

# Co-treatment with gemcitabine and nab-paclitaxel exerts additive effects on pancreatic cancer cell death

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**Abstract.** Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive cancer and current treatments exert small effects on life expectancy. The most common adjuvant treatment for PDAC is gemcitabine. However, relapse almost invariably occurs and most patients develop metastatic, incurable disease. The aim of the present study was to assess the activity of nanoparticle albumin-bound paclitaxel (nab-paclitaxel) alone or in combination with gemcitabine in PDAC cell lines displaying different degrees of sensitivity to gemcitabine treatment. We evaluated the effects of gemcitabine and nab-paclitaxel and their combination on cell proliferation, death, apoptosis and cell cycle distribution in PDAC cell lines either sensitive to gemcitabine, or with primary or secondary resistance to gemcitabine. Our results indicated that the dose-response of PDAC cell lines to nab-paclitaxel was similar, regardless of their sensitivity to gemcitabine. In addition, nab-paclitaxel elicited similar cytotoxic effects on a PDAC cell line highly resistant to gemcitabine that was selected after prolonged exposure to the drug. Notably, we found that combined treatment with gemcitabine and nab-paclitaxel exerted additive effects on cell death, even at lower doses of the drugs. The combined treatment caused an increase in cell death by apoptosis and in cell cycle blockage in S phase, as assessed by flow cytometry and western blot analysis of the PARP-1 cleavage. These results revealed that a combined treatment with nab-paclitaxel may overcome resistance to gemcitabine and may represent a valuable therapeutic approach for PDAC.

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related mortality (1) and it is estimated to become the second by 2030 (2). Less than 20% of PDAC patients are eligible for surgical resection (3) and, since chemotherapy and radiotherapy only marginally improve survival (4), the 5-year survival rate for patients is approximately 5% (1).

Since its approval by the FDA in 1996, the standard treatment for advanced PDAC in the past two decades has been chemotherapy with gemcitabine, a nucleoside analogue of deoxycytidine that has been also extensively used for the treatment of other solid tumors. Gemcitabine, however, offers only a small improvement of survival to patients with advanced PDAC compared to 5-fluorouracil (5-FU) (5). The relative lack of response to gemcitabine treatments is attributed to mechanisms of either primary or acquired resistance, many of which have been investigated extensively (6). For instance, resistance to gemcitabine can be acquired through mechanisms related to its transport, cellular uptake and metabolism within tumor cells. Furthermore, the activation of pro-survival signaling pathways and the expression of specific microRNAs can also influence the response to this drug (6). Recently, we have highlighted the impact of alternative splicing on both short-term and long-term resistance to gemcitabine. Upon brief exposure to the drug, upregulation of the oncogenic splicing factor SRSF1 induces splicing of the MNK2b protein kinase variant and phosphorylation of the translation factor eIF4E, which promote PDAC cell survival under genotoxic stress (7). Conversely, selection of gemcitabine-resistant PDAC clones after chronic exposure to the drug, correlated with increased expression of the polypyrimidine-tract binding protein (PTBPI) and alternative splicing of the pyruvate kinase gene (PKM) resulting in the promotion of the PKM2 isoform (8). The expression of PKM2 was required for the maintenance of gemcitabine-resistance in PDAC cell lines and correlated with worse recurrence-free survival in operated patients treated with adjuvant gemcitabine (8).

Recently, the standard treatment for patients with advanced PDAC has improved due to the positive results of trials with the

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combination of fluorouracil-leucovorin-irinotecan-oxaliplatin (FOLFIRINOX) (9) and the addition of nanoparticle albumin-bound paclitaxel (nab-paclitaxel) to gemcitabine (10,11). These combined regimens are now considered the standard care for patients with advanced PDAC. However, toxicity limits FOLFIRINOX use to patients with a good performance status, while the combination of gemcitabine and nab-paclitaxel is usually more tolerable. Single-agent gemcitabine therapy is still an acceptable treatment in patients with advanced disease and reduced performance status, as well as in the adjuvant setting after surgical resection (12).

Nab-paclitaxel (Abraxane®; Celgene Inc., Odenton, MD, USA) is a specific formulation of paclitaxel that was developed to improve its solubility and to overcome resistance due to the desmoplastic stroma surrounding PDAC cells (13). Paclitaxel is a taxane and acts by reversibly binding to tubulin, causing defects in mitotic functions that lead to blockage of the cell cycle and eventually to apoptosis, with mechanisms that differ from those of gemcitabine. While the clinical use of nab-paclitaxel and gemcitabine has been investigated extensively (14), the available data on the activity of nab-paclitaxel as a single-agent therapy in PDAC both in clinical trials and preclinical models are poor. Therefore, the aim of the present study was to assess the activity of nab-paclitaxel alone or in combination with gemcitabine in PDAC cell lines displaying different degrees of sensitivity to gemcitabine treatment.

## Materials and methods

**Cell cultures and drugs.** All cell lines were obtained from the Centre for Molecular Oncology, Barts Cancer Institute (London, UK) in 2004 and authenticated in 2012. The HPAF-II, Pt45P1, PANC-1 and PANC-1 DR cell lines were cultured in RPMI-1640 (Lonza, Basel, Switzerland) and MiaPaCa-2 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza). All media were supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), gentamycin, penicillin, streptomycin and non-essential amino acids and the cells were maintained at 37°C with 5% CO<sub>2</sub>.

Nab-paclitaxel (Abraxane®; kindly provided by Celgene Inc.) was dissolved in physiological solution. Gemcitabine (Eli Lilly and Company, Clinton, IN, USA) was dissolved in water. The cells were plated at 50% confluency. Twenty-four hours after plating, the cells were treated with nab-paclitaxel and/or gemcitabine at the indicated concentrations for 24, 48 and 72 h before being collected for further analyses.

**Cell viability assays.** The cells were plated at 50% confluency in 96 wells and, after 24 h, treated with nab-paclitaxel at the concentrations indicated in Fig. 1. After 72 h of treatment, the cell viability was evaluated by MTS assay (Promega, Madison, WI, USA) following the manufacturer's instructions and by assessing the optical density (OD) at 490 nm. The results are represented as the mean  $\pm$  standard deviation (SD) of three experiments.

For cell death, the cells were plated at 70% confluency and, after 24 h, treated with gemcitabine and/or nab-paclitaxel at the indicated doses. After an additional 48 h, the cells were washed in phosphate-buffered saline (PBS), trypsinized and incubated with 0.4% Trypan Blue stain (Sigma-Aldrich, St. Louis, MO, USA). Blue positive cells were then counted

using the Countess II Automated Cell Counter (Invitrogen Life Technologies, Carlsbad, CA, USA) and the percentage of cell death was determined. The results are represented as the mean  $\pm$  SD of three experiments.

**BrdU-PI staining and cell cycle analysis.** For the cell cycle analysis, the cells were treated with 10  $\mu$ M BrdU (Sigma-Aldrich) in the final 30 min of treatments. Subsequently, the cells were trypsinized, washed in chilled PBS and resuspended in PBS/ethanol 70%. The samples were incubated at -20°C until use. The cells were then centrifuged at 2,000 rpm for 5 min, washed with PBS and incubated with 2 N HCl/0.5% Triton X-100 at room temperature (RT) for 30 min. The cells were centrifuged at 2,000 rpm for 5 min and then resuspended with 0.1 M NaB<sub>4</sub>O. After incubation for 2 min at RT, the cells were washed with PBS/1% BSA and incubated for 1 h at RT in a solution of 0.5% Tween-20/1% BSA in PBS containing 10  $\mu$ l of anti-BrdU 1 mM (Becton-Dickinson and Company, Franklin Lakes, NJ, USA). Subsequently, the cells were washed with PBS/1% BSA and incubated in a solution of PBS/0.5% Tween-20/1% BSA containing 5  $\mu$ l of Alexa Flour 488 anti-mouse IgG-FITC (polyclonal; cat. no. A-11001; Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at RT. The cells were washed with PBS/1% BSA and incubated with PBS containing 1 mg/ml RNase A (Roche, Basel, Switzerland) and 20  $\mu$ g/ml propidium iodide (PI; Sigma-Aldrich) for 30 min at 37°C. Subsequently the cells stained with BrdU-PI were analyzed by FACS.

**Cell extracts and western blot analysis.** MiaPaCa-2 cells were resuspended in lysis buffer (50 mM HEPES pH 7.4, 10% glycerol, 15 mM MgCl<sub>2</sub>, 150 mM NaCl; 15 mM EGTA; 20 mM  $\beta$ -glycerophosphate; 1 mM dithiothreitol, 0.5 mM NaVO<sub>4</sub>, 1 mM NaF and protease inhibitor cocktail) supplemented with 1% Triton X-100, sonicated for 5 sec and centrifuged for 10 min at 13,000 rpm at 4°C. Supernatants were collected, diluted in sodium dodecyl sulphate (SDS) sample buffer and boiled for 5 min. The proteins were separated on 8 or 12% SDS-PAGE gel and transferred onto PVDF blotting membranes (Amersham Hybond; GE Healthcare, Little Chalfont, UK). The membranes were saturated in 5% non-fat dry milk in PBS plus 0.1% Tween-20 for 1 h at RT and incubated overnight at 4°C with the following primary antibodies: Rabbit anti-PARP1 (1:500; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse anti-actin (1:1,000; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-cyclin E2 (1:1,000; Cell Signaling Technology), rabbit anti-cyclin A2 (1:1,000), rabbit anti-cyclin B1 (1:1,000), mouse anti-cyclin D1 (1,000; cat. no. A-12) (all from Santa Cruz Biotechnology).

## Results

**Nab-paclitaxel exerts cytotoxic effects in PDAC cells displaying different primary sensitivity to gemcitabine.** In order to assess the efficacy of nab-paclitaxel on cell proliferation and viability, we analyzed the dose-response to nab-paclitaxel of the PDAC cells displaying different sensitivity to gemcitabine, with the MiaPaCa-2 and Panc-1 cells demonstrating the highest resistance to gemcitabine, thus offering *in vitro* models of primary resistance to this drug (7,8,15).

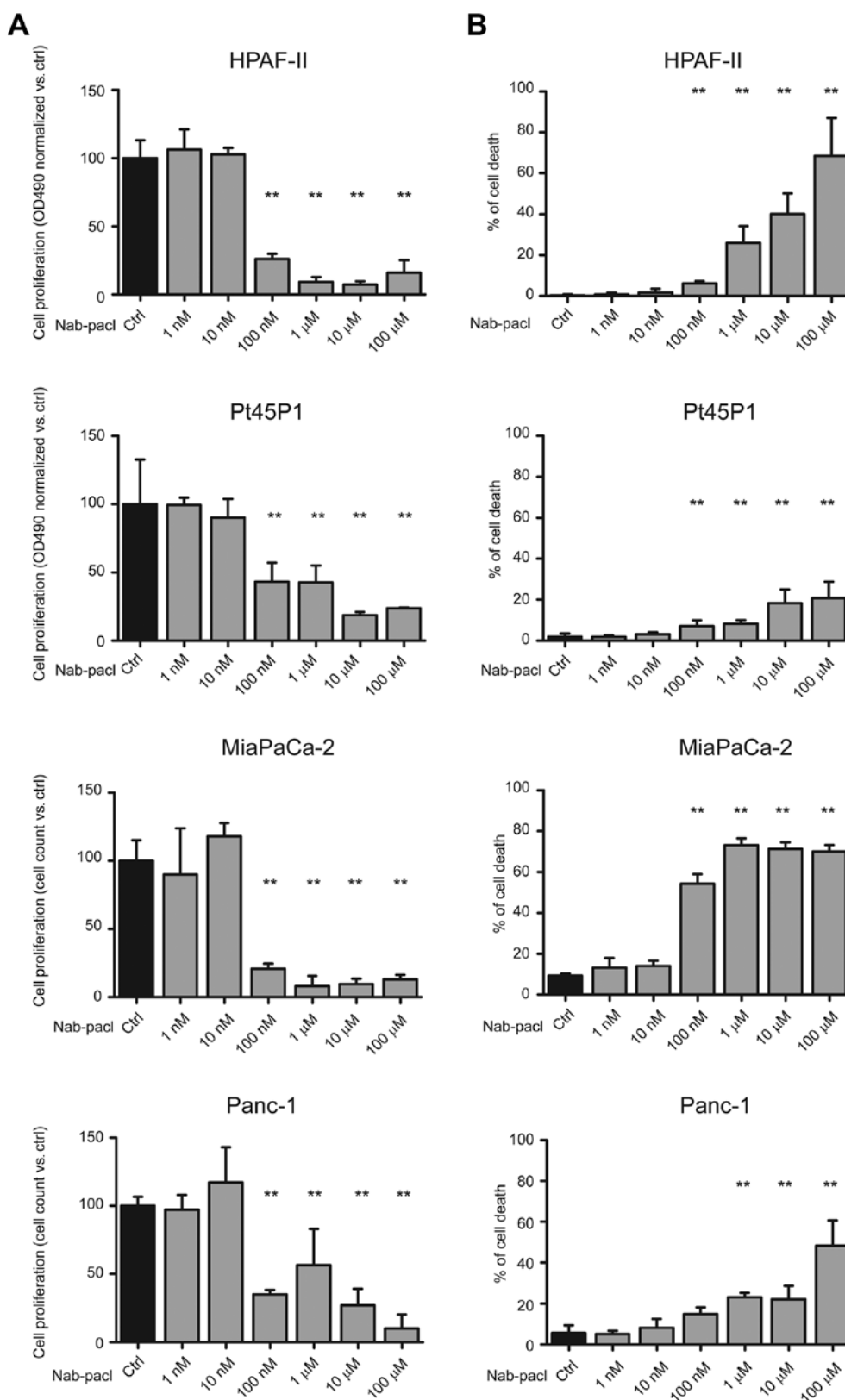


Figure 1. Nab-paclitaxel exerts a similar cytotoxic effect in PDAC cells with different sensitivity to gemcitabine. Histograms display the analysis of (A) cell proliferation performed by an MTS assay (HPAF-II and PT45P1) or by cell count (MiaPaCa-2 and Panc-1) and (B) cell death performed by Trypan blue after 72 h of treatment with the indicated doses of nab-paclitaxel. The results represent the mean  $\pm$  SD of three experiments. Significance vs. control, was determined by Student's t-test: \*\*P<0.01.

Nab-paclitaxel induced a significant reduction of cell proliferation (60-65%) starting from the dose of 100 nM compared to controls in all PDAC cells (Fig. 1A). Furthermore, at this

dose, nab-paclitaxel induced a significant increase of cell death in all cell lines with the exception of Panc-1 cells (Fig. 1B). Notably, the increase of cell death at 100 nM was modest in

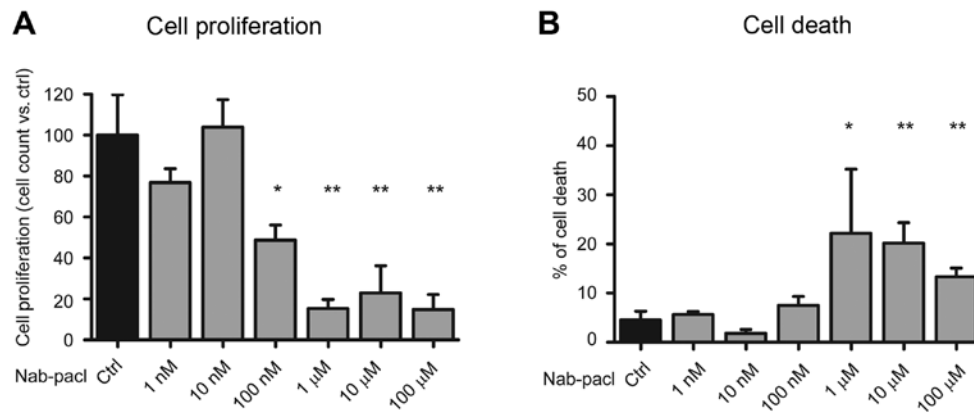


Figure 2. Nab-paclitaxel exerts cytotoxic effect in PANC-1 DR cell line with secondary resistance to gemcitabine. Histograms reveal the analysis of (A) cell proliferation performed by cell count and (B) cell death performed by Trypan blue cell count after 72 h of treatment with the indicated doses of nab-paclitaxel. The results represent the mean  $\pm$  SD of three experiments. Significance vs. control was determined by Student's t-test: \* $P<0.05$  and \*\* $P<0.01$ .

HPAF-II (6%) and Pt45P1 (7%) cells, whereas it was very high in MiaPaCa-2 cells (54%) (Fig. 1B), which displayed higher resistance to gemcitabine (15). Conversely, nab-paclitaxel significantly reduced Panc-1 cell proliferation at this dose without inducing cell death, whereas cell viability was affected only at micromolar doses of the drug (Fig. 1A and B). At these higher doses (1-100  $\mu$ M), nab-paclitaxel led to substantial induction of cell death in the HPAF-II, MiaPaCa-2 and Panc-1 cell lines, while cell death remained at 20% in Pt45P1 even at the highest dose (Fig. 1B).

These results revealed that, regardless of their sensitivity to gemcitabine, the PDAC cells demonstrated similar sensitivity to nab-paclitaxel in terms of inhibition of cell proliferation, however, different response in terms of cell death.

*Nab-paclitaxel exerts a cytotoxic effect in PDAC cells with secondary gemcitabine resistance.* As aforementioned we selected PDAC cells which acquired resistance to gemcitabine after chronic exposure to the drug (8). Notably, these cells were also more resistant to cisplatin (8), another drug exerting genotoxic stress. To examine whether these drug-resistant (DR) cells were still sensitive to nab-paclitaxel, a dose-response study was performed. We found that DR-Panc-1 cells maintained the same sensitivity to nab-paclitaxel as the parental cell line, with significant inhibition of cell proliferation starting at the dose of 100 nM, while cell death increased significantly at the dose of 1  $\mu$ M (Fig. 2A and B). These results confirmed that nab-paclitaxel sensitivity did not correlate with gemcitabine sensitivity and suggested that nab-paclitaxel may overcome acquired resistance to gemcitabine in PDAC cells.

*Combined treatment with Nab-paclitaxel and gemcitabine exerts additive effects on the inhibition of cell proliferation.* In order to understand whether nab-paclitaxel in combination with gemcitabine enhances the cytostatic and cytotoxic effects of the chemotherapeutic treatment, we tested their combined action on cell proliferation and death in MiaPaCa-2 cells, a cell line demonstrating relatively high resistance to gemcitabine (15). The combination of gemcitabine (100 nM) and nab-paclitaxel (10 nM) exerted a significant increase in cell death compared to gemcitabine alone (56 vs. 37%) (Fig. 3A). Notably, the effect of the combined treatment was similar to

that exerted by gemcitabine alone at a dose 100 times higher (i.e. 10  $\mu$ M; Fig. 3A). The combination of gemcitabine (30 nM) and nab-paclitaxel (10 nM) elicited a significant additive effect even when used at a suboptimal dosage (Fig. 3B). Furthermore, similar effects were also obtained with the highly resistant DR-Panc-1 cells, albeit at considerably higher doses (Fig. 3C). These data indicated that combined treatment with nab-paclitaxel and gemcitabine enhanced the cytotoxic effects of both drugs, allowing to lower their doses, thus possibly limiting adverse effects.

*Combination of suboptimal doses of nab-paclitaxel and gemcitabine induces a significant increase in apoptosis.* To investigate the nature of the additive effect of gemcitabine and nab-paclitaxel on PDAC cell death, we analyzed cell cycle progression and cell death in more detail in MiaPaCa-2 cells. Flow cytometry analysis with propidium iodide (PI) of cells treated with suboptimal doses of gemcitabine (30 nM) and nab-paclitaxel (10 nM) for 48 h indicated that gemcitabine strongly affected the cell cycle progression, leading to cell accumulation in S phase, whereas nab-paclitaxel elicited very mild effects. Notably, however, the addition of nab-paclitaxel to gemcitabine led to the appearance of a defined peak in the sub-G1 population of MiaPaCa-2 cells (Fig. 4A), indicating cell death by apoptosis. To confirm the effect of the combined treatment on cell apoptosis, we monitored cleavage of poly(ADP-ribose) polymerase (PARP1) by western blot analysis. Consistent with the appearance of the sub-G1 peak, PARP1 cleavage was noticeably increased in MiaPaCa-2 cells treated with the combination of the two drugs (Fig. 4B).

*Addition of nab-paclitaxel to gemcitabine induces a stronger cell cycle blockage in S phase.* The PI profile indicated that co-treatment with nab-paclitaxel enhanced the accumulation of cells in the S phase of the cycle compared to gemcitabine alone (Fig. 4A). To further investigate this possibility, we analyzed the incorporation of BrdU as a precise marker of DNA duplication in S phase. A short pulse of BrdU was administered to MiaPaCa-2 cells 30 min before harvesting, following 24 h of incubation with the drugs. We observed that both gemcitabine and nab-paclitaxel, used as single agents, caused an accumulation of cells in the S phase (from 45,93

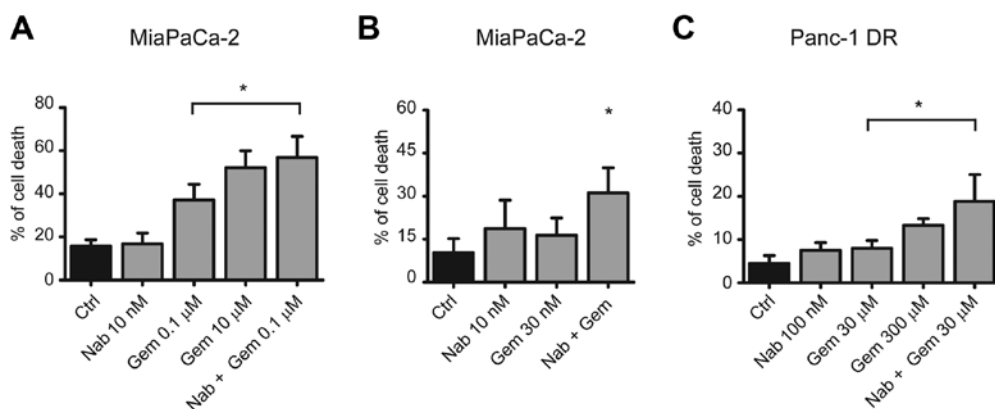


Figure 3. Combined treatment of gemcitabine and nab-paclitaxel exert an additive effect on the inhibition of cell proliferation. Histograms display the analysis of cell death performed by Trypan blue on MiaPaCa-2 cells after (A) 72 h of treatments with the indicated doses of nab-paclitaxel and gemcitabine. Significance vs. control, was determined by Student's t-test: \* $P < 0.05$ . (B) Analysis of cell death after 48 h of treatments with the indicated suboptimal doses of nab-paclitaxel and gemcitabine. Statistical analysis was performed by one-way analysis of variance by ANOVA test followed by Tukey's Multiple Comparison post-test (\* $P < 0.05$ ). (C) Analysis of cell death performed by Trypan blue on Panc-1 DR cells after 72 h of treatments with the indicated doses of nab-paclitaxel and gemcitabine. Significance vs. control, was determined by Student's t-test: \* $P < 0.05$ . All results represent the mean  $\pm$  SD of three experiments.

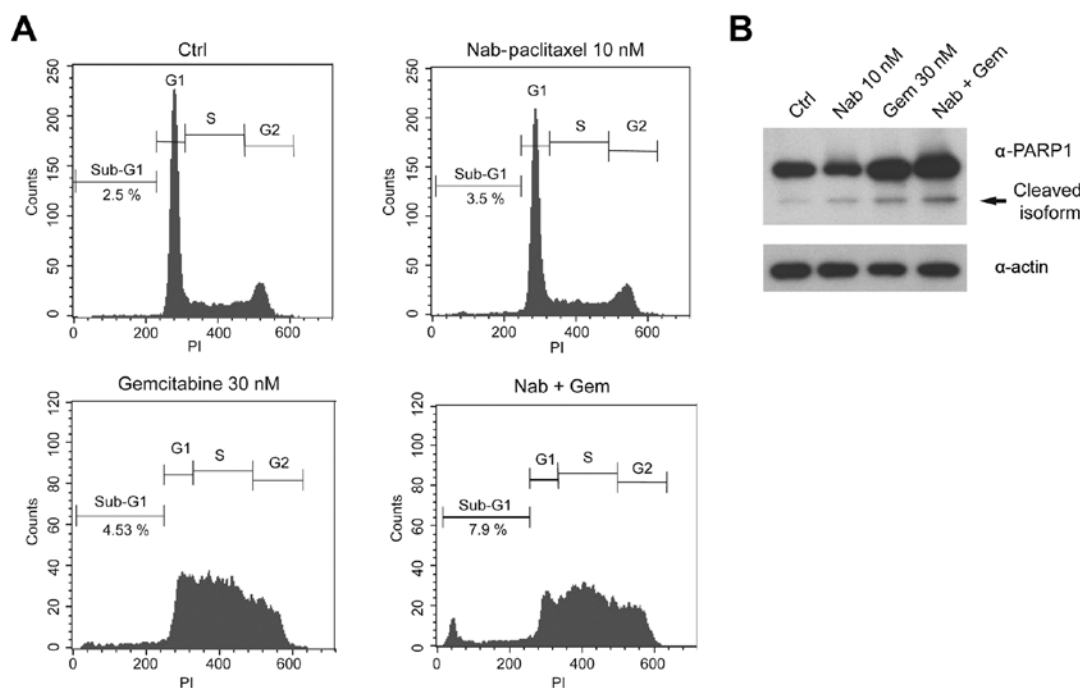


Figure 4. Combination of suboptimal dose of nab-paclitaxel and gemcitabine induces a significant increase of cell death. (A) Panels display the percentage of cells stained for propidium iodide (PI) in Sub-G1, G1, S and G2 phases in MiaPaCa-2 cells treated with nab-paclitaxel, gemcitabine or both drugs for 48 h. (B) Western blot analysis of cleaved protein PARP-1 in MiaPaCa-2 cells treated with nab-paclitaxel, gemcitabine or both drugs for 48 h.

to 78,30 and 74,37%) (Fig. 5A). Notably, the combination of both drugs resulted in an additive effect on the accumulation of cells in S phase, which reached 84.84%. As a consequence of this blockage in cell cycle progression, co-treatment with gemcitabine and nab-paclitaxel resulted in a sharp reduction of cells transiting in the G2 phase (Fig. 5A).

In addition, we checked the changes in cell cycle progression by monitoring the expression levels of phase-specific cyclins. As displayed in Fig. 5B, cyclin D1 levels were not affected by treatments, whereas cyclin A2 and E2 levels increased after gemcitabine administration either alone or in combination with nab-Paclitaxel, confirming that the cells are mainly

blocked in the S phase. Treatment with nab-paclitaxel alone did not cause accumulation of S phase cyclins (Fig. 5B), even though the cells were blocked at this stage of the cycle. Since we noticed that nab-paclitaxel caused accumulation of cells in the left-most region of S phase (Fig. 5A), indicating very little duplication of DNA, it is probable that this drug blocks cells before the accumulation of cyclins E2 and A2. Additionally, we observed that the combined treatment with both drugs reduced the expression of cyclin B1 compared to gemcitabine alone. Since this cyclin is involved in the S-G2 cell cycle transition, its levels reflect the reduction of cells in G2 phase, which was observed in flow cytometric analyses (Figs. 4A and 5A).

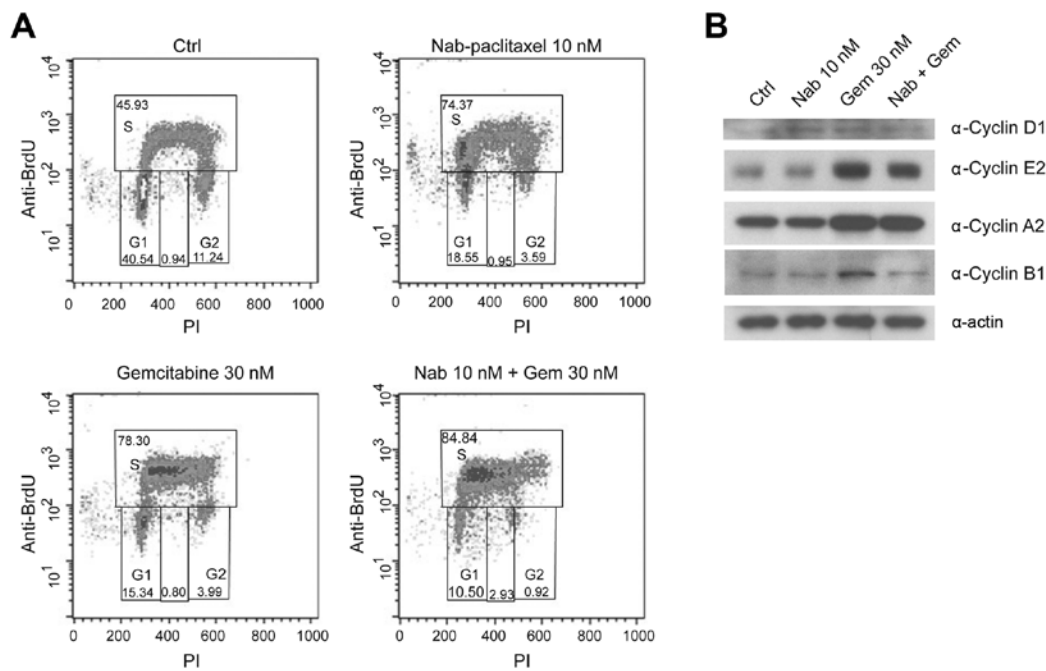


Figure 5. Combination of suboptimal dose of nab-paclitaxel and gemcitabine induces a significant increase of cell death. (A) Panels display the percentage of cells stained for propidium iodide (PI) and anti-BrdU FITC antibody. They are detected in G1, S and G2 phases in MiaPaCa-2 cells treated with nab-paclitaxel, gemcitabine or both drugs for 24 h. (B) Western blot analysis of cyclin D1, A, E and B1 in MiaPaCa-2 cells treated with nab-paclitaxel, gemcitabine or both drugs for 24 h.

## Discussion

The aim of the present study was to examine the activity of nab-paclitaxel alone or in combination with gemcitabine in PDAC cell lines displaying different degree of primary resistance to gemcitabine and in a previously described model of secondary resistance to the drug (7,8).

The results of the present study revealed that nab-paclitaxel is effective in PDAC cells irrespective of their sensitivity to gemcitabine and to the status of primary or secondary (acquired) resistance (Figs. 1 and 2). Notably, both drugs demonstrated an additive effect at suboptimal doses in cell lines with primary or secondary resistance to gemcitabine (Fig. 3).

To investigate the underlying mechanisms of the observed efficacy of nab-paclitaxel, we explored the changes occurring in the cell cycle (Figs. 4 and 5). Our results indicated that nab-paclitaxel blocked cell proliferation in a different manner compared to gemcitabine. Although both drugs caused an arrest in S phase, the cells treated with gemcitabine exhibited a different extent of DNA duplication, whereas the peak of cells treated with nab-paclitaxel is present in the left region of the graph, indicating that cells arrest as soon as they start duplicating their DNA. This difference is also illustrated by the accumulation of S phase cyclins, which is evident in gemcitabine- but not in nab-paclitaxel-treated cells. While the blockage in S phase is expected after gemcitabine exposure, due to depletion of the nucleotide pool required for DNA duplication, cells treated with nab-paclitaxel were expected to arrest in mitosis or late G2 phase due to defects in spindle elongation. However, recent data have revealed that cells treated with paclitaxel often proceeded through mitosis into the next interphase, where the majority of cell deaths occurred (16). In

particular, nab-paclitaxel seemed to interfere with the very early stages of the S phase in PDAC cells (Fig. 5A). This may explain why it was previously found that the interference with the DNA replication origin activity enhanced the response of cells to paclitaxel (16).

The different mechanism of S phase blockage by the two drugs may explain the additive effect observed in the combined treatment. Markedly, such additive effect was observed both at the cell cycle and the cell death level, indicating a causal relationship between the two events. Although the molecular mechanisms involved in such effect need further investigation, our results indicated that nab-paclitaxel strongly enhances the cytotoxicity of gemcitabine and may help to overcome both primary and acquired resistance to this drug.

The *in vitro* results of the present study revealed that nab-paclitaxel, alone or in combination with gemcitabine, is an active drug in preclinical models of gemcitabine-resistant PDAC. Hence, our observations indicated that, in certain clinical scenarios, nab-paclitaxel could be active in patients with PDAC that are not responding to gemcitabine, even in monotherapy. However, clinical data on the use of nab-paclitaxel as a single agent in patients with PDAC that were previously treated with gemcitabine, are limited. In a small phase II trial, 19 patients received nab-paclitaxel after progression under gemcitabine-based therapy (17). One of them (5.3%) had a confirmed partial response and 6 (32%) exhibited stable disease as the best response. Another single-center retrospective study evaluated the use of nab-paclitaxel in 20 patients with advanced PDAC who previously exhibited progression under gemcitabine, 40% of whom also received FOLFIRINOX. Notably, about two thirds of patients had stable disease as best response, although the median OS was

only of 5.2 months (18). Further studies are needed to elucidate whether this approach may be beneficial, possibly in patients with less advanced disease.

The present study is one of the few that aimed to evaluate the efficacy of nab-paclitaxel in preclinical settings, using cell lines that are models of both primary and secondary resistance to gemcitabine. As the investigation is limited to *in vitro* models, the results should be interpreted with caution and the mechanisms of the activity observed in the present study need further experiments to be elucidated. In particular, our data are useful to generate hypotheses that need to be confirmed in other models, such as animal ones, that may better recapitulate the human pathology. Conversely, the additive effect of nab-paclitaxel and gemcitabine observed in these experiments cannot be due to factors that have been extensively investigated in animal models and that are related with tumor stroma and penetration of drugs. Another limitation of the present study concerns the lack of a defined mechanism for the observed effects. Following the revision process, we tested some common signal transduction pathways that could be involved in the response to these chemotherapeutic agents, such as the PI3K-mTOR and ERK pathways (data not shown). However, we did not find a direct correlation with the cytotoxic effect. Thus, further studies are needed to analyze the mechanisms underlying the observed effects. Considering the above-mentioned limitations, our results revealed that treatment with nab-paclitaxel may overcome resistance to gemcitabine and may represent a potentially valuable therapeutic approach for advanced PDAC.

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### Competing interests

The authors declare that they have no competing interests.

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