal therapy started after 2 weeks with PBS (control), NPT (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 the formula \( V = \frac{1}{2} (L \times W) \), where \( L \) = length and \( W \) = width. After completion of treatment, the animals were euthanized, tumors were removed, weighed, dissected, and processed for histologic 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). 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 and then with Ki67 antibody (1:200) overnight at 4°C followed by a 40-minute incubation with Cy3 (1:200) secondary antibody. Slides were mounted with DAPI (4',6-diamidino-2-phenylindole)-containing mounting solution (Invitrogen). Proliferative activity was evaluated by counting Ki67-positive cells from five different high-power fields (HPF) in a blinded manner. Intratumoral apoptosis was assessed 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 sections with PECAM-1 (1:100) antibody (BD Pharmingen) overnight at 4°C followed by a 40-minute 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 smooth muscle actin (α-SMA) or collagen I (1:200) antibody overnight at 4°C followed by a 40-minute 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) with a DSU spinning confocal unit using Slidebook software (Intelligent Imaging Innovations).

Statistical analysis

In vitro cell proliferation data are expressed as mean ± SD. Statistical significance was analyzed by the two-tailed Student t test using GraphPad Prism 6.0 software (GraphPad Software) for individual group comparison. Statistical analysis for in vivo tumor growth studies was performed by one-way ANOVA for multiple-group comparison and the Student t test for the individual-group comparison. Survival study statistics were evaluated using log-rank group comparison (GraphPad Prism 6.0). Values of \( P < 0.05 \) were considered to represent statistically significant group differences.

Results

Effects of NPT, bevacizumab, and sunitinib on animal survival

Animal survival benefit was evaluated using an intraperitoneal PDAC murine xenograft model in NOD/SCID mice after 2 weeks of therapy. The median animal survival was 19 days in the control group and was significantly improved by single agent NPT (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 NPT 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 NPT), whereas the combination of NPT 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 NPT). Importantly, the combination of NPT 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; Fig. 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 (Supplementary Fig. S1A).

NPT, 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 (Fig. 2A). However, some inhibition of local tumor growth in vivo was observed after all treatments; compared with controls (100 ± 22.8), percentage net local tumor growth was 28.2 ± 23.4 with NPT, 35.6 ± 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; Fig. 2B). There was no significant change in mouse body weight during the course of therapy (Fig. 3A). Combination therapy groups Bev+Su, NPT+Su, NPT+Bev+Su, and NPT+Bev+Su showed a significant improvement in median survival compared with controls (100 ± 22.8) (Fig. 4A) with a 68% increase for combination therapy group (Supplementary Fig. S1A).
weight during the experiment (Supplementary Fig. S1B). The tumor weight measurement at completion of therapy was very comparable to the final day tumor volume data (Supplementary Fig. S2). We also evaluated NPT, bevacizumab, and sunitinib 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 with the percentage 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; Supplementary Fig. 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 NPT, bevacizumab, and sunitinib, either alone or in combination, were investigated by immunohistologic and immunoblot analyses of tumor tissues obtained from AsPC-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 with the percentage 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; Supplementary Fig. S3). Overall, group differences were highly statistically significant as determined by one-way ANOVA (P < 0.0001).

Ki67 staining to analyze intratumoral proliferative activity demonstrated that NPT 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 with the Bev+Su or NPT+Bev groups. Compared with 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%), bevacizumab (69% ± 6%), sunitinib (36% ± 11%), Bev+Su (34% ± 9%), NPT+Bev (36% ± 8%), NPT+Su (17% ± 10%), and NPT+Bev+Su (13% ± 6%) therapy groups (Fig. 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 NPT 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 (Fig. 3B and Supplementary Fig. S4).

TUNEL staining was performed to analyze the effects of NPT, 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 NPT and bevacizumab monotherapy. Combinations of NPT with bevacizumab or sunitinib were also effective but not significantly better than sunitinib alone (Fig. 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; Fig. 4).

The effects of NPT 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 MVD, sunitinib being the 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 (Fig. 5). Mean microvessel counts per HPF were as follows: 21/4 (control), 12.9/2 (NPT), 71/ (Bev), 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; Fig. 5). MVD in the setting of NPT therapy was only significantly reduced by the addition of sunitinib, but not after bevacizumab.

The effects of NPT and antiangiogenic agents on tumor stroma were examined by fluorescent immunohistochemical staining of stromal marker proteins collagen I and α-SMA. Relative to control, collagen I pixel intensity was decreased in treatment groups by (in percentage) 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; Supplementary Fig. S5). Pixel intensity of α-SMA expression was also significantly decreased by NPT (68%, P < 0.0003) but less affected by bevacizumab and sunitinib; combination treatment groups were not significantly different from the NPT monotherapy group (Supplementary Fig. S6).

**Effects on in vitro cell proliferation and related protein expression**

To delineate the antitumor mechanism of the addition of bevacizumab and sunitinib to NPT 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, NPT IC₅₀ 5.1 μmol/L was reduced to 41 μmol/L by bevacizumab and to 63 μmol/L by sunitinib. In WI-38 cells, the NPT IC₅₀ of 7.2 μmol/L was decreased to 7.8 μmol/L by sunitinib, whereas no significant decrease was observed after bevacizumab addition (Fig. 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. NPT treatment caused a significant increase in phospho-stathmin and...
cleaved PARP-1 in all the three cell types. NPT 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 the three cell types (Fig. 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 patients with pancreatic cancer. 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). NPT 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 NPT and gemcitabine combination, as observed in the recent clinical PDAC trial (31). Recent studies in our laboratory have shown that NPT is the most effective single cytotoxic agent when compared with gemcitabine or docetaxel in experimental PDAC (10). In addition, in a breast cancer model, NPT 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 radiotherapy and targeted therapy (32, 33). Therefore, the evaluation of combination treatment benefits of NPT with antiangiogenic agents seems logical and plausible in advanced and metastatic cancers. Because 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 NPT 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 NPT 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 NPT was combined with both bevacizumab and sunitinib. Advantages in combining NPT 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 laboratory have shown that in

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**Figure 5.** Tumor vasculature analysis after NPT, bevacizumab, or sunitinib therapy. AsPC-1 tumor-bearing athymic nude mice were treated for 3 weeks and 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 HPFs. The data are expressed as the mean ± SD.
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 NPT, giving support to a rationale for using poly-mechanistic antiangiogenic therapy independent from the underlying cytotoxic agent’s mechanism. In addition, this strategy seems to function even in the setting of enhanced intratumoral delivery, a characteristic for NPT over other unbound taxanes.

Subcutaneous murine xenograft studies revealed that NPT inhibited local tumor growth, but in contrast with 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 NPT and sunitinib, either alone or in combination, indicates 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 NPT 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 bevacizumab. High PECAM-1 staining, a well-established marker of MVD/angiogenesis, in control tumor tissues confirmed an abnormal

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Figure 6. NPT, 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 NPT with or without IC50 dose of bevacizumab or sunitinib. After 72 hours of 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 IC50 values were calculated using GraphPad Prism 6.0 software. B, antiproliferative effects of NPT, bevacizumab, and sunitinib correlate with increased phospho-stathmin and cleaved PARP-1 expression. Subconfluent cell monolayers were treated with NPT (10 μmol/L), bevacizumab (1 mg/mL), or sunitinib (10 μmol/L), 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.
hypermorphy of PDAC. A decrease in MVD indicating antiangiogenic effects was observed by all therapies via NPT, bevacizumab, and sunitinib; this effect was more pronounced in combination therapy groups. Furthermore, NPT decreased the tumor stromal density markers, collagen I and α-SMA, that corroborate with previously published report that NPT disrupts stromal architecture (31). NPT-containing combination therapy groups were also effective in decreasing collagen I and α-SMA content. Overall, all these tissue-based analyses showed differential effects of NPT, bevacizumab, and sunitinib, but in general correlate with in vitro 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 NPT combination with bevacizumab and sunitinib, in vitro cell viability studies revealed that NPT 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, whereas sunitinib addition effectively decreased the NPT IC50, supporting the notion that sunitinib has indeed direct antiproliferative effects in addition to its antiangiogenic properties (22). Targeting endothelial cells and fibroblast for solid tumor treatment has been shown to be a potentially effective strategy (45, 46). Furthermore, NPT inhibited representative endothelial cells 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, NPT effects were further improved by bevacizumab and sunitinib, but in WI-38 fibroblasts NPT 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, NPT induced phospho-stathmin and cleaved PARP-1 in all three cell-types. NPT decreased α-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 NPT. Also, as represented in Fig. 3B, there was no significant change in α-tubulin expression in tumor lysates, probably due to the presence of multiple nonepithelial cell types within the samples derived from tumor tissues. In all the three cell types, sunitinib increased cleaved PARP-1 expression, whereas bevacizumab had no effect, again supporting aspects of the multitargeting nature of sunitinib. The in vitro expression pattern of cleaved PARP-1 by NPT, bevacizumab, and sunitinib in all three cell types was very comparable with the 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 NPT 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 NPT activity by bevacizumab or sunitinib remains unclear, it could be attributed to a decrease in angiogenesis, reduction of desmoplastic stroma formation, increased delivery of NPT to tumor, and greater efficacy in growth inhibition of multiple cell types within the tumor microenvironment (49). These results strongly support a rationale for combining polymechanistic, multitargeting antiangiogenic agents with effective systemic therapy such as NPT for clinical PDAC treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: N. Awasthi, R.E. Schwarz
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Awasthi, C. Zhang, A.M. Schwarz, S. Hinz, M.A. Schwarz, R.E. Schwarz
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