

## Original Article

# PAK inhibition by PF-3758309 enhanced the sensitivity of multiple chemotherapeutic reagents in patient-derived pancreatic cancer cell lines

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**Abstract:** Background/objective: Pancreatic ductal adenocarcinoma (PDA) remains the most lethal malignancy due to lack of an effective treatment. P21-activated kinases (PAKs) play a key role not only in cell proliferation and migration, but also in mediating chemo-resistance in PDA. The aim of this study was to investigate the combined effect of a PAK inhibitor PF-3758309 with multiple chemotherapeutic reagents on a panel of patient-derived PDA cell lines, and potential mechanisms involved. Methods: Cells were treated with PF-3758309 plus or minus gemcitabine, 5-fluorouracil (5-FU) or abraxane, and cell growth was determined using a cell proliferation assay kit. Protein expression profiles were measured by Western blot. PDA cells were subcutaneously injected into the flanks of SCID mice which were then treated with saline, gemcitabine, PF-3758309, gemcitabine plus PF-3758309 or abraxane. Tumour growth was measured by volume and weight. Results: PAK1 was correlated with CK19 expression, and PAK4 with  $\alpha$ -SMA and palladin expression. Combination of PF-3758309 with 5-FU, gemcitabine or abraxane further suppressed cell growth of patient-derived PDA cell lines *in vitro*. The combination of PF-3758309 with gemcitabine maximally inhibited tumour growth *in vivo* by suppressing cell proliferation. PF-3758309 inhibited the expression of HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA both *in vitro* and *in vivo*. Conclusions: PAK inhibitor PF-3758309 can enhance anti-tumour effects of multiple chemotherapeutic reagents on a panel of patient-derived PDA cell lines. Combination of PF-3758309 with gemcitabine achieves comparable efficacy to combination of gemcitabine with abraxane, and thus provides a potential targeted therapy in the management of PDA.

**Keywords:** p21-activated kinase, PF-3758309, chemotherapy, gemcitabine, pancreatic cancer

## Introduction

The low survival rate of pancreatic ductal adenocarcinoma (PDA) is largely caused by resistance to chemotherapy. Due to lack of effective early diagnosis of PDA, only 20% of patients are suitable for surgical resection, which makes chemotherapy a critical component in the clinical management of PDA. Gemcitabine has been the mainstay of systemic treatment for most stages of PDA. With the discovery of active multi-agent chemotherapeutic regimens, such as FOLFIRINOX (a combination of leucovorin (folinic acid), fluorouracil (5-FU), irinotecan and oxaliplatin) and gemcitabine plus nab-paclitax-

el (abraxane), the treatment landscape of PDA is slowly evolving. FOLFIRINOX and gemcitabine plus abraxane are now utilized as standard first line treatment options in local advanced and metastatic PDA [1]. FOLFIRINOX treatment offers a median survival of 11.1 months in patients with advanced disease [2], while gemcitabine plus abraxane treatment provides a median survival of 8.5 months [3]. Compared to gemcitabine monotherapy, the survival rate of PDA patients has been marginally improved with the combination treatment.

The unique desmoplastic stroma in PDA forms a barrier of fibrotic tissue which prevents the

penetration of chemotherapeutic reagents and thus compromises the efficacy [4]. The stroma contains a large number of pancreatic stellate cells (PSCs), which are characterized by the expression of desmin, glial fibrillary acidic protein (GFAP), vimentin and nestin [5]. PSCs are activated by PDA cells, which is characterized by absence of vitamin A droplets in the cytoplasm and presence of myofibroblast phenotype with increased expression of alpha-smooth muscle actin ( $\alpha$ -SMA). The interaction between PDA cells and PSCs plays an important role in tumour growth, metastasis and chemo-resistance. Palladin, an actin binding protein, has been reported to be expressed in both PDA and stroma cells. The stromal expression of palladin is an independent prognostic factor for predicting PDA patient's survival and a surrogate indicator for the effectiveness of chemoradiation therapy [6].

P21-activated kinases (PAKs) are critical downstream effectors of mutated Kras signalling, and contribute to initiation and progression of PDA. The six known members of the PAK family can be categorized by similarities in their sequence and structure into two groups: group I (PAK1-3) and group II (PAK4-6). Over-expression of PAK1 [7] and PAK4 [8] has been reported in PDA patients, and both PAK1 and PAK4 are involved in the proliferation and migration/invasion of PDA cells [9]. Inhibition of PAK1 synergistically enhanced the inhibitory effects of gemcitabine on the growth of PDA cells both *in vitro* and *in vivo* [10], and activation of PAK1 contributed to gemcitabine resistance of PDA cells [11]. Furthermore, inhibition of PAK1 also suppressed the activation and proliferation of PSCs while promoting the apoptosis, and deletion of stromal PAK1 increased the survival of mice with orthotopic PDA [12]. PAK4 promoted the proliferation and survival of PDA cells by activation of NF- $\kappa$ B pathway [13], and inhibition of PAK4 increased the sensitivity of PDA cells to gemcitabine [14]. In addition, inhibition of PAK4 also decreased PDA cell proliferation via down-regulation of Bad phosphorylation and up-regulation of tumour suppressive miRNA [15]. A novel PAK4 allosteric inhibitor suppressed PDA cell proliferation on its own or synergistically with gemcitabine and oxaliplatin while promoting the apoptosis of PDA cells [16]. These important roles of PAKs in PDA and stromal cells make PAKs emerge as promising targets in PDA treatment.

The affinity of PF-3758309, a pan-PAK inhibitor, is highest for PAK4 ( $IC_{50}$  2.7-4.5 nM), and second highest for PAK1 ( $IC_{50}$  14 nM) [17]. PF-3758309 suppressed the proliferation and migration/invasion of melanoma and lung cancer by targeting both PAK1 [18] and PAK4 [19]. PF-3758309 inhibited the growth of colorectal cancer cells both *in vitro* and *in vivo* by decreasing the activity of PAK1, and enhanced the inhibitory effect of 5-FU on the proliferation and tumourigenesis of colorectal cancer cells [20]. The aims of this study were to determine the effects of PF-3758309, in combination with multiple chemotherapeutic reagents, on a panel of patient-derived PDA cell lines, and to investigate the possible mechanisms involved.

## Methods

### Cells and reagents

The patient-derived cell lines (TKCC 2.1, TKCC15, TKCC18, TKCC22, TKCC23, TKCC26) used in this study were isolated at The Kinghorn Cancer Centre (TKCC) from primary patient-derived pancreatic ductal adenocarcinoma xenografts [21] and cultured under hypoxia (5%  $O_2$ ) as described previously [22]. Cells were tested regularly for mycoplasma contamination and were not passaged more than 30 times after resuscitation.

Gemcitabine and 5-fluorouracil were purchased from Sigma-Aldrich (Melbourne, Australia), PF-3758309 from Active Biochemical Co. (Maplewood, NJ), and Nab-paclitaxel (Abraxane) from Abraxis Bioscience Australia Pty Ltd (East Kew, Victoria, Australia).

### Cell proliferation

Cells ( $5 \times 10^3$  cells/well) were seeded in the 96-well plate, and were incubated with different concentrations of PF-3758309 for 48 h or pre-treated with PF-3758309 at the  $IC_{50}$  calculated for proliferation (**Table 1**) for 48 h, followed by treatment with gemcitabine, 5-FU or Abraxane for another 48 h in the absence of PF-3758309. Cell proliferation was measured using a CyQUANT Direct Cell Proliferation Assay Kit (Thermo Fisher, Melbourne, Australia) according to the manufacturer's instruction. Fluorescence intensity was determined using a plate reader (FLUOstar OPTIMA, BMG Labtech, Melbourne, Australia).

**Table 1.** IC<sub>50</sub> values for a panel of patient-derived PDA cell lines

	PF-3758309 (nM)	5-FU ( $\mu$ M)	Gemcitabine (nM)	Abraxane (nM)
TKCC 15	5	10	10	10
TKCC 18	5	5	20	25
TKCC 2.1	400	5	5	15
TKCC 22	400	100	60	40 ( $\mu$ M)
TKCC 23	200	150	1300 ( $\mu$ M)	70
TKCC 26	5	150	45	10 ( $\mu$ M)

#### Western blot

Cells were lysed in SDS sample buffer. Proteins extracts were obtained from tumour tissues by homogenising in 1% Triton X100 lysis buffer (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100 mM NaF, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin and 1 mM PMSF). All the samples were subjected to SDS-PAGE for immunoblotting and were detected with antibodies against GAPDH, total and phospho-PAK1 and PAK4 (Cell Signaling Technology, Arundel, Australia), cytokeratin 19 (CK19), alpha-smooth muscle actin ( $\alpha$ -SMA), palladin (Abcam, Cambridge, MA) and hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ , BD Biosciences, North Ryde, Australia). Bound antibodies were visualized using ECL reagents (GE Healthcare, Amersham, UK), and the density of each band was analysed using Multigauge computer software (Berthold, Bundoora, Australia). The relative amount of each protein was calculated as the ratio of the density of its band to the density of the GAPDH band.

#### Mouse study

All mouse experiments were approved by the Austin Health Animal Ethics Committee (A20-15/05269). SCID mice were purchased from the Animal Resource Centre (Perth, Australia). TKCC 15 cells ( $3 \times 10^6$  cells/100  $\mu$ l/site) were injected subcutaneously into the opposite flanks of 8-week old SCID mice. On day 25 after tumour induction, the mice were randomly divided into 5 groups (3 mice/group): control (saline), gemcitabine alone (40 mg/kg), PF-3758309 alone (25 mg/kg), gemcitabine plus abraxane (10 mg/kg), and gemcitabine plus PF-3758309. The mice were treated by intraperitoneal injection (i.p.) as shown in **Figure 5A** for four weeks. Tumour dimensions were mea-

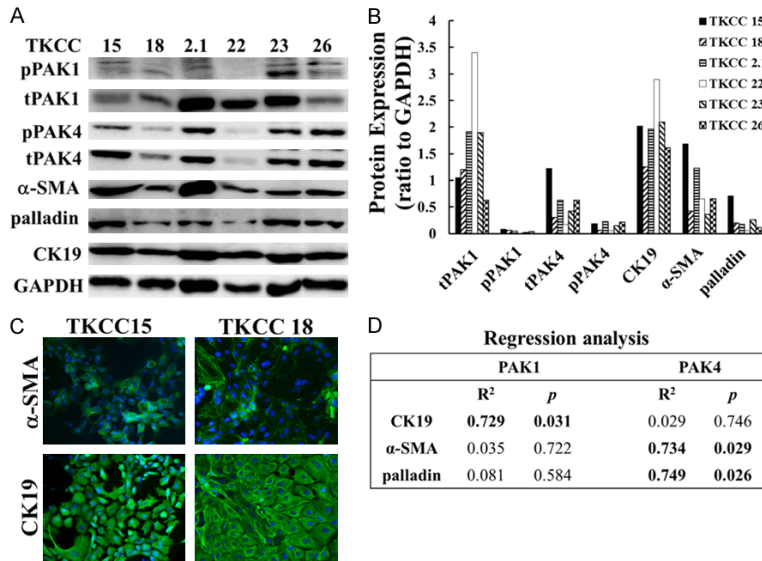
sured twice/week using a caliper, and volumes were calculated using the formula  $w^2 \cdot L/2$ , where w and L are the shortest and longest tumour diameters, respectively. The tumour volumes on day 25 in each group were taken as 1. Mice were euthanized on day 53, and tumour tissues were weighed and collected for paraffin-embedding for the following immuno-staining.

#### Immunohistochemistry

Tumour tissues, collected from the animal model as described above, were cut into 4- $\mu$ m thick sections. Antigens were retrieved by treating sections in boiling citrate buffer (10 mM, citric acid, pH6, Sigma-Aldrich) for 30 min. Sample sections were incubated with hydrogen peroxidase for 15 min and 5% normal goat serum for 30 min at room temperature for endogenous peroxidase quenching and protein blocking, respectively. After incubation with Ki67 antibody (ThermoFisher Scientific, Australia) for proliferation, cleaved caspase 3 antibody (Cell Signaling Technology, Arundel, Australia) for apoptosis, sections were visualized by using an EnVision System kit (Dako, Botany, Australia) and were counterstained with hematoxylin. Samples were imaged using a Leica microscope for at least 10 fields at  $\times 20$  magnification. For quantification, the percentage of positive stained cells in each field was calculated.

#### Immunofluorescent staining

TKCC cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% normal goat serum in 1% bovine serum albumin (BSA) TBST. For mouse tumour sections, samples were quenched, blocked and antigen retrieved as described above. Then samples were incubated with primary antibodies against CK19 (1:300),  $\alpha$ -SMA (1:300) (Abcam, Cambridge, MA) overnight at 4°C, followed by incubation with secondary AlexaFlour 488 anti-rabbit or AlexaFlour 546 anti-mouse antibodies (1:1000) in 1% BSA TBST in the dark. After counterstained with DAPI (Life Technologies, Scoresby, Australia) (1:5000), samples were mounted using fluorescence mounting medium. All images were captured using a Nikon fluorescent microscope (Eclipse Ti-E Model) at  $\times 40$  magnification.



**Figure 1.** PAK1 expression was correlated with CK19, while PAK4 expression was correlated with α-SMA and palladin. The expression of multiple proteins in six TKCC cell lines was determined by Western blot (A), and results were analysed and quantified (B) as described in Materials and Methods. The intracellular localization of CK19, α-SMA were determined by immunofluorescent staining in two selected TKCC cell lines (TKCC 15, 18) (C). The fluorescent images were taken using a Nikon fluorescent microscope at × 40 magnification. The correlations between PAK1 or PAK4 expression with the expression of CK19, α-SMA and palladin were assessed by regression analysis, and R<sup>2</sup> and p values were presented (D). pPAK1, phospho-PAK1; tPAK1, total PAK1; pPAK4, phospho-PAK4; tPAK4, total PAK4; CK19, cytokeratin 19; α-SMA, alpha-smooth muscle actin.

As an epithelial marker, CK19 was highly expressed, meanwhile the stromal markers including α-SMA, palladin were also observed in all these cell lines. The whole protein profile is shown in **Figure 1A, 1B**. Additionally, immunofluorescent staining was performed to further confirm the expression and localization of CK19, α-SMA in two selected cell lines (TKCC 15, 18) (**Figure 1C**). Regression analysis showed that the expression of PAK1 was positively correlated with the expression of CK19, whereas the protein level of PAK4 was positively correlated with α-SMA, palladin (**Figure 1D**). These results indicate that PAK1 and PAK4 mediate the regulation of proteins differently in these TKCC cell lines. The interaction between PAKs proteins and the tumoural and stromal marker proteins requires further investigation.

*Statistical analysis*

All values were expressed as mean ± standard error. The results were summarized from three independent experiments. Results were analysed by one-way ANOVA or student’s t-test. The correlation of PAK1 or PAK4 expression with CK19, α-SMA and palladin expression was determined by regression analysis. Differences between two means with P < 0.05 were considered significant. All the statistical analyses were performed using SPSS software (v16.0, SPSS Inc., Chicago, IL).

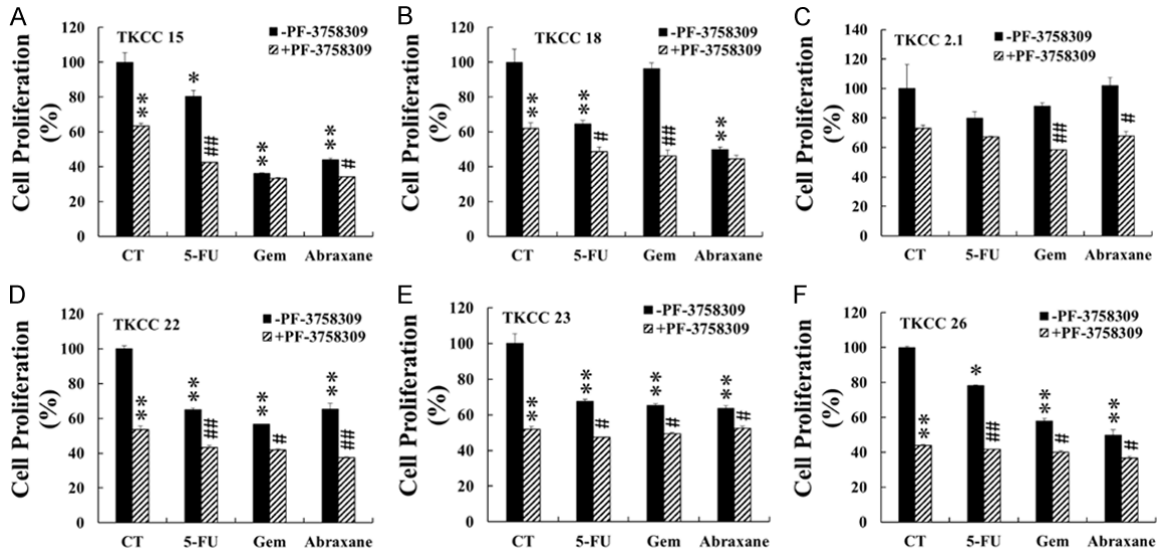
**Results**

*PAK1 expression is correlated with CK19, and PAK4 expression is correlated with α-SMA and palladin*

The protein expression profile of six TKCC cell lines were determined by Western blots. The expression of total and phospho-PAK1 and PAK4 varied in the panel of six TKCC cell lines.

*PF-3758309 enhanced the inhibitory effects of 5-FU, gemcitabine and abraxane on the proliferation of TKCC cells in vitro*

The TKCC cells were incubated with increasing concentrations of PF-3758309 for 48 h under hypoxia (5% O<sub>2</sub>). PF-3758309 inhibited the proliferation of all TKCC cell lines in a dose-dependent manner (data not shown). The IC<sub>50</sub> values of PF-3758309 for each cell line were calculated and listed in **Table 1**. Similarly, the IC<sub>50</sub> values for 5-FU, gemcitabine and abraxane were also determined and were listed in **Table 1**. Then all six TKCC cell lines were pre-treated with PF-3758309 at concentrations around the IC<sub>50</sub> values for 48 h, and followed by another 48 h treatment with 5-FU, gemcitabine or abraxane at concentrations around the IC<sub>50</sub> values in the absence of PF-3758309. PF-3758309 pre-treatment enhanced the inhibitory effects of 5-FU on TKCC 15, 18, 22, 23 and 26 cells, of gemcitabine on TKCC 18, 2.1, 22, 23 and 26 cells, and of abraxane on TKCC 15, 2.1, 22, 23 and 26 cells (**Figure 2**) compared to each cor-



**Figure 2.** PF-3758309 enhanced the inhibitory effects of 5-FU, gemcitabine and abraxane on TKCC cell lines. Six TKCC cell lines (TKCC 15 (A); TKCC 18 (B); TKCC 2.1 (C); TKCC 22 (D); TKCC 23 (E); TKCC 26 (F)) were pre-incubated with PF-3758309 at the  $IC_{50}$  concentrations listed in **Table 1** for 48 h, followed by another 48 h incubation with 5-FU, gemcitabine (Gem) or abraxane at the  $IC_{50}$  concentrations listed in **Table 1**. Cell proliferation was determined as described in Materials and Methods. The values obtained from controls (CT) without any treatment were taken as 100%. The data were summarized from at least three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , compared to the values obtained for the controls of non-PF-3758309 pre-treated cells. # $P < 0.05$ , ## $P < 0.01$ , compared to the values obtained from individual chemo-reagent (non-PF-3758309)-treated cells. 5-FU, 5-fluorouracil; Gem, gemcitabine.

responding single-agent treatment. These results indicate that PF-3758309 inhibited TKCC cell growth synergistically with multiple chemotherapeutic reagents.

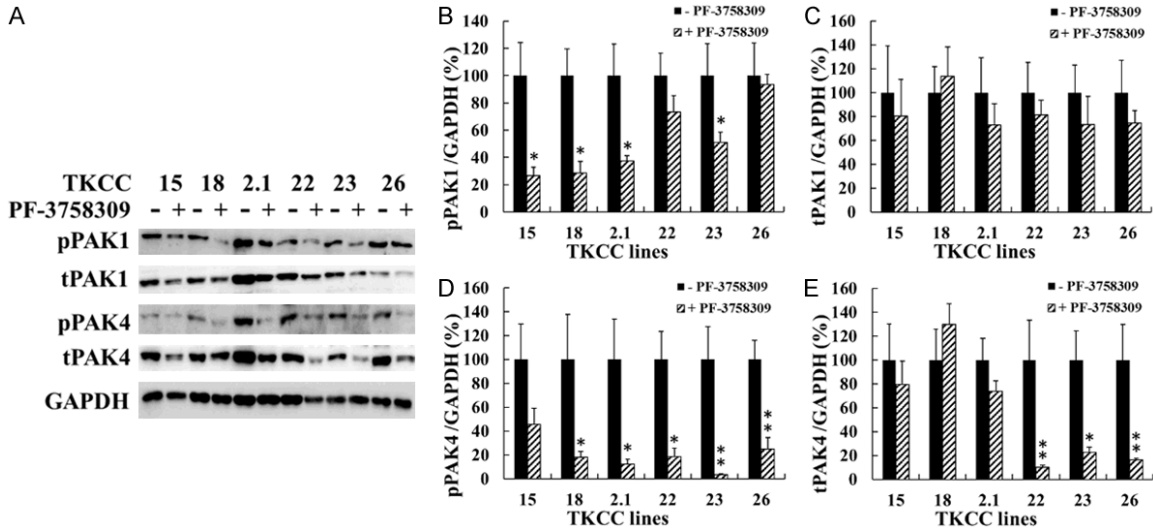
*PAKs inhibition by PF-3758309 caused reduced expression of HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA in TKCC cell lines in vitro*

After 48 h treatment with PF-3758309 at the  $IC_{50}$  concentrations determined in a proliferation assay (**Table 1**), the expression of pPAK1 (TKCC 15, 18, 2.1, 23) and pPAK4 (TKCC 18, 2.1, 22, 23, 26) were significantly reduced in the majority of these cell lines (**Figure 3A, 3B, 3D**), whereas no significant reduction in tPAK1 and tPAK4 were observed except for tPAK4 in TKCC 22, 23 and 26 cell lines (**Figure 3A, 3C, 3E**). All six TKCC cell lines were cultured under hypoxia (5%  $O_2$ ), the inhibitory effect of PF-3758309 on the expression of HIF-1 $\alpha$  which is critical for cell survival under hypoxia, was determined. PF-3758309 significantly inhibited the expression of HIF-1 $\alpha$  in all six TKCC lines (**Figure 4A, 4B**). Additionally, decreased expression of palladin (TKCC 15, 18, 2.1) and  $\alpha$ -SMA (TKCC 15, 26) were also observed in the select-

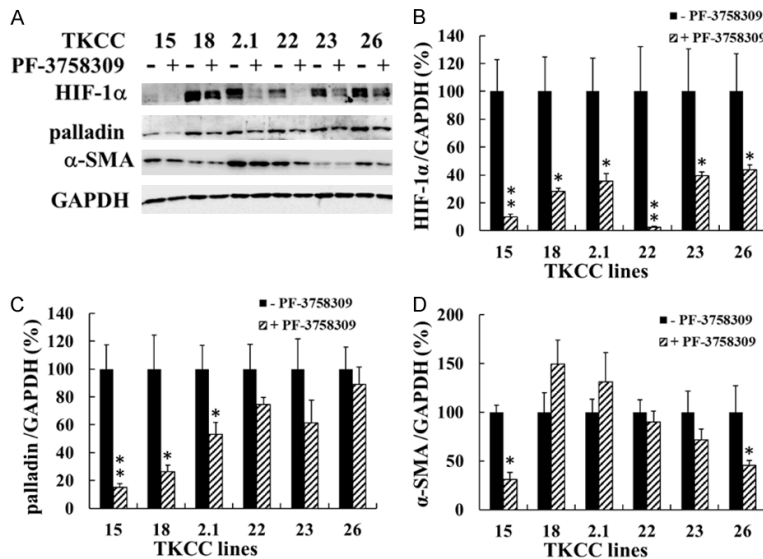
ed TKCC cell lines (**Figure 4A, 4C, 4D**). These data suggest that PAK1 and/or PAK4 play an important role in regulating the expression of HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA. Inhibition of PAKs by PF-3758309 leads to down-regulation of HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA expression.

*The combination of gemcitabine with PF-3758309 maximally inhibited tumour growth by suppressing cell proliferation in vivo*

SCID mice bearing TKCC 15 xenografted tumours were divided into the following five groups: control, gemcitabine alone, PF-3758309 alone, gemcitabine plus abraxane and gemcitabine plus PF-3758309. The schematic diagram of tumour induction and treatment was shown in **Figure 5A**. At the beginning of the treatments (day 25), the tumour volumes (measured in  $mm^3$ ) were  $72 \pm 12$ ,  $82 \pm 11$ ,  $80 \pm 13$ ,  $81 \pm 11$ , and  $83 \pm 13$  for control, gemcitabine alone, PF-3758309 alone, gemcitabine plus abraxane and gemcitabine plus PF-3758309, respectively. Tumour volume on day 25 was taken as 1 in each group. The following tumour volume was calculated as the ratio to that on day 25 (**Figure 5B**). After 4-week treat-



**Figure 3.** PF-3758309 suppressed the activities of PAK1 and PAK4. The six TKCC cell lines were incubated with PF-3758309 at the concentrations listed in **Table 1** under hypoxia (5% O<sub>2</sub>) for 48 h. The protein levels of total and phospho-PAK1 and PAK4 were determined by Western blot, and representative images were presented (A). Relative protein amount was calculated as described in Materials and Methods, and the values for untreated cells were taken as 100% (B-E). The results were summarized from three independent experiments. \*P < 0.05, \*\*P < 0.01, compared to the values for non-PF-3758309-treated cells. pPAK1, phospho-PAK1; tPAK1, total PAK1; pPAK4, phospho-PAK4; tPAK4, total PAK4.

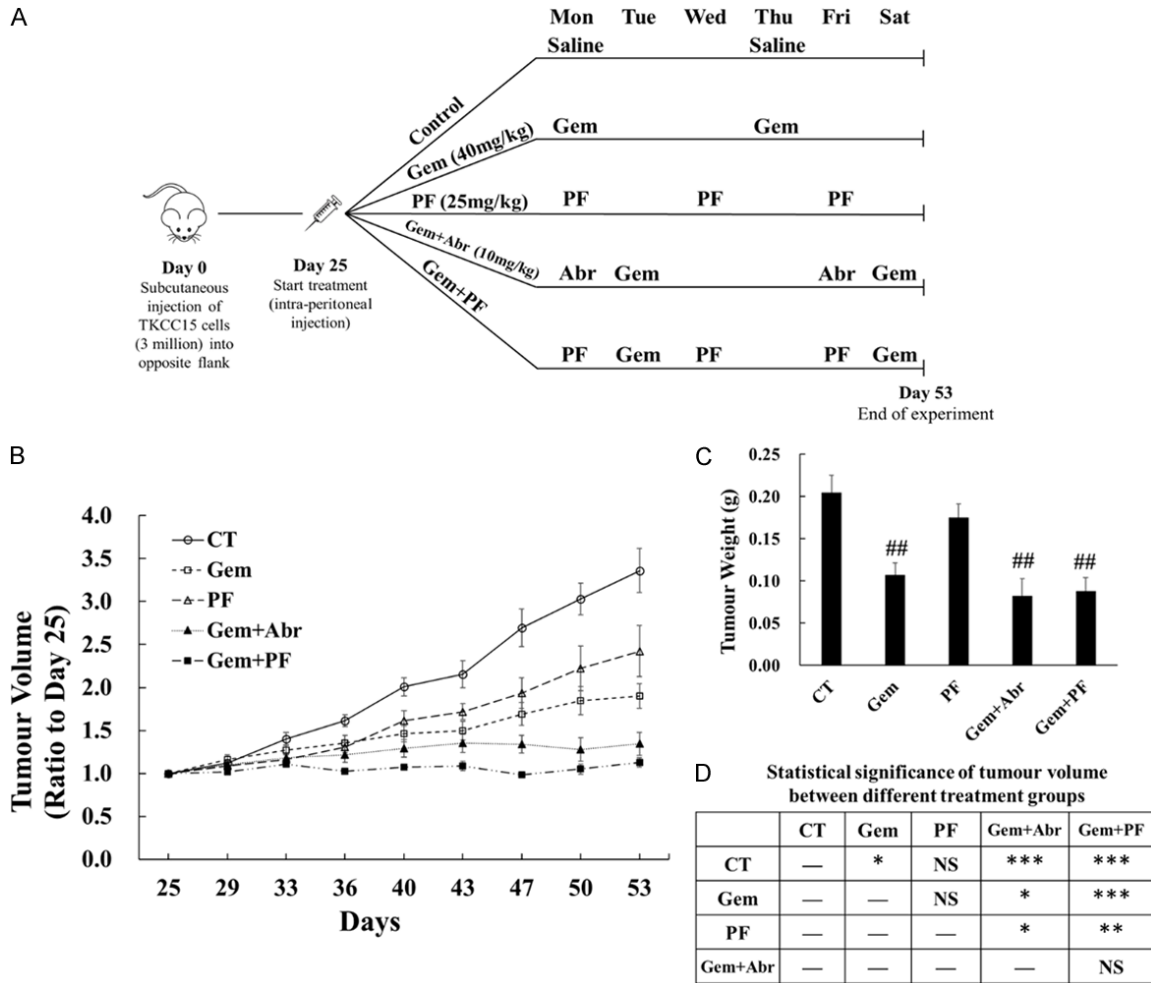


**Figure 4.** PF-3758309 inhibited the expression of HIF-1α, palladin and α-SMA *in vitro*. The six TKCC cell lines were incubated with PF-3758309 at the concentrations listed in **Table 1** under hypoxia (5% O<sub>2</sub>) for 48 h. The protein levels of HIF-1α, palladin and α-SMA were determined by Western blot, and representative images were presented (A). Relative protein amount was calculated as described in Materials and Methods, and the values for untreated cells were taken as 100% (B-D). The results were summarized from three independent experiments. \*P < 0.05, \*\*P < 0.01, compared to the values for non-PF-3758309-treated cells. HIF-1α, hypoxia-inducible factor 1-α; α-SMA, alpha-smooth muscle actin.

compared to control. No significant difference was observed between gemcitabine alone and PF-3758309 alone treatment group. Both combination treatment groups (gemcitabine plus abraxane or gemcitabine plus PF-3758309) showed further inhibition on tumour growth compared to both single-agent treatment groups (gemcitabine or PF-3758309) (**Figure 5B, 5D**). Among all four drug treatment groups, gemcitabine plus PF-3758309 inhibited the tumour growth maximally, but no significant difference was observed when compared to gemcitabine plus abraxane. All treatment groups except for PF-3758309 alone showed significantly decreased tumour weights compared to control. However, neither of combination treatment showed further significant decrement in tumour weight compared to gemcitabine alone treatment (**Figure 5C**). Presumably, the difference between the tumour volume and

ment, tumour volumes in all groups (except for PF-3758309 alone) were significantly reduced

compared to control. No significant difference was observed between gemcitabine alone and PF-3758309 alone treatment group. Both combination treatment groups (gemcitabine plus abraxane or gemcitabine plus PF-3758309) showed further inhibition on tumour growth compared to both single-agent treatment groups (gemcitabine or PF-3758309) (**Figure 5B, 5D**). Among all four drug treatment groups, gemcitabine plus PF-3758309 inhibited the tumour growth maximally, but no significant difference was observed when compared to gemcitabine plus abraxane. All treatment groups except for PF-3758309 alone showed significantly decreased tumour weights compared to control. However, neither of combination treatment showed further significant decrement in tumour weight compared to gemcitabine alone treatment (**Figure 5C**). Presumably, the difference between the tumour volume and

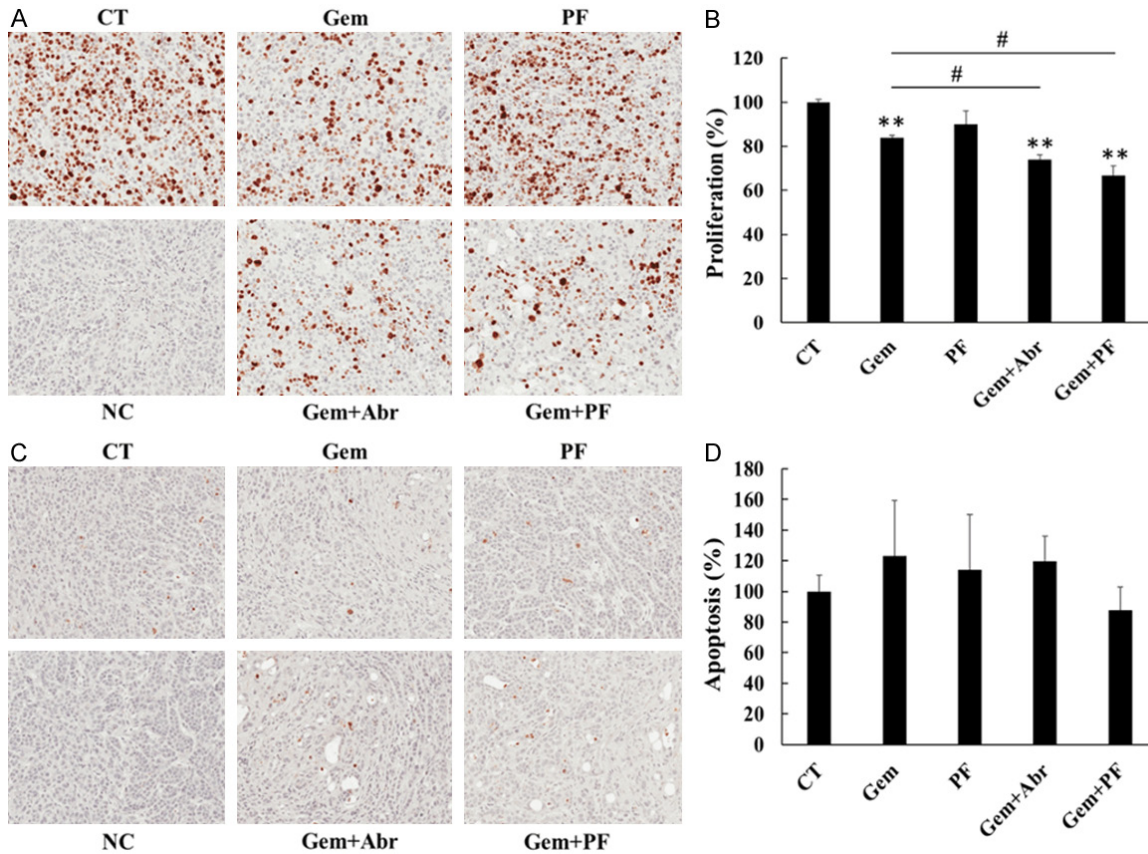


**Figure 5.** Combination of gemcitabine with PF-3758309 maximally inhibited tumour growth *in vivo*. TKCC 15 cells ( $3 \times 10^6/100 \mu\text{l}/\text{site}$ ) were injected subcutaneously into the opposite flanks of 8-week old male SCID mice. On day 25 after tumour induction, when the xenografted tumours had grown to approximate  $80 \text{ mm}^3$ , the mice were randomly divided into the following 5 treatment groups: control (CT), PF-3758309 (PF), gemcitabine (Gem), gemcitabine plus abraxane (Gem+Abr) and gemcitabine plus PF-3758309 (Gem+PF). Each group had 3 mice bearing 2 tumours each mouse, making 6 tumours in total. The treatments were given by intra-peritoneal (i.p.) injection for four weeks, and the mice were euthanized on day 53. The schematic diagram of tumour induction and treatment was summarized and presented (A). The tumour volume (B) and weight (C) were measured and calculated as described in Material and Methods. The average tumour volume in each group on day 25 were taken as 1 (B). The statistical significance of tumour volumes between different groups were summarized in the table (D). NS, not significant; CT, control treatment with saline; Gem, gemcitabine; PF, PF-3758309; Abr, abraxane. ## $P < 0.01$  compared to CT. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  as indicated in the table.

tumour weight was caused by necrosis where liquification was presented leading to weight loss in some tumours.

Proliferation and apoptosis within xenograft tumour were determined by immunohistochemical staining of Ki67 and cleaved caspase-3, respectively. Gemcitabine alone, but not PF-3758309 alone, significantly inhibited cell proliferation to 83% of control. Combination treatment further reduced proliferation to 74% and

67% of control for gemcitabine plus abraxane and gemcitabine plus PF-3758309, respectively, and the reductions were significantly greater than that of gemcitabine alone (Figure 6A, 6B). There was no significant difference in apoptosis in all four treatment groups compared to control (Figure 6C, 6D). These results indicated that combination of gemcitabine with PF-3758309 synergistically reduced tumour growth via suppressing cell proliferation. This combination could achieve a comparable or even great-



**Figure 6.** PF-3758309 promoted anti-tumour effect of gemcitabine *in vivo* by decreasing cell proliferation without affecting apoptosis. Tumour sections originated from TKCC 15 xenograft were immunohistochemically stained with antibody against Ki67 (A) and cleaved caspase-3 (C) to determine cell proliferation and apoptosis, respectively. The images were taken using a Leica microscope at  $\times 20$  magnification. The data were analysed as described in the Materials and Methods. The values for CT in both Ki67 (B) and cleaved caspase-3 (D) were taken as 100%. NC, negative control stained without the primary antibody; CT, control treatment with saline; Gem, gemcitabine; PF, PF-3758309; Abr, abraxane. \*\* $P < 0.01$  compared to CT. # $P < 0.05$  compared to gemcitabine alone.

er therapeutic efficacy compared to gemcitabine plus abraxane.

*PF-3758309 reduced the expression HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA *in vivo**

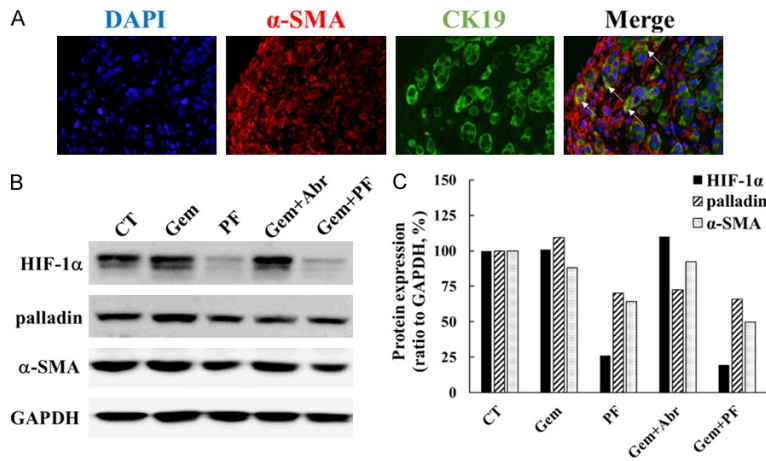
Co-expression of CK19 and  $\alpha$ -SMA was observed by dual immunofluorescent staining in tumour sections of TKCC15 xenograft (Figure 7A). Consistent to the *in vitro* results where PF-3758309 treatment significantly reduced the expression of HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA in TKCC 15 cells (Figure 4), PF-3758309 (PF-3758309 alone and gemcitabine plus PF-3758309 treatment group) also greatly decreased the expression of HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA in protein extracts of tumour tissues originated from TKCC 15 xenograft. Gemcitabine alone treatment slightly decreased  $\alpha$ -SMA, but had no effects on HIF-1 $\alpha$  and palladin expression.

Gemcitabine plus abraxane treatment suppressed the level of palladin and  $\alpha$ -SMA, but not HIF-1 $\alpha$ . These results further confirmed the expression of  $\alpha$ -SMA in PDA cells (TKCC 15), and PAK inhibition by PF-3758309 treatment also down-regulated HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA expression *in vivo*.

**Discussion**

The data presented here indicates that the pan-PAK inhibitor PF-3758309 [23] is an attractive therapeutic reagent for targeting pancreatic cancer. In this study, PF-3758309 not only inhibited cell proliferation on its own, but also enhanced the inhibitory effects of multiple chemotherapeutic reagents including 5-FU, gemcitabine and abraxane in a panel of established cell lines derived from patients with pancreatic cancer [22]. In xenograft animal model, combi-





**Figure 7.** PF-3758309 reduced the expression of HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA *in vivo*. A dual immunofluorescent staining of CK19 (green) and  $\alpha$ -SMA (red) was conducted on tumour tissue sections generated from TKCC 15 xenografts (A). Co-localization of CK19 and  $\alpha$ -SMA (yellow, arrows) was observed in several tumour cells. The images were taken using a Nikon fluorescent microscope at  $\times$  40 magnification. The amounts of HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA were determined by Western blotting in protein extracts obtained from TKCC 15 xenograft tumour tissue, and representative images were presented (B). Relative protein amount was calculated as described in Materials and Methods, and the values for CT were taken as 100% (C). CK19, cytokeratin 19;  $\alpha$ -SMA, alpha-smooth muscle actin; CT, control treatment with saline; Gem, gemcitabine; PF, PF-3758309; Abr, abraxane; HIF-1 $\alpha$ , hypoxia-inducible factor 1-alpha;  $\alpha$ -SMA, alpha-smooth muscle actin.

nation of gemcitabine with PF-3758309 achieved a comparable or even greater therapeutic efficacy compared to gemcitabine plus abraxane *via* suppression of cell proliferation rather than apoptosis. In agreement, our previous studies also demonstrated that genetically or pharmacologically inhibition of PAK1 and/or PAK4 synergistically enhanced gemcitabine sensitivity both *in vitro* and *in vivo* [10, 24]. Additionally, it has been reported that both PAK1 and PAK4 play pivotal roles in mediating gemcitabine resistance [11, 14]. Here, our data showing that PF-3758309 enhanced the inhibitory effects of multiple chemotherapeutic reagents on a panel of patient's derived PDA cell lines *in vitro* and *in vivo*, make PF-3758309 a potential reagent in adjunctive or combination therapy in the management of pancreatic cancer.

Protein profiling by Western blot showed that TKCC cell lines not only expressed the cancer cell marker CK19, but also stromal cell markers including  $\alpha$ -SMA and palladin. Total and phospho-PAK1 and PAK4 were also expressed in all six cell lines. Interestingly, the expression of PAK1 was significantly correlated with CK19, while the expression of PAK4 was significantly

correlated with palladin and  $\alpha$ -SMA (Figure 1D). Presumably, this result suggests that PAK1 and PAK4 contribute to regulation or maintenance of an "epithelial" or "mesenchymal"-like state differently in tumour development. Furthermore, a dual immunofluorescent staining also confirmed the co-expression of CK19 and  $\alpha$ -SMA within tumour tissues. To our knowledge, this is the first study showing the expression of  $\alpha$ -SMA in PDA cells *in vitro* and *in vivo*.  $\alpha$ -SMA is an established marker for PSC activation [25], and is reported to be a negative prognostic factor for patients with resectable PDA [26, 27]. In PSCs, all-trans retinoic acid induced down-regulation of  $\alpha$ -SMA attenuated its pro-tumour interaction with PDA cells and suppressed the tumour progression [28]. Our previous

study also demonstrated that inhibition of PAK1 suppressed  $\alpha$ -SMA expression in PSCs, leading to decreased cell proliferation and increased apoptosis [12]. In this study, PF-3758309 significantly reduced the expression of  $\alpha$ -SMA both *in vitro* and *in vivo*. The different inhibitory effect of PF-3758309 on  $\alpha$ -SMA expression in selected TKCC cell lines may be due to the heterogeneous nature of pancreatic cancer. Colocalization of CK19 and  $\alpha$ -SMA in some tumour cells *in vivo*, particularly in the interphase where tumour cells meet stromal cells (Figure 7A), suggested that these dual stained cells might play an important role in metastasis. Similarly,  $\alpha$ -SMA has also been reported to be involved in tumour cell metastasis in lung cancer [29]. Targeting  $\alpha$ -SMA in both PSC and PDA cells by PAK inhibition might be a potential approach in PDA treatment. The role of  $\alpha$ -SMA in PDA tumour biology is still not fully understood, further investigation is required to unravel the underlying mechanisms linking PAKs to  $\alpha$ -SMA regulation.

The expression of HIF-1 $\alpha$  and palladin, which was involved in cell survival and cytoskeletal re-organization, was also significantly reduced

by PF-3758309 treatment *in vitro* and *in vivo*. HIF-1 $\alpha$  was reported to mediate gemcitabine resistance in pancreatic cancer [30]. Inhibition of HIF-1 $\alpha$  could sensitize cancer cells to gemcitabine treatment [31], whereas high expression and activity of HIF-1 $\alpha$  inhibited the transcription of hENT1 and hENT2, leading to decreased gemcitabine uptake [32]. The critical role of PAK1 in regulating HIF-1 $\alpha$  has been demonstrated in our previous studies, showing that PAK1 enhanced cancer cell survival by up-regulation of HIF-1 $\alpha$  [33] and PAK1 knockdown by shRNA decreased expression of HIF-1 $\alpha$  and tumour growth [10]. Another recent study also reported that PAK4 inhibition reduced expression of HIF-1 $\alpha$  via the AKT-mTOR-4E-BP1 axis [34]. Therefore, PAKs could mediate cell survival and gemcitabine resistance by up-regulation of HIF-1 $\alpha$  signalling. It was reported that palladin stimulated cell invasion of PDA by promoting invadopodia formation in cancer-associated fibroblasts [35]. One recent clinical study analysed tissue microarrays of resected PDA samples from 167 patients, and found that palladin expression was correlated with a significantly lower survival rate and was an independent poor prognostic factor [6]. Although the mechanisms involved in the relationship between PAKs and palladin have not been well elucidated, the inhibitory effect of PAK inhibitor on palladin expression was observed in this study. Treatment with PAK inhibition could suppress tumour metastasis and improve patient's survival via down-regulation of palladin.

In conclusion, we have demonstrated here that the combination of the PAK inhibitor PF-3758309 with multiple chemotherapeutic reagents further suppressed the growth of a panel of patient-derived pancreatic cancer cell lines compared to single-agent chemotherapies. The combination of PF-3758309 with gemcitabine showed a comparable or even greater inhibition of the tumour growth *in vivo* when compared to gemcitabine plus abraxane, and thus provided a potential treatment regime in the management of PDA. Furthermore, we firstly reported that PDA tumour cells not only expressed epithelial marker, but also expressed stromal marker both *in vitro* and *in vivo*. PAK inhibition by PF-3758309 reduced the expression of HIF-1 $\alpha$ , palladin and  $\alpha$ -SMA, which were critical effector proteins in cell growth, survival and chemo-resistance in PDA.

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## Disclosure of conflict of interest

None.

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