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Superior therapeutic efficacy of *nab*-paclitaxel over cremophor-based paclitaxel in locally advanced and metastatic models of human pancreatic cancer

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Background: Albumin-bound paclitaxel (*nab*-paclitaxel, *nab*-PTX) plus gemcitabine (GEM) combination has demonstrated efficient antitumour activity and statistically significant overall survival of patients with metastatic pancreatic ductal adenocarcinoma (PDAC) compared with GEM monotherapy. This regimen is currently approved as a standard of care treatment option for patients with metastatic PDAC. It is unclear whether cremophor-based PTX combined with GEM provide a similar level of therapeutic efficacy in PDAC.

Methods: We comprehensively explored the antitumour efficacy, effect on metastatic dissemination, tumour stroma and survival advantage following GEM, PTX and *nab*-PTX as monotherapy or in combination with GEM in a locally advanced, and a highly metastatic orthotopic model of human PDAC.

Results: *Nab*-PTX treatment resulted in significantly higher paclitaxel tumour plasma ratio (1.98-fold), robust stromal depletion, antitumour efficacy (3.79-fold) and survival benefit compared with PTX treatment. PTX plus GEM treatment showed no survival gain over GEM monotherapy. However, *nab*-PTX in combination with GEM decreased primary tumour burden, metastatic dissemination and significantly increased median survival of animals compared with either agents alone. These therapeutic effects were accompanied by depletion of dense fibrotic tumour stroma and decreased proliferation of carcinoma cells. Notably, *nab*-PTX monotherapy was equivalent to *nab*-PTX plus GEM in providing survival advantage to mice in a highly aggressive metastatic PDAC model, indicating that *nab*-PTX could potentially stop the progression of late-stage pancreatic cancer.

Conclusions: Our data confirmed that therapeutic efficacy of PTX and *nab*-PTX vary widely, and the contention that these agents elicit similar antitumour response was not supported. The addition of PTX to GEM showed no survival advantage, concluding that a clinical combination of PTX and GEM may unlikely to provide significant survival advantage over GEM monotherapy and may not be a viable alternative to the current standard-of-care *nab*-PTX plus GEM regimen for the treatment of PDAC patients.

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Pancreatic ductal adenocarcinoma (PDAC) is well known for its aggressive clinical course and remains as one of the most deadly malignancies in the world with a 5-year mortality of 95% (Hidalgo, 2010; Oettle, 2014). The unusually poor prognosis of the disease is due to the aggressively invasive and metastatic nature of these tumours, and their being impervious to chemotherapy and radiotherapy. A constant hallmark of PDAC is the presence of a dense desmoplastic reaction that consists largely of fibroblasts, myofibroblasts and extracellular matrix proteins including fibronectin and collagens (Maitra and Hruban, 2008). The stromal compartment is conscripted to provide protection to carcinoma cells and contributes to cancer progression and therapeutic resistance (Ijichi *et al*, 2011; Kadaba *et al*, 2013; Whatcott *et al*, 2013). Gemcitabine (GEM; Gemzar) has been established as the standard first-line chemotherapeutic drug for the treatment of patients with advanced PDAC (Burriss *et al*, 1997). Despite laboratory evidence of robust combination activity, numerous phase III trials of GEM in combination with different cytotoxic or molecularly targeted agents have resulted in no substantial clinical improvement over the use of GEM alone (Van Cutsem *et al*, 2009; Colucci *et al*, 2010; Philip *et al*, 2010). Combinations focused to the tumour cell compartment have not proven clinically impactful, perhaps reflecting the invulnerability of this highly *KRAS*-mutated tumour to signaling inhibitors. This has led to an increased focus on targeting the desmoplastic stroma to improve clinical outcomes of patients with this deadly disease (Beatty *et al*, 2011; Provenzano *et al*, 2012; Kadaba *et al*, 2013).

We previously performed pilot studies in which several advanced PDAC patients were successfully treated on the basis of robust anticancer response in the xenografts (patient-derived xenografts; PDXs) derived from the resected primary tumours of those patients (Hidalgo *et al*, 2011; Villarroel *et al*, 2011). Preclinical and early clinical studies conducted by us confirmed that albumin-bound cremophor-free formulation of Paclitaxel (*nab*-PTX, Abraxane) exerts substantial antitumour activity in PDAC (Maitra *et al*, 2009; Von Hoff *et al*, 2011). Our previous studies, conducted in mice with established PDXs (subcutaneous) derived from 11 individual PDAC patients, confirmed that *nab*-PTX treatment in combination with GEM provided a high antitumour efficacy compared with GEM alone (Maitra *et al*, 2009; Von Hoff *et al*, 2011). This synergy was later confirmed by another group of researchers, utilising a genetically engineered mouse model of PDAC (Frese *et al*, 2012). Positive findings from these studies later led to a large randomised phase III clinical trial ($N=861$ metastatic PDAC patients) showed that *nab*-PTX plus GEM was superior in efficacy to GEM alone in terms of progression-free survival and overall survival in the treatment of metastatic PDAC (Von Hoff *et al*, 2013; Goldstein *et al*, 2015). In addition, our studies showed that one of the mechanisms by which *nab*-PTX works in PDAC is by eliminating the extensive desmoplastic reaction, which is considered as a key hallmark of PDAC and barrier to drug diffusion and treatment efficacy (Von Hoff *et al*, 2011; Xie and Xie, 2015).

Cremophor EL (CreEL)-based Paclitaxel (PTX, Taxol) is a key chemotherapy component for the treatment of several human malignancies (Von Hoff, 1997). Despite the clinical benefit achieved with solvent-based taxanes, the treatment often produce significant side effects (Gradishar *et al*, 2009). *Nab*-PTX is a cremophor-free and water-soluble albumin-based formulation of PTX consisting of 130nm albumin-paclitaxel nanoparticles. *Nab*-PTX treatment showed higher response rates and improved tolerability compared with solvent-based formulations in patients with advanced metastatic breast cancer and non-small cell-lung cancer (Montana *et al*, 2011; Viudez *et al*, 2014). Clinical experience with solvent-based traditional taxanes such as PTX and docetaxel in PDAC patients were disappointing (Saif *et al*, 2010).

Nab-PTX plus GEM combination is currently being implemented in national and international guidelines as a standard of care treatment option for patients with metastatic PDAC. It is unclear

whether PTX when combined with GEM provide similar therapeutic efficacy in PDAC. Since PTX is widely available and the treatment cost of *nab*-PTX is relatively higher than PTX, clinicians, third party payers and regulatory agencies have a substantial interest in understanding whether these drugs share a similar level of pharmacological activities in PDAC.

In the present study, we utilised orthotopic models of human PDAC, which were shown to better recapitulate the histologic and metastatic characteristics of disease than the subcutaneous cell line xenograft models (Hoffman, 2015), and directly compared the anticancer activity, effect on tumour stroma modulation, metastatic spreading to distant organs and survival following GEM, PTX, *nab*-PTX and combinations of GEM plus PTX or *nab*-PTX. To our knowledge, this is the first study which used a large number of mice (300) with established orthotopic tumours, and comprehensively explored the therapeutic efficacy of PTX and *nab*-PTX alone and in combination with GEM in human PDAC models.

MATERIALS AND METHODS

Establishment of orthotopic PDX models of pancreatic cancer.

Eight- to ten-week-old male athymic (nu/nu) nude mice (Harlan) were used for the study. Animals were maintained in a sterile environment and had access to autoclaved laboratory rodent diet and water *ad libitum*. All animal experiments were conducted following approval and in accordance with the Animal Care and Use Committee guidelines of the Johns Hopkins University, which is in line with guidelines for the welfare and use of animals in cancer research (Workman *et al*, 2010). Two human pancreatic cancer xenografts (Panc185 and Panc265) from the 'PancXenoBank', a collection of patient-derived human pancreatic cancer xenografts established from the resected primary tumours of patients with PDAC, were used for the present study (Rubio-Viqueira *et al*, 2006). We used Panc185 tumour as a representative model of locally advanced primary PDAC and Panc265 tumour as a representative model of highly aggressive and metastatic PDAC. Briefly, subcutaneously maintained Panc185 and Panc265 tumours in mice were resected aseptically at the exponential growth phase and used as the source of tumours for surgical orthotopic implantation into the pancreas. Tumours were minced with a sterile razor and cut into cubes of $\sim 1.5\text{ mm}^3$. Mice were anaesthetised using isoflurane inhalation. A 1-cm left lateral abdominal incision was made on the splenic silhouette, without injury to underlying organs, with a sterile microscissor. The pancreas was identified and laterally externalised. A small cut was made at the middle of pancreas and a 1.5-mm^3 tumour fragment, dipped in Matrigel (BD Biosciences, Bedford, MA, USA), was immediately implanted into the middle of the pancreas and sutured using 3-0 surgical sutures. The pancreas was returned into the peritoneal cavity. The abdominal wall and skin were sutured in two layers using 3-0 surgical sutures (Kim *et al*, 2009). Orthotopic tumour establishment was assessed initially by transabdominal palpations, confirmed by an ultrasound scan (Vevo 660 VisualSonics, Toronto, ON, Canada).

Drugs. Gemcitabine (Eli Lilly, Indianapolis, IN, USA) and cremophor-based paclitaxel (Bristol-Myers Squibb Company, Princeton, NJ, USA) were purchased from the Johns Hopkins Hospital Pharmacy (Baltimore, MD, USA). *Nab*-paclitaxel (Abraxis BioScience, LLC, a wholly owned subsidiary of Celgene Corporation) was supplied by Celgene Corporation (Summit, NJ, USA). All three drugs were reconstituted separately in normal saline, prepared fresh daily as required and administered to tumour harbouring mice within 1 h of preparation.

Evaluation of the *in vivo* efficacy of drug regimens in orthotopic PDAC harbouring mice. Mice were orthotopically implanted with Panc185 and Panc265 tumours. Engrafted tumours were

allowed to grow over a period of 2–7 weeks (Panc265 and Panc185, respectively). In order to confirm orthotopic tumour growth in mouse pancreas, determine the weight of primary tumours and any metastatic dissemination (baseline), five animals in Panc185 and Panc265 were randomly selected and exploratory laparotomy was performed. Mice were screened visually for metastatic lesions in the spleen, liver, lungs, kidneys, lymph nodes and peritoneum using a $\times 2.5$ lens. Primary tumour was excised from pancreas, weighed and measured using a digital caliper. Spleen, liver, lungs, kidneys, lymph nodes were excised. Tumours and tissues were formalin-fixed, paraffin-embedded, sectioned and were used for haematoxylin and eosin (H&E) staining.

In order to determine the efficacy of treatment regimens, mice with demonstrable primary tumours (200–300 mm³) were selected and randomly allocated into following arms: (1) Control (untreated); (2) GEM (100 mg kg⁻¹, i.p. twice weekly for 4 weeks); (3) PTX (13.4 mg kg⁻¹, i.v. once daily for 5 consecutive days, only on first week); (4) nab-PTX (22.3 mg kg⁻¹, i.v. once daily for 5 consecutive days, only on first week); (5) PTX and GEM at the above-mentioned dose and schedule; (6) nab-PTX plus GEM at the above-mentioned dose and schedule ($N=8-10$ mice/group). Drug doses were selected based on previous studies (Desai *et al*, 2006; Rajeshkumar *et al*, 2011). Mice were evaluated for signs of discomfort or morbidity. Tumour growth was monitored by periodic transabdominal palpations. Animals were killed on day 28. Upon autopsy, mice were screened visually for metastatic lesions in the spleen, liver, lungs, kidneys, lymph nodes and peritoneum using a $\times 2.5$ lens. After excision, the suspected metastatic lesions in organs were fixed in 10% buffered formalin, paraffin-embedded, sectioned and stained with H&E. Visible macroscopic metastatic lesions verified histologically were counted towards metastatic incidence. Primary tumours were excised from the pancreas, weighed and measured using a digital caliper. Tumour volume was calculated using the following formula: tumour volume = (length \times width²)/2. Tumour pieces fixed in 10% buffered formalin were used for histological and immunohistochemical analysis.

Treatment effect on desmoplastic stroma, tumour cell proliferation and angiogenesis. We performed H&E staining on formalin-fixed, paraffin-embedded primary tumours and tissues (spleen, liver, lungs, kidneys and lymph nodes) harvested at the termination of experiment one (day 28). Tumours and tissues were sectioned (5 μ m) and de-paraffinised. H&E staining was conducted using standard procedures. Evaluable tumours from four to five separate animals were used for assessing the intratumour vascularity, tumour cell proliferation and stromal desmoplasia. Intratumour vascularity and tumour cell proliferation were assessed using anti-CD31 antibody (Cat # DIA 310, Dianova GmbH, Hamburg, Germany) and Ki-67 staining (Cat # 790-4286, Ventana Medical Systems Inc., Tucson, AZ, USA), respectively, as previously described (Yabuuchi *et al*, 2013). Masson's trichrome (Rajeshkumar *et al*, 2015) and collagen IV staining (Cat # M0785, Dako, Carpinteria, CA, USA) were used to assess the extent of stromal desmoplasia. After staining, sections were observed under a light microscope and photographed using a digital camera.

Evaluation of the survival benefit of drug regimens in orthotopic PDAC harbouring mice. A separate study was conducted for assessing the survival advantage of treatment regimens. Mice with established orthotopic Panc185 and Panc265 tumours (200–300 mm³) were randomly allocated to six groups and treated with drug doses and schedule as mentioned in the *in vivo* efficacy study design ($N=10$ mice/group). Animals were monitored twice daily. Survival end points were reached and recorded when mice began to appear moribund with cachexia, abdominal distension, showed signs of hunched posture, hindlimb paralysis or laboured breathing, whichever occurred first. Mice

were humanely killed by standard CO₂ asphyxiation. Median survival was estimated and compared using Kaplan–Meier method.

Determination of the plasma and intratumour concentration of paclitaxel. Mice harbouring established Panc265 orthotopic tumours ($\sim 200-300$ mm³) were either untreated or treated with PTX (13.4 mg kg⁻¹) or nab-PTX (22.3 mg kg⁻¹), i.v. once daily for five consecutive days ($N=9$ mice per group). On day 5, blood and tumour samples were collected 2 h after the dosing of nab-PTX or PTX. Paclitaxel concentration was analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described elsewhere (Zhang *et al*, 2013). For the calibration curve, an aliquot of paclitaxel stock solution (0.2 mg ml⁻¹ in acetonitrile) was spiked into plasma to make the initial plasma stock at 4000 ng ml⁻¹, which was then diluted to make calibration standard samples at concentrations ranging from 2 to 4000 ng ml⁻¹ in plasma. Quality control (QC) samples were made similarly with concentrations of low QC at 6 ng ml⁻¹, medium QC at 100 ng ml⁻¹ and high QC at 3200 ng ml⁻¹. Dilution QC at 20 000 ng ml⁻¹ was made by spiking the stock solution into blank plasma and then diluted 10-fold with blank plasma. Aliquots (50 μ l) of plasma and tumour samples (including standards, blanks, QCs) were transferred into a 96-well plate. To each well, 150 μ l of a solvent mixture of acetonitrile:methanol (9:1) containing 100 ng ml⁻¹ of d⁵-paclitaxel (as the internal standard) was added. The plate was capped, vortex mixed and centrifuged. A 150 μ l aliquot of the supernatant was transferred into a clean 96-well plate for LC-MS/MS analysis. Chromatographic separation was carried out using a Shimadzu LC20 system equipped with an Agilent Pursuit XRs 3 C₁₈ column (100 \times 2 mm). Mobile phases consisted of 5 mM ammonia acetate in water containing 0.1% formic acid (A) and 5 mM ammonia acetate in acetonitrile:water (9:1) mixture containing 0.1% formic acid (B), at a flow rate of 0.35 ml min⁻¹. The column was eluted with a gradient of 25% B for 0.9 min, linearly increased to 100% B over 1.5 min and then maintained at 100% B for another 3.5 min. The LC elute was connected directly to a Sciex API4000 QTrap mass spectrometer (Ab Sciex, Framingham, MA, USA) equipped with an electrospray ionisation (ESI) ion source. The ratio of intratumoural vs plasma concentrations of paclitaxel was calculated and compared between nab-PTX and PTX treatment groups.

Evaluation of the acute effect of taxanes on tumour desmoplastic stroma. Mice harbouring established Panc185 and Panc265 orthotopic tumours ($\sim 200-300$ mm³) were either untreated or treated with PTX (13.4 mg kg⁻¹) or nab-PTX (22.3 mg kg⁻¹), i.v. once daily for five consecutive days ($N=5$ mice per group). Tumours were resected 24 h post the last dose of PTX or nab-PTX. Tumours were formalin-fixed, paraffin-embedded, sectioned and stained with Mason's Trichrome or Collagen IV. Sections were evaluated under a light microscope and photographed using a digital camera.

Statistical analysis. Data were analysed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). Primary tumour volumes were compared and analysed using Mann–Whitney *U*-test. Survival data were estimated and compared using Kaplan–Meier method and statistical significance was determined using Log-rank (Mantel–Cox) test. Differences and associations were considered statistically significant where $P < 0.05$.

RESULTS

Characteristics of orthotopic PDAC mouse models. Upon orthotopic implantation in mouse pancreas, Panc185 tumours grew as locally advanced primary tumours, lacking metastatic dissemination to other organs. In contrast, Panc265 tumours showed aggressive tumour growth and metastatic spreading to

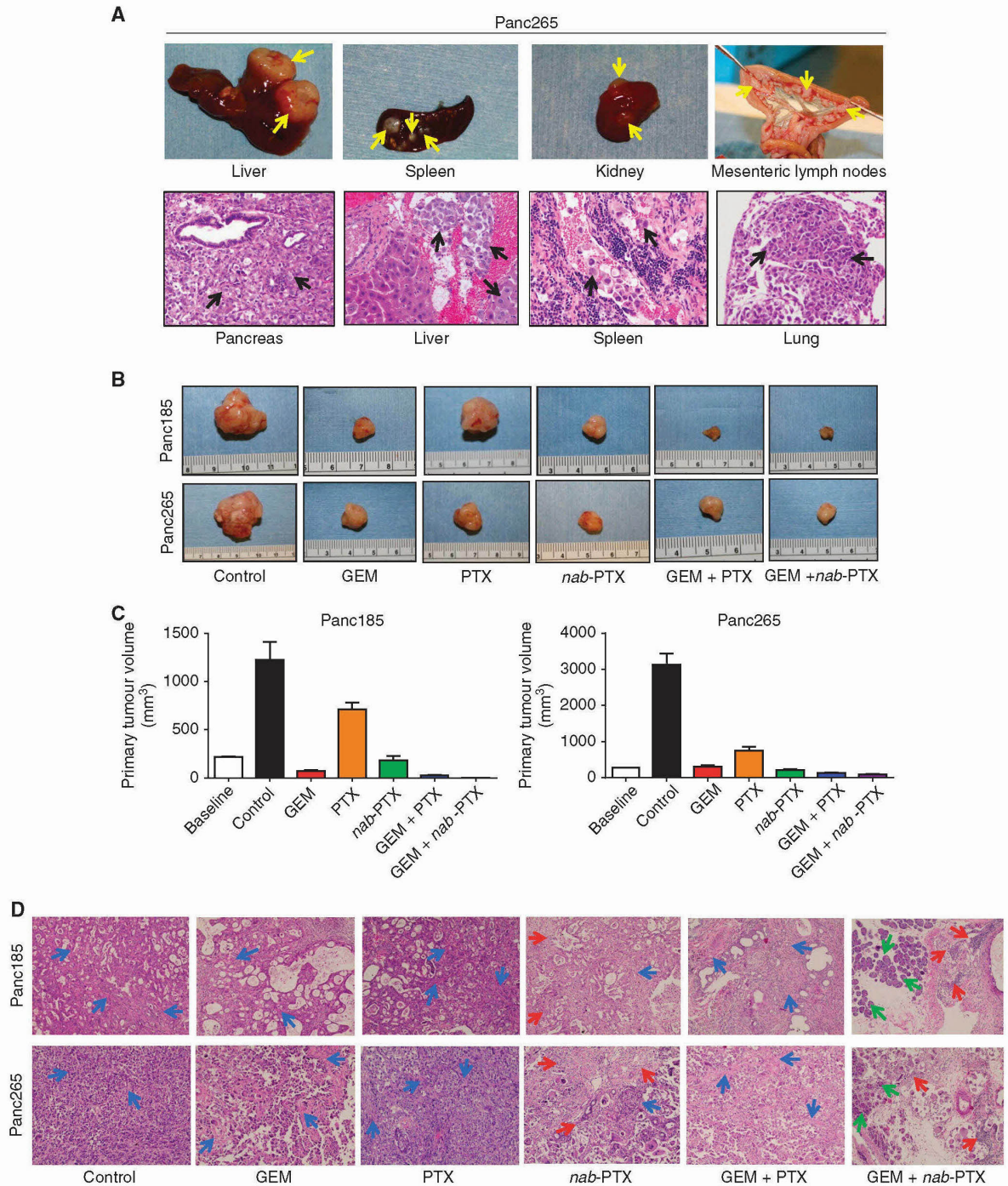


Figure 1. Characteristics of orthotopically implanted PDAC tumours in mice and therapeutic efficacy of treatment regimens. Upon implantation in mouse pancreas, untreated Panc265 tumour showed more aggressive tumour growth and metastatic dissemination in mouse organs. (A) Photographs of resected liver, spleen and mesenteric lymph nodes from an untreated Panc265 tumour harbouring mouse showing prominent metastatic lesions in organs (upper panel). Yellow arrows point towards metastatic lesions. Representative photomicrographs (H&E, $\times 40$) showing primary tumour growth in mouse pancreas, metastatic dissemination in liver, spleen and lungs. Black arrows point towards metastatic deposits in organs. (B) Gross morphology representative of the resected primary tumours at the termination of efficacy experiment (D28). Primary tumour volumes in different treatment regimens are shown in (C). Data represent mean primary tumour volumes \pm s.e.m. $N = 9$ –10 mice per group in Panc185 and 8–10 mice per group in Panc265. Baseline mean tumour volumes ($N = 5$) are shown on the left side of graphs. Statistical comparison of primary tumour volumes and P -values are shown in Supplementary Table S3. (D) Representative photomicrographs (H&E, $\times 20$) of primary tumour sections. Histological evaluation of Panc185 and Panc265 tumours showed moderately differentiated and poorly differentiated pancreatic ductal adenocarcinoma, respectively. Blue arrows point towards desmoplastic stroma. Red arrows point towards areas of neutrophil infiltrate. Green arrows point towards normal pancreatic acinar cells.

Table 1. Treatment effect of regimens on metastatic dissemination to organs and peritoneum in the Panc265 PDAC model

Group	Spleen	Liver	Lungs	Kidneys	LN	Peritoneum
Vehicle	10/10	4/10	1/10	5/10	10/10	8/10
GEM	9/10	1/10	0/10	0/10	6/10	3/10
PTX	10/10	0/10	0/10	0/10	3/10	1/10
nab-PTX	5/10	0/10	0/10	0/10	1/10	0/10
GEM + PTX	5/10	0/10	0/10	0/10	1/10	0/10
GEM + nab-PTX	3/8	0/8	0/8	0/8	0/8	0/8

Abbreviations: GEM = gemcitabine; LN = lymph nodes; nab-PTX = nab-paclitaxel; PTX = paclitaxel. N = 8–10 mice/group.

liver, spleen, lungs, kidneys, lymph nodes and peritoneum (Figure 1A and B and Table 1). Cachexia was evident in control animals harbouring Panc265 tumours and their abdomens were enlarged due to tumour burden and ascites. There was a 11.47-fold increase in primary tumour volumes of control animals compared with baseline tumour volumes, indicating the highly aggressive phenotype of Panc265 tumours (Figure 1C and Supplementary Table S2). Both Panc185 and Panc265 tumours harbour activating mutations of *KRAS* oncogene and inactivating mutations in *p53* tumour suppressor gene, the two most frequently found genetic alterations in human PDAC (Jones *et al*, 2008; Biankin *et al*, 2012; Rajeshkumar *et al*, 2015).

Determination of the therapeutic efficacy of treatment regimens. We first determined the antitumour efficacy of each drug alone and in combination with GEM. Mean primary tumour volumes, tumour weights and complete response of treatment groups are shown in Supplementary Tables S1 and S2. All treatment regimens tested in both Panc185 and Panc265 tumour models showed statistically significant reductions in primary tumour volume compared with control animals (Figure 1B and C and Supplementary Tables S1–S3). The primary tumour volumes increased substantially in the control and PTX-treated animals of Panc185 ($P=0.0007$ and 0.0007 , respectively) compared with the baseline (pre-treatment) tumour volumes (Figure 1C and Supplementary Tables S1 and S3). GEM and nab-PTX monotherapy were highly effective in reducing the primary tumour volumes ($P<0.0001$) compared with the tumour volumes of control animals (Figure 1C and Supplementary Tables S1 and S3). Although the both combination treatments were remarkably effective in abolishing primary tumour growth, the rate of complete responses were higher in the GEM plus nab-PTX (89%) vs GEM plus PTX (22%) treatment, supporting the higher efficacy of GEM plus PTX regimen (Supplementary Table S1).

Baseline mean tumour volume of Panc265 tumours increased from 273 to 3123 mm^3 ($P=0.0007$) and 751 mm^3 ($P=0.008$) by day 28 in the control and PTX-treated groups, respectively (Figure 1C and Supplementary Tables S2 and S3). Treatment of the combination regimens resulted in statistically significant reductions in tumour size, but neither combination induced complete regressions (Supplementary Tables S2 and S3). GEM plus nab-PTX treatment produced statistically significant reduction in tumour volumes compared with GEM treatment ($P=0.0004$).

In both Panc185 and Panc265 tumour models, nab-PTX monotherapy resulted in primary tumour regression compared with baseline tumours (Figure 1C). Nab-PTX treatment was remarkably effective in blocking primary tumour progression compared with PTX monotherapy (3.944- and 3.645-fold; $P<0.0001$ and $P=0.0001$, respectively; Figure 1C and Supplementary Tables S1–S3). In both tumour models, one mouse each treated with nab-PTX achieved a complete response, whereas no complete

responses were observed with PTX treatment (Supplementary Tables S1 and S2). Results of exploratory laparotomies conducted in mice before randomisation (baseline) confirmed the tumour growth confined only in pancreas.

Tumour histology. Histological evaluation of Panc185 and Panc265 tumours revealed moderately differentiated and poorly differentiated pancreatic ductal adenocarcinoma, respectively (Figure 1D). Tumours of control mice showed cribriform pattern of neoplastic cells admixed with necrotic debris, moderate to severe nuclear atypia, nuclear overcrowding, aberrant mitoses and abundant desmoplastic stroma (Figure 1D). While there was a reduction in neoplastic cells, desmoplastic stroma persisted in the tumours of animals treated with GEM or PTX. Depletion of desmoplastic stroma was clearly evident in the tumours of animals treated with nab-PTX alone and in combination with GEM. Tumours harvested from animals treated with nab-PTX alone and in combination with GEM displayed mild to moderate neutrophil infiltration in tumour microenvironment compared with control tumours and other treatment regimens. Nab-PTX alone and in combination with GEM treatment culminated in a sharp reduction of tumour cellularity and progressive loss of desmoplastic stroma. Islands of mouse pancreatic acinar cells were frequently noticed in the Panc185 and Panc265 tumours harvested from the animals administered with GEM plus nab-PTX (Figure 1D), indicating the therapy effectiveness of this regimen in destroying tumour cells and desmoplasia. H&E staining performed on the organs (spleen, liver, lungs, kidneys, lymph nodes) of mice killed prior to randomisation (baseline) did not show any metastatic infiltration (data not shown).

Effect of drug treatments on metastatic dissemination to organs and peritoneum. Panc265 tumours are highly metastatic when implanted orthotopically into mouse pancreas, showing metastatic dissemination in spleen, liver, lungs, kidneys, lymph nodes and peritoneum with frequencies ranging from 10 to 100% of animals (Figure 1A and Table 1). Treatment effects of agents alone and in combination with GEM on metastatic dissemination to distant organs and peritoneum are summarised in Table 1. All animals untreated or treated with PTX developed metastasis in at least one organ (Table 1). GEM treatment was ineffective in controlling metastatic spreading as evidenced by metastatic lesions in the spleen of nine out of ten (90%) mice (Table 1). However, nab-PTX prevented the metastatic spreading to spleen in 5 out of 10 mice (50%). Combined treatment of GEM and nab-PTX decreased metastatic tumour burden compared with either agent used alone (Table 1).

Impact of drug treatments on carcinoma cell proliferation, tumour vascularity and desmoplastic tumour stroma. We determined the treatment effects of agents alone and in combination with GEM on tumour cell proliferation and desmoplastic stroma. Previous studies conducted by us revealed the presence of desmoplastic stroma in subcutaneously grown PDXs and metastatic lesions of human PDAC (Rajeshkumar *et al*, 2015; Whatcott *et al*, 2015). Tumours harvested from untreated animals of Panc265 PDX showed higher number of carcinoma cells expressing Ki-67, compared with tumours in Panc185, indicating the aggressiveness of Panc265 tumours (Figure 2A). The GEM plus nab-PTX combination effectively suppressed tumour cell proliferation in both Panc185 and Panc265 PDXs (Figure 2A). Comparable CD31-positive endothelial cell populations were noticed in the blood vessels of untreated and drug treated tumours, indicating that drug treatments did not alter the tumour vascularity (Figure 2B). Abundance of desmoplastic stroma, a hallmark of human PDAC, was evident in tumours harvested from untreated animals (Figure 3A and B). While GEM and PTX treatments were largely ineffective in reducing tumour stroma, nab-PTX

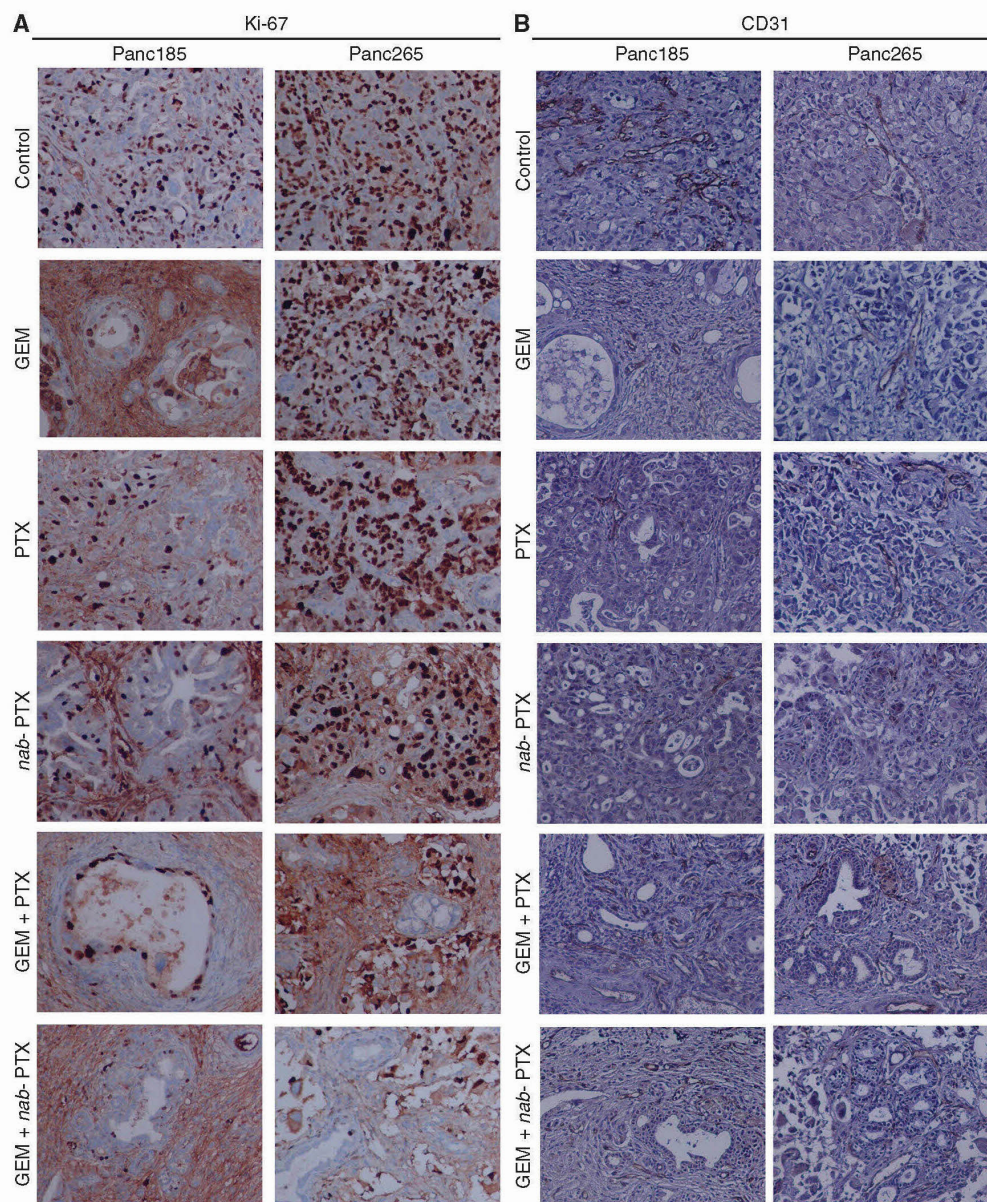


Figure 2. Treatment effect of agents alone and in combinations on tumour cell proliferation and tumour vascularity. The nab-PTX plus GEM combination was highly effective in controlling tumour cell proliferation, as evidenced by reduced number of Ki-67-stained carcinoma cells. Tumours harvested from four to five mice of Panc185 and Panc265 on day 28 (first experiment) were processed and stained with Ki-67 or CD31 antibodies. Ki-67, a nuclear protein, which is expressed in the proliferating carcinoma cells is visualised as dark brown. There was no difference in various sized blood vessel density in tumours harvested from drug-treated animals compared with tumours in the untreated animals. CD31-stained endothelial cells in blood vessels are visualised as brown stain. (A, B) Representative photomicrographs of Ki-67 or CD31 stained tumour sections ($\times 20$).

monotherapy and in combination with GEM facilitated the destruction of dense tumour stroma (Figure 3A and B). These results are in agreement with our previous preclinical and clinical results (Maitra *et al*, 2009; Alvarez *et al*, 2013).

Therapeutic effect of regimens on the survival of mice harbouring human PDAC. We evaluated the impact of drug treatments on the survival of mice harbouring established PDAC. Kaplan–Meier survival curves of different treatment groups and

the comparisons are shown in Figure 4 and Supplementary Figures S1 and S2. In the locally advanced model (Panc185), GEM monotherapy prolonged the median survival from 58 to 114 days (Figure 4A). PTX monotherapy also showed a positive, albeit smaller effect and extended median survival to 81.5 days. Nab-PTX monotherapy showed significant advantage compared with PTX monotherapy by extending the median survival to 115 days ($P=0.0024$; Figure 4A and Supplementary Figure S1). The combination of PTX and GEM showed a median survival of 125

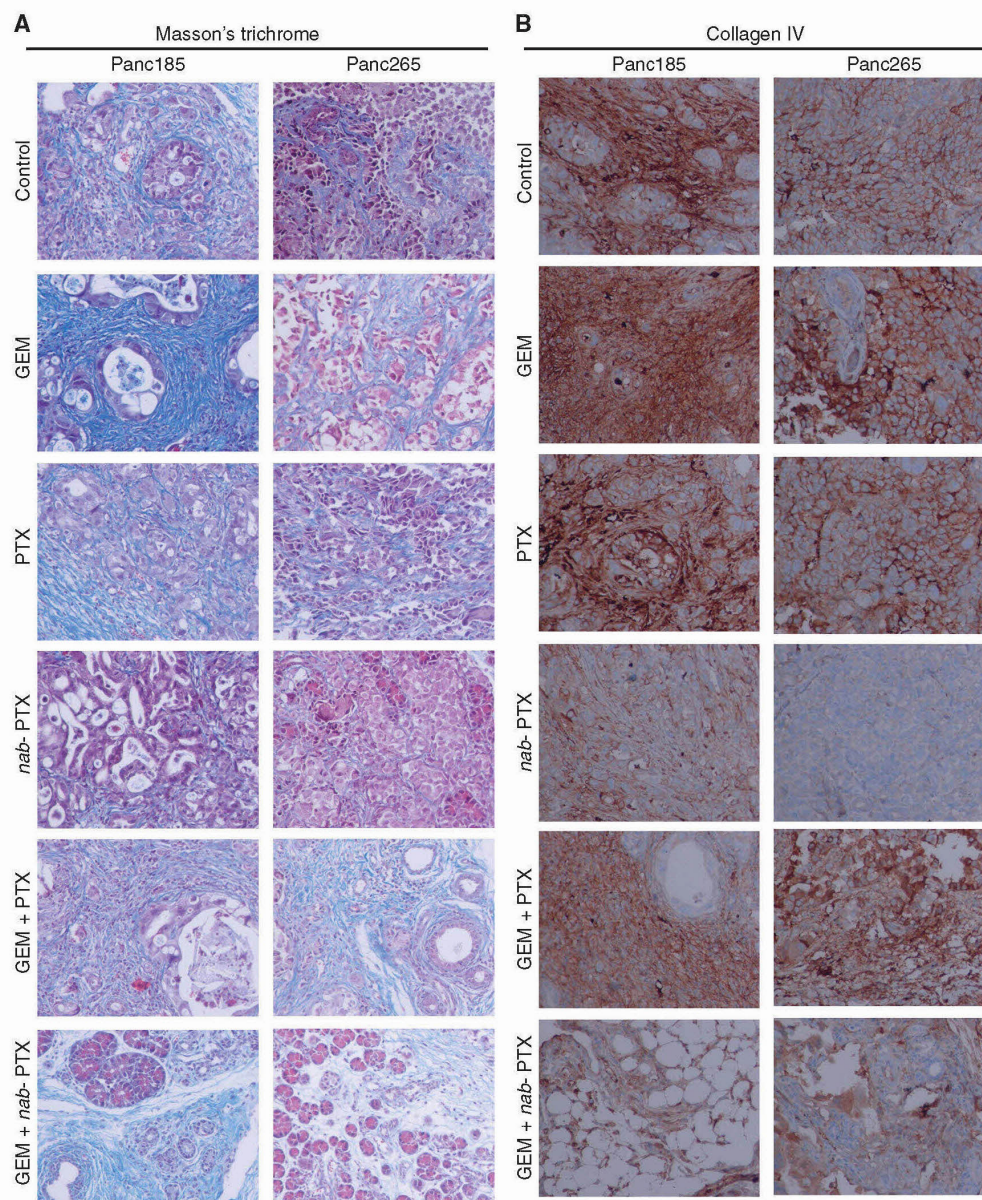


Figure 3. Treatment effect of agents alone and in combinations on desmoplastic tumour stroma. Nab-PTX monotherapy or combined with GEM robustly depleted the dense tumour stroma as evidenced by less number of collagen fibres in the primary tumours. We performed immunohistochemistry on primary tumours harvested from untreated or treated with GEM, PTX, nab-PTX and combinations of GEM plus PTX or GEM plus nab-PTX (D28 samples, first experiment). The fibrotic tissue is visualised as blue (Mason's trichrome) or brown stain (Collagen IV). (A, B) Representative photomicrographs of Mason's trichrome and Collagen IV stained tumour sections ($\times 20$).

days. The combination of nab-PTX and GEM was the most effective treatment. Median survival in this group was 161 days (Figure 4A), and the increase was statistically significant compared with PTX plus GEM treatment ($P = 0.0455$; Figure 4A). Finally, the nab-PTX plus GEM combination treatment resulted in a statistically significant improvement in median survival compared with GEM treatment ($P = 0.0039$; Figure 4A and Supplementary Figure S1).

Mice implanted with Panc265 tumours showed a more aggressive disease course with a median survival of 38 days in untreated mice (Figure 4B). Both GEM and PTX monotherapy resulted in the

median survivals of 56 days (Figure 4B and Supplementary Figure S2). Nab-PTX treatment resulted in a near doubling in median survival to 75.5 days (Figure 4B), and the difference was statistically significant compared with PTX monotherapy ($P = 0.0121$; Figure 4B and Supplementary Figure S2). Neither GEM plus PTX nor GEM plus nab-PTX combination was better than single agent nab-PTX (Figure 4B). The nab-PTX plus GEM combination treatment resulted in a statistically significant improvement in median survival compared with GEM treatment ($P = 0.0228$; Figure 4B and Supplementary Figure S2). Remarkably, the survival of animals in the nab-PTX monotherapy was equivalent to those of animals

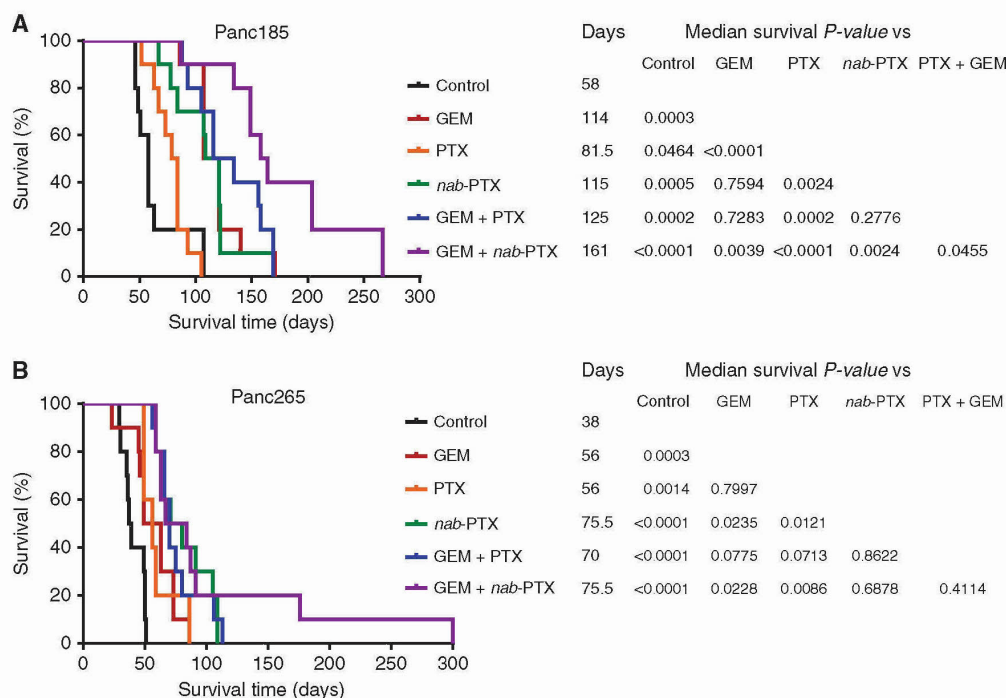


Figure 4. Treatment effect of regimens on the survival of mice with established PDAC. *Nab*-PTX monotherapy and in combination with GEM significantly improved the survival of mice with orthotopically implanted human PDAC. **(A and B)** Kaplan–Meir survival curves of mice with orthotopically implanted Panc185 tumour and Panc265 tumours. Briefly, tumour harbouring mice were treated with GEM, PTX, *nab*-PTX and combinations of GEM and PTX or GEM and *nab*-PTX as described in the study design. Survival data were analysed using GraphPad Prism 6 software. *P*-values for survival differences were calculated using the log-rank (Mantel-Cox) test. Tables shown on the right side of figure panels depict the median survival days and statistical significance. *N* = 10 mice per group in both Panc185 and Panc265 PDXs.

received *nab*-PTX plus GEM treatment in the highly aggressive metastatic Panc265 PDAC model, indicating that *nab*-PTX could potentially stop the progression of late-stage pancreatic cancer and its utility as a backbone for combinations with existing or investigational agents.

Acute effect of taxanes on plasma and intratumour paclitaxel concentrations and desmoplastic stroma. We determined the plasma and intratumour concentrations of paclitaxel following 5-day PTX or *nab*-PTX treatment in the Panc265 tumour model. Plasma paclitaxel concentrations in animals administered with PTX or *nab*-PTX were 280.9 and 265.8 ng ml⁻¹, respectively. Plasma paclitaxel concentrations did not statistically differ between the two treatment groups (*P* = 0.8357; Figure 5A). However, animals receiving *nab*-PTX treatment was associated with significantly higher (30.158%) tumour paclitaxel concentrations compared with PTX treatment (*P* = 0.0014; Figure 5B). As shown in Figure 5C, the paclitaxel tumour plasma ratio was significantly higher (1.98-fold) in animals administered with *nab*-PTX compared with PTX treatment (*P* = 0.0311). While PTX treatment failed to impart stromal depletion, *nab*-PTX treatment drastically lowered the levels of desmoplastic stroma in the tumour microenvironment (Figure 5D).

DISCUSSION

Advanced PDAC is deadly and difficult to treat with success (Wolfgang *et al*, 2013). The incidence of PDAC is increasing and is expected to be the second deadliest malignancy in the USA by 2020

(Garrido-Laguna and Hidalgo, 2015). Recently, an intensive cytotoxic regimen comprising oxaliplatin, irinotecan, fluorouracil and leucovorin (FOLFIRINOX) compared with GEM as first-line therapy in patients with metastatic PDAC has been shown to significantly improve the survival in patients with good performance status (Conroy *et al*, 2011). However, the safety profile of FOLFIRINOX was less favorable than that of GEM and many patients are not eligible to receive FOLFIRINOX because of poor performance status and advanced age (Ko, 2011).

The taxanes, PTX and docetaxel, were introduced more than two decades ago. These two drugs represented a revolution in cancer chemotherapy and have become core components of standard-of-care treatments in several cancer types such as breast, lung and ovary (Chiorean and Von Hoff, 2014). One of the drawbacks of PTX is the cremophor-ethanol, which has been shown to alter the pharmacokinetics of this agent and contribute to side effects such as hypersensitivity reactions in humans (Yared and Tkaczuk, 2012). In an effort to develop more effective and safe taxanes, *nab*-PTX was created as a solvent-free albumin-paclitaxel nanoparticle (Hawkins *et al*, 2008). *Nab*-PTX has demonstrated higher response rates and improved tolerability when compared with solvent-based formulations of PTX in patients with advanced metastatic breast cancer and advanced non-small-cell lung cancer and approved in certain settings and combinations for the treatment of these malignancies (Viudez *et al*, 2014).

Preclinical and early clinical trials conducted by us showed promising antitumour activity of *nab*-PTX and its potential to modulate dense desmoplastic stroma (Maitra *et al*, 2009; Von Hoff *et al*, 2011). These studies were followed by MPACT trial, a large randomised phase III study, which demonstrated the superior

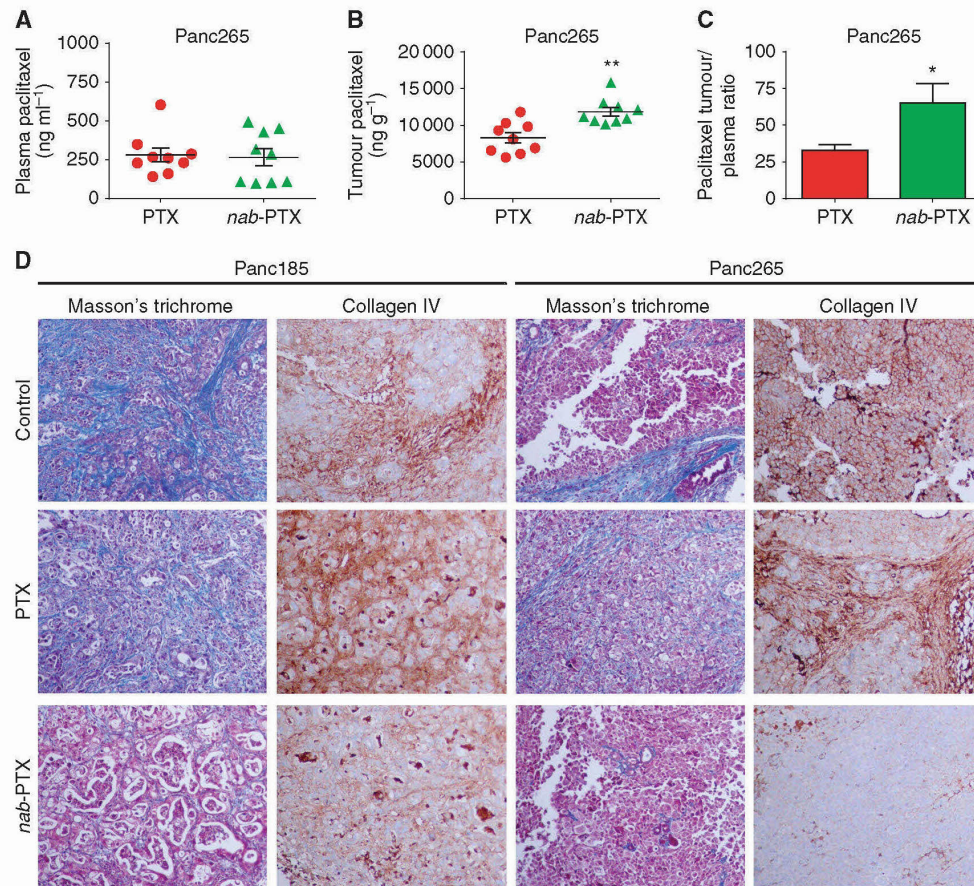


Figure 5. Acute effect of taxanes on plasma and intratumour paclitaxel concentrations and desmoplastic stroma. Nab-PTX treatment resulted in higher intratumour paclitaxel concentration, higher paclitaxel tumour plasma ratio and facilitated depletion of tumour desmoplastic stroma, compared with PTX treatment. For the quantitation of plasma and intratumour paclitaxel, mice with established Panc265 orthotopic tumours were either untreated or treated with PTX/nab-PTX once daily, i.v. for five consecutive days as described in the Materials and Methods. Blood was collected and tumours were resected 2h post the last dose of PTX or nab-PTX on day 5 ($N=9$ mice/group). Tumour and plasma paclitaxel concentrations were analysed by LC/MS/MS. **(A)** Scatter plots showing plasma paclitaxel concentrations of animals administered with PTX or nab-PTX. Plasma paclitaxel concentrations of PTX was not statistically significant compared with nab-PTX ($P=0.8357$). As shown in panel **B**, nab-PTX treatment was associated with significantly higher (30.158%) tumour paclitaxel concentrations compared with PTX treatment (** $P=0.0014$). The paclitaxel tumour plasma ratio was significantly higher (1.98-fold) in animals administered with nab-PTX compared with PTX treatment (**C**, * $P=0.0311$). In order to determine the acute effect of taxanes on tumour stroma, mice with established Panc185 and Panc265 orthotopic tumours were either untreated or treated with PTX/nab-PTX once daily, i.v. for five consecutive days as described in the Materials and Methods. Tumours were resected 24h post the last dose of PTX or nab-PTX on day 5 ($N=5$ mice/group). **(D)** Representative photomicrographs ($\times 20$) of Masson's trichrome and Collagen IV staining performed on formalin-fixed paraffin-embedded primary tumour tissues. Treatment with nab-PTX resulted in a higher depletion of tumour desmoplastic stroma compared with PTX treatment. Fibrotic tissue is visualised as blue, nuclei are stained dark red/purple and cytoplasm is stained red/pink (Masson's trichrome). Collagen IV enriched fibrotic areas are visualised as brown stain.

efficacy of nab-PTX in combination with GEM vs GEM alone (Von Hoff *et al.*, 2013). Positive results from this study have led to the regulatory approval of this regimen, which is currently being implemented as a standard of care regimen for the treatment of patients with metastatic PDAC (Von Hoff *et al.*, 2013). While the clinical efficacy of nab-PTX in PDAC is beyond any doubt, the mechanism of action is less clear. The extracellular matrix protein secreted protein, acidic and rich in cysteine (SPARC) had been put forward as a potential biomarker associated with superior nab-PTX activity. Despite support from a Phase I/II study (Von Hoff *et al.*, 2011), a *post hoc* exploratory study of the MPACT trial did not detect any association between SPARC positivity in tumour tissue or plasma and treatment outcomes (Hidalgo *et al.*, 2015). Two

independent preclinical investigations using SPARC knockout mice and genetically engineered mice have corroborated this result (Alvarez *et al.*, 2013; Neesse *et al.*, 2014). Thus the notion that nab-PTX is effective in pancreatic cancer because the albumin binds to SPARC may not be correct (Hidalgo *et al.*, 2015). A recent study demonstrated that efficacy of nab-PTX in metastatic breast cancer do not appears to be associated with expression of SPARC in primary or metastatic tumour tissue or in the serum (Schneeweiss *et al.*, 2014). We recently showed that nab-PTX and GEM treatment effectively decreased the density of cancer-associated fibroblasts and induced a marked alteration in cancer stroma that results in primary tumour softening of patients with operable PDAC (Alvarez *et al.*, 2013).

We performed studies to explore the efficacy and survival benefit of taxanes alone and in combination with GEM and determined the treatment effect on tumour cell proliferation, tumour desmoplasia and metastatic spreading to distant organs. We utilised orthotopic PDX models, which have been shown to better mimic metastasis than subcutaneously grown xenografts (Herrerros-Villanueva *et al*, 2012; Hoffman, 2015; Hwang *et al*, 2016). Our results showed that PTX as a single agent was not significantly effective in reducing primary tumour burden or enhancing mouse survival compared with the activity of *nab*-PTX or GEM monotherapy. When compared with either agent used alone, combined treatment of GEM plus *nab*-PTX decreased metastatic tumour burden and increased median survival of animals (Table 1 and Figure 4). Masson's trichrome and Collagen IV staining performed on tumours harvested from untreated animals showed abundant levels of fibrotic tissue in tumour microenvironment (Figure 3). Visually striking reduction in fibrotic tissue was observed in tumour tissues harvested from the *nab*-PTX-treated animals compared with the tumours harvested from PTX-treated animals (Figure 3). As previously reported in mouse models and patient tissues (Maitra *et al*, 2009; Von Hoff *et al*, 2011), *nab*-PTX treatment effectively dismantled PDAC stroma (Figure 3). *Nab*-PTX treatment reduced the number of proliferating carcinoma cells compared with PTX treatment as evidenced by reduced number of carcinoma cells expressing Ki-67, a nuclear protein, expressed in proliferating cells (Figure 2A). GEM plus *nab*-PTX treatment was highly effective in reducing the Ki-67-positive carcinoma cells compared with GEM plus PTX treatment (Figure 2A).

In order to explore the potential mechanism of enhanced therapeutic efficacy of *nab*-PTX over PTX, we determined the plasma and intratumour concentrations of paclitaxel following *nab*-PTX or PTX treatment. We also evaluated the acute effect of taxanes on tumour desmoplastic stroma. Our results showed a 30.158% increase in tumour paclitaxel concentration and 1.98-fold higher paclitaxel tumour plasma ratio, following 5-day *nab*-PTX treatment compared with PTX treatment (Figure 5B and C).

Nab-PTX treatment was associated with higher depletion desmoplastic stroma in the tumour microenvironment compared with untreated or PTX-treated tumours. Considerable evidence from both preclinical and clinical studies demonstrated that multiple factors including favorable pharmacokinetics contribute to the augmented antitumour efficacy of *nab*-PTX over cremophor-based PTX (Gardner *et al*, 2008; Ma and Hidalgo, 2013). A previous preclinical study demonstrated that tumour paclitaxel area under the curve was 33% higher for *nab*-PTX treated mice compared with PTX treatment in a breast tumour model (Desai *et al*, 2006). Recent reports demonstrated that human pancreatic cancer cells display macropinocytosis and albumin internalisation occur through tumour macropinocytosis (Commisso *et al*, 2013; Kamphorst *et al*, 2015). Our *in vivo* studies did not investigate whether tumour macropinocytosis contribute to the uptake of albumin cocoon from *nab*-PTX or facilitate the bioavailability of paclitaxel in tumour microenvironment. Our data demonstrated that *nab*-PTX treatment resulted in a higher intratumour paclitaxel concentration, leading to enhanced killing of neoplastic cells and depletion of tumour desmoplastic stroma, which may be responsible for the significant therapeutic advantage of *nab*-PTX over PTX treatment in pancreatic cancer.

Our results convincingly demonstrated that *nab*-PTX treatment was remarkably effective in blocking primary tumour progression, depletion of dense tumour stroma and consistently achieved greater antitumour response, resulting in enhanced survival of tumour harbouring animals compared with PTX treatment. Combined treatment of GEM plus *nab*-PTX decreased metastatic tumour burden and increased overall survival of animals when compared with either agent used alone. Our findings clarified no benefit of adding PTX to GEM therapy for locally advanced and metastatic

pancreatic cancer. Our studies confirmed that therapeutic efficacy of PTX and *nab*-PTX vary widely, and the contention that these agents elicit similar antitumour response was not supported. In this regard, clinical investigation of PTX alone and in combination with GEM in PDAC is not supported by our results.

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CONFLICT OF INTEREST

ZT, SH, SB, DWP and CH are employees of Celgene Corporation. The remaining authors declare no conflict of interest.

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