List of supplementary materials

Supplementary methods

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Supplementary methods

Drug preparations

Gemcitabine powder (a 48% preparation of difluorodeoxycytidine, dFdC) was provided by Addenbrooke's Hospital Pharmacy (Cambridge, UK) and resuspended in sterile normal saline at 10.2mg/ml. The drug was administered at 100mg/kg by intraperitoneal injections at day 0, day 4 and day 8. *nab*-paclitaxel (Abraxane®) was provided by Celgene (USA) and resuspended in sterile normal saline at 24mg/ml and intravenously administered at 120mg/kg at day 0, day 4 and day 8. Human albumin (Baxter Healthcare, USA) was administered intravenously for vehicle and gemcitabine cohorts. Paclitaxel was provided by Addenbrooke's Hospital Pharmacy (Cambridge, UK), resuspended in Cremophor at 6mg/ml, and administered at 30mg/kg.

Therapeutic intervention study

For the purpose of enrollment, mice were subjected to high-contrast ultrasound screening using the Vevo 770 System with a 35MHz scanhead (Visual Sonics, Inc.) as described [38]. Mice with mean tumor diameters of 6-9mm were enrolled. During the study, tumor growth was quantified on day 3 and day 7 by measuring tumor volumes using reconstructed 3D ultrasonography with the integrated Vevo 770 software package. All mice were sacrificed on day 8, 4h after the last *nab*-paclitaxel injection and 2h after the last gemcitabine administration. One half of the tumor was fixed in 10% neutral buffered formalin for histology, the other half was sliced in 4-6 pieces and either snap frozen in liquid nitrogen or placed in RNA later (Qiagen) for 24h and subsequently stored at -80º. Full blood counts were obtained on terminal cardiac bleeds using a MS9-5 Veterinary Hematology Analyzer (Woodley Equipment Company Ltd.)

Picrosirius red staining

The picrosirius red staining kit was obtained from Polysciences Inc., and staining performed according to the manufacturer's protocol.

ELISA

96 well plates were coated overnight at 4°C with 5 μg/mL human serum albumin in carbonate buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.6) and blocked in 5% milk/PBS. Mouse plasma diluted in blocking buffer was incubated overnight at 4°C. After washing, plates were incubated with goat anti-mouse HRP antibodies in blocking buffer for 1 hour. After washing, reactions were developed with TMB (T0440, Sigma), terminated with H_2SO_4, and the OD_{450} was read on a Powerwave XS plate reader (Biotek).

Methods of quantification

Automated quantification

Automated quantification of IHC for cleaved caspase-3 and phospho-histone H3 was performed on 30 randomly chosen fields using the Ariol SL-50 imaging system and analysis software (Genetix). For picosirius staining and αSMA or SPARC immunoreactivity, positive stained areas and total tumor area were assessed by the Ariol imaging system using a low threshold to account for background staining of necrotic tumor areas. Alternatively, complete tumor cross sections were scanned using the Aperio XT scanner and analyzed for necrosis or vascular parameters via Genie Histology Pattern Recognition software or the Microvessel Analysis Algorithm (Aperio), respectively.

Manual quantification:

Tumor cell apoptosis as assessed by co-immunofluorescence of CC3 and E-cadherin was determined by the number of CC3 and E-cadherin positive cells per 40X field. Images were captured on a Leica SP5 confocal microscope. At least 10 fields were quantified per tumor.

Metastasis quantification:

Two lobes of liver were sliced into a total of 4-6 strips and embedded in paraffin. For each mouse, 10 sections at 30-50µm distance were H&E stained and microscopically analysed for metastases. Metastatic lesions were classified in micro, small and large, and burden was calculated by the sum of all metastatic lesions multiplied 1x (micro), 2x (small) and 3x (large).

Quantitative PCR

FAM labelled assays were used in conjunction with TaqmanFast mastermix 384-wellplates. Actin was used as the endogenous control.

Actb	Mm00607939_s1
Mrp1	Mm0456156_m1
Mrp3	Mm00551550_m1
Mrp5	Mm00443360_m1
Cnt1	Mm01315355_m1
Cnt2	Mm04212034_m1
Cnt3	Mm00627874_m1
Ent1	mM00452176_m1
Ent2	mM00432817_m1
Dctd	Mm00618904_m1
Nt5c1a	Mm01192248_m1
Nt5c3	Mm0046604_m1
Cda	mM01341706_m1

Dck	Mm00432794_m1
Rrm1	Mm00485870_m1
Rrm2	Mm00485881_g1
Rrm2b	Mm01165702_gH
Tk2	Mm00445175_m1

siRNA

KPC tumor cells were transfected with Lipofectamine2000 (11668-019, Invitrogen) according to manufacturers recommended protocols with indicated gene-specific siRNA ON-TARGETplus SMART pools (Dharmacon).

NT	D-001810-10
Cda	L-050833-01
Cnt3	L-059421-01
Ent1	L-050990-01

Supplementary figure legends

Table S1. Intratumoral gemcitabine metabolites (ng/mg tumor tissue) in mice treated with (A) gemcitabine/vehicle or (B) nab-paclitaxel+gemcitabine for 8 days.

Figure S1. Clinical study parameters. **A**) Mice received ultrasounds on day -2, 3, and 7 and were treated on day 0, 4, and 8. All samples were taken 4 hours after the last dose of *nab*-paclitaxel and 2 hours after the last dose of gemcitabine. **B**) Initial tumor volumes, as assessed on day -2 were similar among all cohorts. ($n \geq 8$) **C**) Pre-treatment mouse weights were recorded on days 0, 4, and 8. Data represents cohort means +/- SEM. ($n \geq 8$) **D**) Cohorts of mice were treated with indicated compounds and tumor growth was monitored twice weekly. Data represents the mean relative tumor volumes +/- SEM. *nab*-paclitaxel monotherapy is significantly better than vehicle, but not gemcitabine ($p=0.007$ and $p=0.120$, respectively). (n=8) **E**) To detect mouse anti-human albumin antibodies, an ELISA format revealed the titre by using indicated dilutions of plasma from mice from each cohort. The largest dilution that yielded a positive result is indicated for each mouse. All mice (n=6) were positive due to the presence of antibodies against either human albumin vehicle or the human albumin component of *nab*-paclitaxel.

Figure S2. Cell biological effects of *nab*-paclitaxel. **A**) Mice were treated with a maximum tolerated dose of either paclitaxel (30mg/kg) or *nab*-paclitaxel (120mg/kg). 4 hours later tissues were harvested and assessed for intratumoural paclitaxel levels ($n \geq 6$). **B**) Representative H&E section from a *nab*-paclitaxel-treated mouse with aberrant mitotic figures (arrowheads). Scale bar = 50μm **C**) Quantification of phospho-histone H3 immunohistochemistry reveals elevated aberrant mitotic figures in tumors from *nab*-paclitaxel-treated mice. (n=8) **D**) Quantification of necrosis in H&E sections of tumors from mice in each cohort revealed no significant differences. (n=8) **E**) Representative H&E

sections from each cohort do not reveal stromal depletion. Scale bar = 100μm. (n=8) **F**) Co-immunofluorescence of E-cadherin or αSMA (green) and cleaved caspase 3 (red) reveals selective apoptosis in the epithelial compartment. Scale bar = 100μm. (n≥9)

Figure S3. *nab*-paclitaxel does not cause stromal collapse in PDA tumors from KPC mice. αSMA immunofluorescence (**A**) and quantification (**B**) as a measure of stromal content in tumors from each cohort (n=8). Picosirius red staining (**C**) and quantification (**D**) as a measure of collagen content in tumors from each cohort (n=8). SPARC immunohistochemistry (**E**) and quantification (**F**) in tumors from each cohort (n=8). Analysis reveals no statistical differences that implicate *nab*-paclitaxel-mediated stromal depletion.

Figure S4. *nab*-paclitaxel does not alter vascular density or structure. **A**) Microvessel density was measured via automated quantification of CD31 immunohistochemistry. (n=8) **B**) Vessel lumen area was measured via automated quantification of unstained area surrounded by CD31 positivity. Analysis reveals no statistical differences between the cohorts. (n=8)

Figure S5. *nab*-paclitaxel does not affect transcription of most gemcitabine metabolic enzymes. **A**) qRT-PCR was performed on RNA isolated from bulk tumors from each cohort. Expression values were normalized to actin. * $p < 0.05$. (n≥7) **B**) KPC cells were transfected with either non-targeting (NT) siRNA or siRNA's directed against Cda, Ent1, or Cnt3. Cells were treated for 2 days with indicated concentrations of gemcitabine and assayed for cell viability. Data are presented as the mean relative cell viability +/- one standard deviation. Cda knockdown significantly increased sensitivity to gemcitabine (GI_{50} = 56 +/- 15 nM) compared to NT siRNA (GI_{50} = 95 +/- 12 nM), whereas Ent1 knockdown decreased sensitivity to gemcitabine (GI_{50} = 193 +/- 28 nM) (n = 4).

Figure S6. Chemotherapy-induced ROS promotes gemcitabine activation. **A**) KPC cells were pre-treated with 10μM paclitaxel, cisplatin, or gefitinib for 4 hours and assessed for intracellular ROS with CM-H2DCFDA. (n=3) **B**) KPC cells were pre-treated with 10μM paclitaxel, cisplatin, or gefitinib for 36 hours and incubated with 1μM gemcitabine for 1 hour. Intracellular dFdCTP was measured (n=3). **C**) Protein lysates were generated from KPC cells treated for 36 hours with 10μM paclitaxel or cisplatin and immunoblotted for indicated proteins. **D**) Protein lysates were generated from KPC cells treated for 1 hour with 10μM gefitinib and immunoblotted for indicated proteins.