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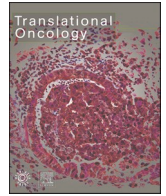
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## Original Research

# A novel PAK4 inhibitor suppresses pancreatic cancer growth and enhances the inhibitory effect of gemcitabine

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## ABSTRACT

Over 95% of Pancreatic ductal adenocarcinomas (PDA) carry mutations in the oncogene KRas which has been proven to be a difficult drug target. P21-activated kinase 4 (PAK4), acts downstream of KRas, and is overexpressed in PDA contributing to its growth and chemoresistance, and thus becomes an attractive therapeutic target. We have developed a new PAK4 inhibitor, PAKib and tested its effect on pancreatic cancer (PC) cell growth *in vitro* and in a syngeneic mouse model of PC. PAKib suppressed PC cell growth by inducing cell death and cycle arrest. PAKib inhibited PC growth and enhanced the inhibition by gemcitabine of PC in cell culture and in PC mouse model. PAKib acted through multiple signaling pathways involved in cell cycle checkpoints, apoptosis, cell junction, and focal adhesion. These proof-of-concept studies demonstrated the anti-cancer effect of PAKib alone and in combination with gemcitabine and warrant a further clinical investigation.

## Introduction

Pancreatic ductal adenocarcinoma (PDA) remains one of the deadliest diseases, with a 5-year survival <10% for decades [1], largely due to lack of effective treatments. Over 95% of PDA carry mutations in the oncogene KRas [2], which is a difficult druggable target [3]. P21-activated kinases (PAKs) act downstream of KRas. PAK1 and PAK4, overexpressed and/or hyperactivated in PDA, contribute to the growth and metastasis of PDA [4–6], and to chemotherapeutic resistance of PDA [7–9]. Reduction in activity of both PAK1 and PAK4 increases sensitivity of PDA to gemcitabine chemotherapy *in vitro* and *in vivo* [10,11]. Inhibition of PAK1 and PAK4 stimulated anti-tumor immunity by increasing tumor infiltrating lymphocytes and decreasing PD-L1 (programed death-ligand 1) expression by pancreatic cancer (PC) cells [12]. These findings have highlighted the prominence of PAKs in PDA and indicated that targeting PAKs would present an exciting therapeutic opportunity for PC treatment.

Several PAK inhibitors have been shown to suppress pancreatic cancer in cell culture and in mouse models [4,8,9]. However, none have successfully gone through clinical trials due to off-target effects [6] or

unknown causes. In pancreatic cancer, ATP-competitive inhibitors selective for PAK1 (FRAX597) and PAK4 (PF3758309) have been shown to suppress tumor growth in cell culture and mouse models [9,11]. However, the FRAX-related compounds also show strong adverse inhibition of hERG potassium channels, and they have poor cellular permeability, which blocked their passage through early clinical trials in other types of disease [13]. PF-3758309 failed in an early-phase clinical study in PDA due to unwarranted pharmacokinetic effects [6]. In addition, in pre-clinical settings, both FRAX597 and PF3758309 have been demonstrated to enhance the effects of chemotherapeutic agents currently used in clinic [9,11], indicating the application of PAK inhibition in combination with chemotherapy. Recently developed PAK4 allosteric modulators, KPT-9274 and its analogues, have been shown to inhibit PDA in cell-based and xenografted tumour mouse models, and KPT-9274 has been entered in an early-phase clinical trial (NCT02702492) [8]. The preclinical results from the development of PAK inhibitors are encouraging. Positioned downstream of difficult-to-target oncogenes such as Ras, PAKs remain promising druggable therapeutic targets. PAK inhibitors should open a new avenue for disruption of Ras-driven tumorigenesis as is the case with PDA.

Abbreviations: PAK, p-21 activated kinase; PDA, pancreatic ductal adenocarcinoma.

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We have developed a novel PAK4 inhibitor, PAKib. We have reported here that PAKib inhibited the activity of PAK4, that PAKib suppressed pancreatic cancer growth and further enhanced the inhibitory effect of gemcitabine on pancreatic cancer *in vitro* and in a mouse model, that PAKib acted through multiple signaling molecules involved in cell cycle checkpoints, apoptosis, cell junction, and focal adhesion.

## Materials and methods

### Reagents and cell culture

The human pancreatic cancer (PC) cell lines PANC-1, MiaPaCa-2 and BxPC3 were purchased from the American Type Culture Collection. The murine pancreatic cancer cell lines KPCPAK1WT833 was isolated and characterized from KPC mice as described previously [12]. PAKib was synthesized and provided by PAKinax Pty. Ltd (Melbourne, Australia). Gemcitabine was purchased from Sigma-Aldrich (Sydney, Australia). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Hyclone Laboratories, Melbourne, Australia) in a 37°C incubator with a humidified atmosphere of 5% CO<sub>2</sub>. p21 activated kinase 4 (PAK4) recombinant protein was purchased from MyBioSource, delivered by Resolving Images Australia (Melbourne, Australia). Anti-PAK4, were purchased from Cell Signaling Technology (Danvers, MA). Protein A-Agarose from Abcam (Melbourne, Australia), and [ $\gamma$ -P<sup>32</sup>]-adenosine triphosphate ( $\gamma$ -P<sup>32</sup>-ATP) from Perkin Elmer (Melbourne, Australia).

### Kinase assay

Both purified recombinant PAK4 and PAK4 proteins immunoprecipitated (using anti-PAK4 antibody at 1:200) from PANC-1 cell lysates were subjected to a kinase assay. Both types of PAK4 were incubated with 2  $\mu$ M cold ATP and 5  $\mu$ g of myelin basic protein (MBP) in kinase buffer (40 mM HEPES pH 7.4, 10 mM MgCl<sub>2</sub>) on ice for 5 min before 10  $\mu$ Ci  $\gamma$ -P<sup>32</sup>-ATP was added and rotated at room temperature for 10 minutes. 2x SDS sample buffer was added to stop the reaction and the samples were then boiled at 95°C for 5 minutes before loaded to a 12% SDS-PAGE. The gel containing separated proteins was fixed in 7% acetic acid at room temperature for 30 minutes, and then dried. The phosphorylated protein bands were developed and visualized through Fuji-film Bas-1800 II (Berthold, Australia) and the density of a band was calculated and analyzed using Multi Gauge (version 2.3) software.

### Cell proliferation assay

Human and murine PC cells were incubated with different concentrations of PAKib in DMEM in the presence or absence of 5% FBS for 24 h. For the combination of PAKib with gemcitabine, the cells were pre-incubated with PAKib (10  $\mu$ M) for 24h, followed by incubation with different concentrations of gemcitabine for another 24h. Cell proliferation was measured by MTT assay.

### Measurements of cell death and cell cycle

Cell death and cell cycle were assayed using propidium iodide (PI) and flow cytometry. All floating and adhering cells were trypsinized and then washed two times with cold phosphate-buffered saline (PBS). For death assays, cells were harvested and resuspended in PBS containing 1  $\mu$ g/mL PI. For cell cycle analysis, cells were harvested and resuspended in PBS containing 0.1% Triton X-100, 50  $\mu$ g/mL PI, and 10  $\mu$ g/mL RNaseA (Thermo Scientific), and then incubated at 37°C for 15 min. Samples were processed on with FACSymphony A3 flow cytometer (BD Biosciences, Melbourne, Australia) and the acquired data analysed with the FlowJo software (BD Biosciences, San Jose, CA).

### Proteomic analysis

PANC-1 (1.25  $\times$  10<sup>6</sup> cells) were seeded in a 10 cm culture dish, allowed to adhere for 2 h and then treated with 10  $\mu$ M PAKib for 48 h and then lysed with radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris HCl, pH 8, 150 mM NaCl, 1% Triton x-100, 1% Na deoxycholate, 0.5% SDS, 1 mM EGTA, protease inhibitor, pHos sTop). The supernatant was precipitated with ice-cold acetone overnight at -20°C before resuspension in 8 M urea (in 50mM triethylammonium bicarbonate + 10mM TCEP) followed by incubation at 37°C for 30mins. Protein concentration was quantified using BCA protein assay and samples (containing equivalent amounts of total protein) were alkylated with 55 mM iodoacetamide for 45 mins at room temperature. The solution was diluted to a final concentration of 1M urea followed by overnight digestion at 37°C with sequencing-grade modified trypsin (ThermoFisher) at a ratio of 1:50 (enzyme:protein). Digestion was stopped by addition of formic acid to a final concentration of 1% (v/v). The peptide mix was purified through solid phase extraction (SPE) with Oasis HLB cartridges (Waters) according to manufacturer's instructions. Eluted peptides were split into two tubes, Tube 1 (global mass spec) and Tube 2 (phospho-enrichment) and freeze-dried. Tube 1 was resuspended in 2% acetonitrile and 0.05% trifluoroacetic acid before analysis by mass spectroscopy. Phosphorylated peptides from Tube 2 were purified using a titanium dioxide (TiO<sub>2</sub>) enrichment method. Peptides were mixed at a ratio of 1:6 (peptides: TiO<sub>2</sub> beads) with TiO<sub>2</sub> beads (GL sciences) in loading buffer (2M Lactic acid in 5% trifluoroacetic acid (TFA), 50% acetonitrile) and incubated for 1 hour at room temperature. The beads were washed twice with loading buffer and then three times with washing buffer (50% acetonitrile, 5% TFA). The phosphorylated peptides were eluted with 1% (v/v) ammonia solution (pH 11.3) and 30% (v/v) acetonitrile, and then acidified with 1 $\mu$ L formic acid per 10 $\mu$ L eluent. Eluted phosphorylated peptides were freeze-dried, resuspended with 2% acetonitrile and 0.05% TFA for mass spectroscopy. The data obtained from mass spectroscopy were processed through MaxQuant (version 1.6.17.0) [14] and search against the Homo Sapiens database (SwissProt Taxonomy ID 9606, updated Dec 2020). The search parameters are Trypsin as the cleavage enzyme and a maximum of 2 missed cleavages. Instrument parameters are based on the default Maxquant setup. Carbamidomethyl cysteine was set as fixed modification. The LFQ algorithm was used for label free quantitation and match between run was activated. Oxidation of methionine, acetylation of the protein N-terminus and phosphorylation of serine, threonine and tyrosine were considered as variable modifications. Protein and peptides groups were set to a maximum false discovery rate (FDR) of < 0.01. Statistical and network analysis of the search results was carried out before using Perseus software (version 1.6.14.0) [15] and Cytoscape (version 3.8.2) [16].

### Mouse study

All mouse experiments were approved by the Austin Health Animal Ethics Committee (A2019/05654). Experimental mice were housed and cared in the BioResource Facility at Austin Health and monitored according to health criteria under the guideline of the Austin Health Animal Ethics Committee. Murine PC cells KPCPAK1WT833 (5  $\times$  10<sup>4</sup> cells/100  $\mu$ l culture medium) were subcutaneously injected into the flanks of 6-week-old, male C57BL6 mice. When a tumour reached 70 mm<sup>3</sup> (in about 9 days) in set one experiment: control and PAKib, PAKib (40 mg/Kg) was given by subcutaneous injection (s.i.) every other day for two weeks. The control mice were given same volume of PEG400 (s.i.) which was used to dissolve PAKib. In set two experiment where mice were divided into three groups: control, gemcitabine alone, and gemcitabine plus PAKib, when a tumour reached 100 mm<sup>3</sup>, gemcitabine (25 mg/Kg) was given by intraperitoneal injection (i.p.) every fourth day, and PAKib was given by s.i. every other day in the group with combination treatment of gemcitabine plus PAKib. The control mice were given same

volume of PEG400 (s.i.). The mice were treated for two weeks. Tumour growth was determined by tumour volume measured with a calliper every other day and by tumour weight measured at the end of each experiment.

#### Statistical analysis

All values are expressed as mean  $\pm$  standard error. The *in vitro* data are from three independent experiments each in triplicate. The *in vivo* data were collated according to numbers of tumour samples. Data were analysed by one-way ANOVA or t-test (SPSS, IBM, New York, NY). Differences between two means with  $p < 0.05$  were considered significant.

## Results

### PAKib inhibited the activity of PAK4

PAKib was synthesized and provided by PAKinax Pty. Ltd (Melbourne, Australia). PAKib suppressed the kinase activity of PAK4 with an  $IC_{50}$  value around 500 nM in an *in vitro* kinase assays using PAK4 immuno-precipitated from PC cells (Fig. 1A&B). PAKib also decreased the kinase activity of purified GST-PAK4 fusion protein (Fig. 1 C&D). The inhibition of PAKib on PAK4 was confirmed in a kinase assay using a Kinome panel containing about 400 kinases from Reaction Biology (Malvern, USA, data not shown). These results have demonstrated PAKib as a PAK4 inhibitor. Since PAK4 plays important role in PC development and progression, we have further investigated the role of PAKib in PC.

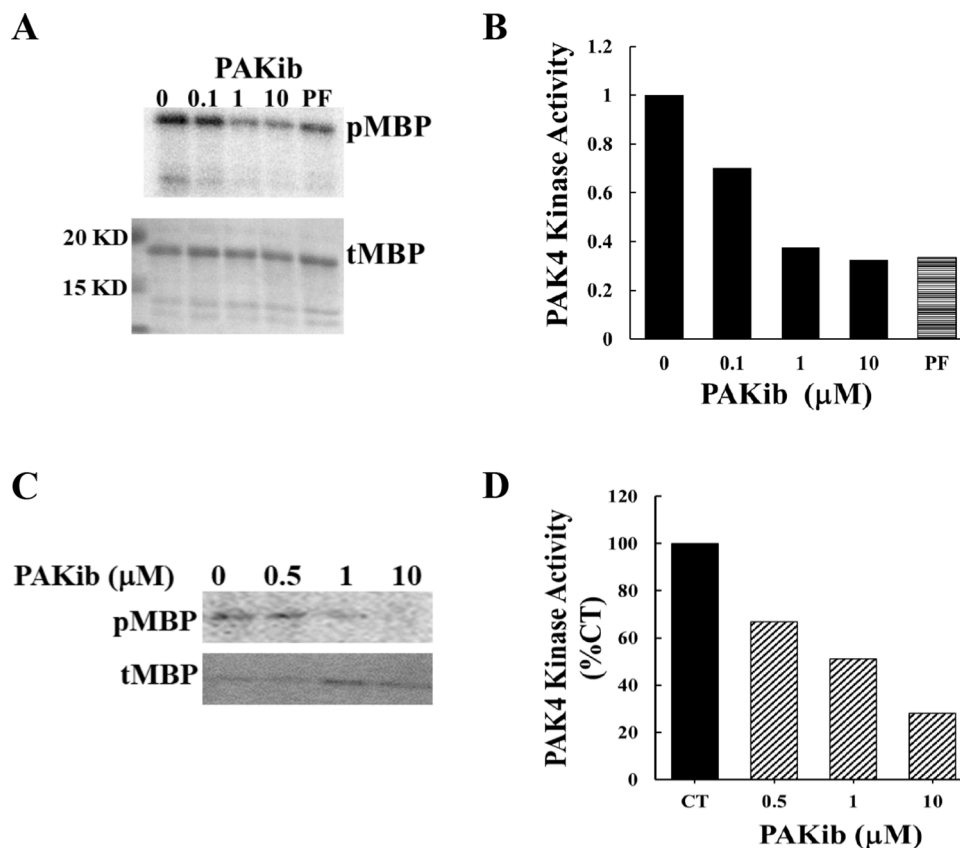
### PAKib inhibited PC cell growth and promoted the inhibition of gemcitabine on PC cells by inducing cell cycle arrest and cell death

Gemcitabine has been considered as a standard treatment and widely used as a first-line drug in palliative treatment of pancreatic cancer [17]. On-going trials have been carried on testing the efficacy of different drugs, either alone or in combination with gemcitabine. Likewise, the effect of PAKib on cell proliferation with or without gemcitabine was determined by MTT assay. PAKib suppressed the proliferation of both human (PANC-1, MiaPaCa-2 and BxPC3) and murine (KPCPAK1WT833) PC cells (Fig. 2A-D, and Table 1) in the presence or absence of serum, with more potent inhibition in the absence of serum. PAKib decreased the proliferation of both KRas-mutated (PANC-1 and MiaPaCa-2) and KRas wild type (BxPC3) PC cells. Pre-treatment of PC cells with PAKib promoted the inhibitory effect of gemcitabine on PC cell by further reduction of proliferation (Fig. 2E-F).

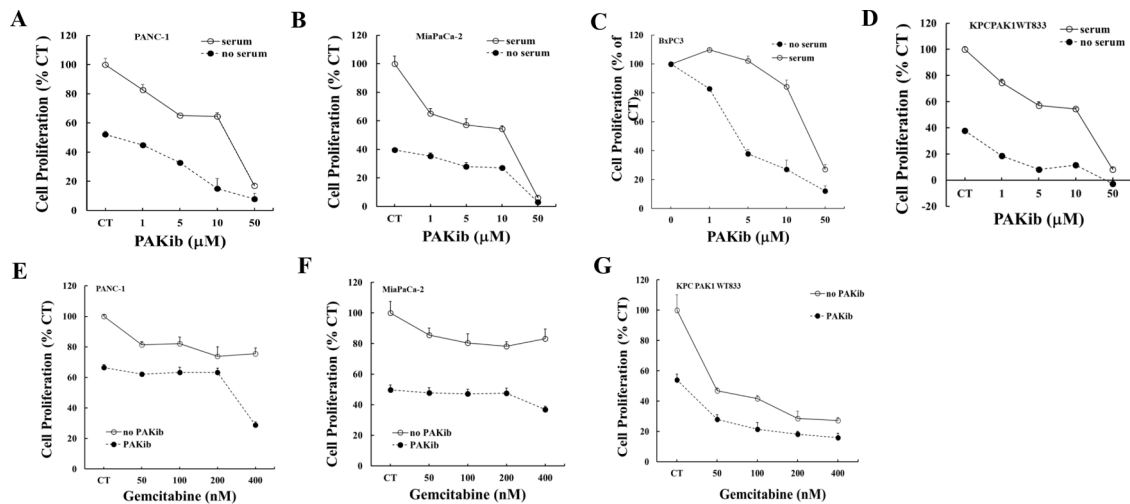
To determine the intracellular mechanism(s) of PAKib in its inhibition of PC cell growth, both human (PANC-1) and murine (KPCPAK1WT833) were treated with PAKib in the presence or absence of serum, changes in cell cycle and cell death were measured by PI staining followed by flow cytometry analysis. By 24 h treatment in serum, PAKib, from 2.5  $\mu$ M, induced cell cycle arrest at G2/M phase in PANC-1 cells (Fig. 3A). In the absence of serum, PAKib caused cell death dose-dependently in KPCPAK1WT833 cells (Fig. 3B). These results indicated that PAKib inhibited PC cell growth by inducing cell cycle arrest (in human PC cells) or cell death (in murine PC cells).

### PAKib suppressed PC growth and enhanced the inhibitory effect of gemcitabine on PC *in vivo*

The effect of PAKib on pancreatic tumour growth was determined



**Fig. 1.** PAKib inhibited the kinase activity of PAK4. In an *in vitro* kinase assay using myelin basic protein (MBP) as substrate, PAKib suppressed the kinase activity of PAK4 (immuno-precipitated from PANC-1 cell) with an  $IC_{50}$  value about 500 nM (A, B), and of a recombinant PAK4 (C, D). pMBP: phosphorylated MBP; tMBP: total MBP; CT: control. The density of pMBP from 0 (PAKib, B) or CT (D) were taken as 1 or 100% respectively.



**Fig. 2.** PAKib decreased PC cell proliferation and enhanced the inhibitory effect of gemcitabine. Human PANC-1 (A), MiaPaCa-2 (B) and BxPC3 (C), and murine KPCPAK1WT833 (D) PC cell lines were treated with PAKib with concentrations indicated in the figures (A-D) with or without serum for 24h. The cell proliferation was determined by MTT assay as described in the Materials and Methods. PAKib dose-dependently decreased the proliferation of both human and murine PC cells. In the combination of PAKib and gemcitabine treatment, PANC-1 (E), MiaPaCa-2 (F) and KPCPAK1WT833 (G) were pre-treated with PAKib (10  $\mu$ M) for 24h and followed by another 24h treatment of gemcitabine (Gem) with concentrations indicated in the figures (E-G). The data was summarized from three independent experiments. CT: control. The values from non-treated control were taken as 100%.

**Table 1**  
PAKib IC50 (mM) on cell proliferation

	PANC-1	MiaPaCa-2	BXPC3	KPCPAKWT833
+serum	13.77 $\pm$ 0.32	6.88	11.02 $\pm$ 0.89	9.49 $\pm$ 0.89
-serum	6.02 $\pm$ 0.23	9.63 $\pm$ 5.24	4.68 $\pm$ 0.20	1.23 $\pm$ 0.20

using a syngeneic mouse model where a murine PC cell line (KPCPAKWT833) was subcutaneously injected into the back of a hind leg of a mouse. PAKib (40 mg/Kg) was given by subcutaneous injection (s.i.) every second day when tumour volumes reached above 70 mm<sup>3</sup> in one set of experiments (Fig. 4A-D). In another set of experiments, when tumour volumes were around 100 mm<sup>3</sup>, gemcitabine (25 mg/Kg) was given every fourth day by intraperitoneal injection (i.p.) alone or plus PAKib (40 mg/Kg, s.i. every second day) (Fig. 4E-H). PAKib suppressed tumour growth by reducing the average tumour volume to 47% of the control maximally at day 20 (Fig. 4B), and by decreasing the tumour weight to 32% of the control (Fig. 4C). No toxicity of PAKib was observed as shown by a stable mouse body weight (Fig. 4D).

Gemcitabine significantly reduced the tumour volume from 12 days after starting treatment till the end of experiment, compared to control (Fig. 4F). Gemcitabine decreased the average tumour volume maximally to 33% (Fig. 4F) and the tumour weight to 15% (Fig. 4G) of the control respectively by the end of experiment. When compared to gemcitabine, the combined treatment of gemcitabine and PAKib further suppressed the tumour growth by reducing the tumour volume and weight to 21% (Fig. 4F) and 26% (Fig. 4G) of gemcitabine treated alone respectively. No significant toxicity was observed from the treatments of either gemcitabine alone or gemcitabine plus PAKib as shown by a stable mouse body weight (Fig. 4H). These results indicated that PAKib suppressed pancreatic tumour growth on its own and stimulated the inhibition by gemcitabine of PC growth *in vivo*.

#### PAKib inhibited PC via multiple signaling pathways

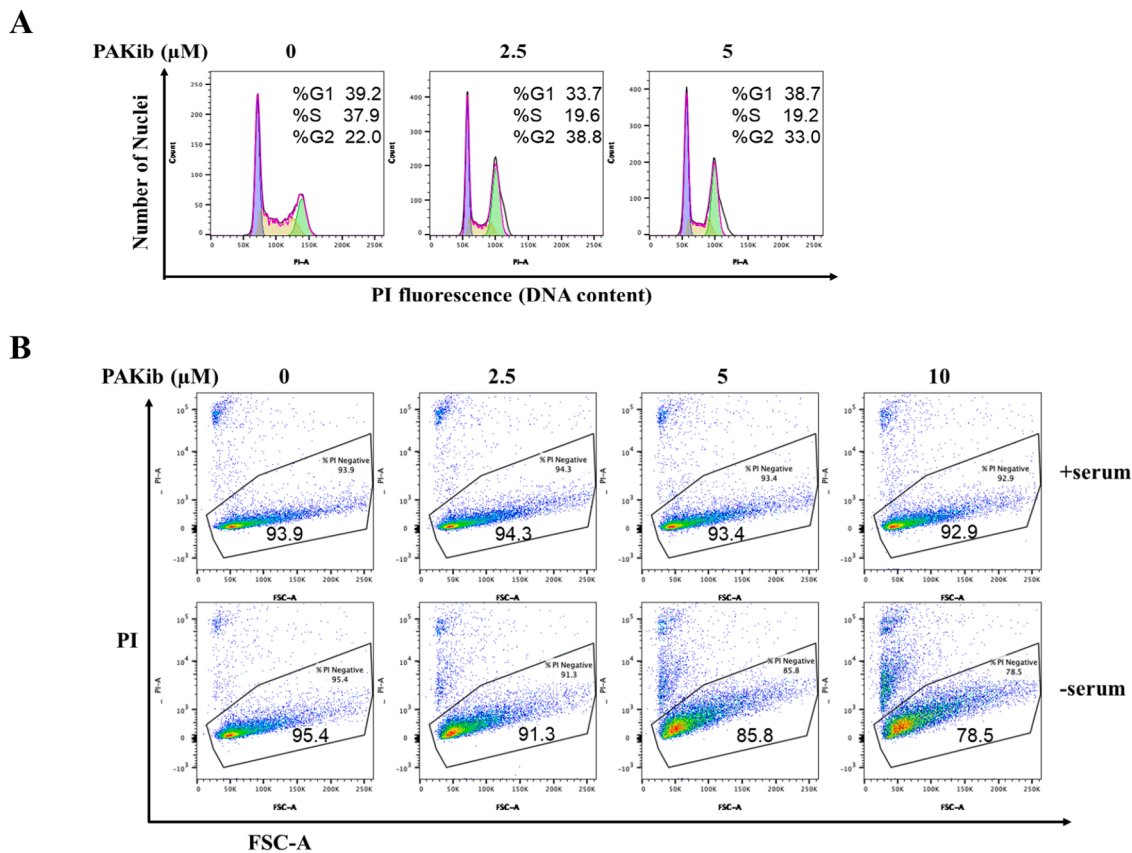
To discover the mechanism(s) involved in the effect of PAKib, PC cells were treated with PAKib for 48 h and then were lysed. The cell lysates were subjected to proteomic analysis as described in the Materials and Methods. There was no significant difference in the total protein abundances between control and PAKib-treated cells as shown from

a global protein profile obtained from a mass spectrometry followed by a statistical analysis using Perseus (version 1.6.14.0). An unbiased phosphorylated peptide profile was also generated from the TiO<sub>2</sub> enriched samples to determine changes in phosphorylated peptides and their related proteins. Upon statistical analysis using Perseus, a total of 285 phosphosites were significantly changed (Supplementary File 1) and they were mapped to 198 proteins (Fig. 5A). A heatmap was generated using the significant phosphosites after normalized by Z-score (Fig. 5B). 43 proteins out of 198 proteins with significant changes were identified in KEGG pathways. The phosphorylation of 16 proteins decreased by PAKib were identified in a distinctive pathway of KEGG (Table 2). The phosphorylation of 27 proteins were increased by PAKib and were involved in a distinctive pathway of KEGG (Table 3). Cell cycle, focal adhesion, tight junction, insulin-signaling, chemokine-signaling, MAPK-signaling, mTOR-signaling, p53-signaling, ErbB and Jak-STAT signaling pathways are among those KEGG pathways identified. The data with significant changes in phosphosites generated through Perseus was formatted and entered to Cytoscape [16] for analysis of interaction networks. 15 pathway networks were built up through a network analysis of Cytoscape (Fig. 6). Consistent with the findings from Perseus software analysis, cell cycle checkpoints, apoptosis, cell junction, focal adhesion, insulin signaling, and glucose metabolism networks were identified (Fig. 6). In addition, Rho-, Rac-activated pathways were identified. A few virus infection and anti-virus networks also showed up (Fig. 6).

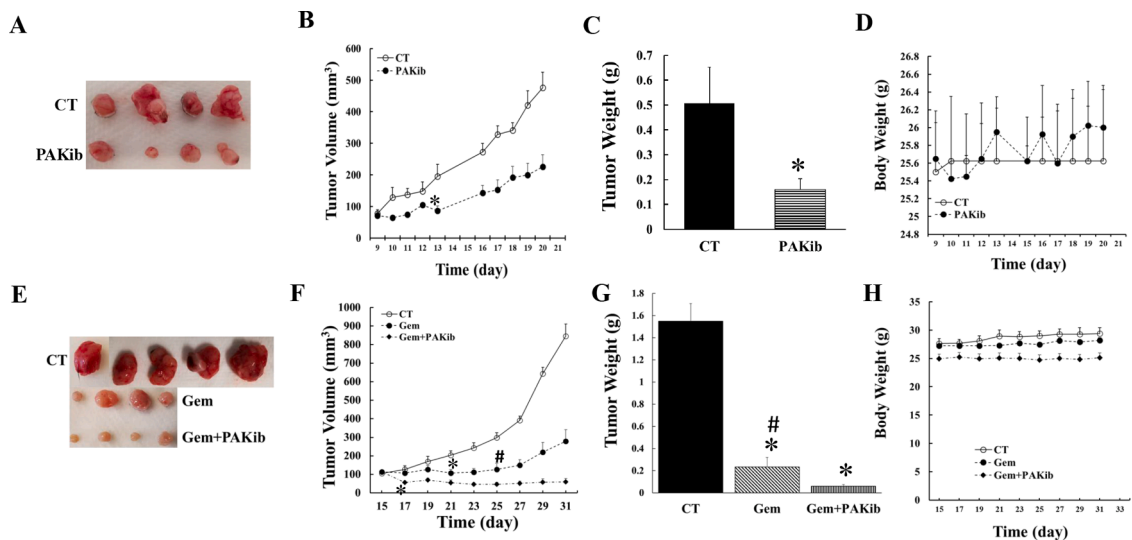
In summary, PAKib could affect cell cycle and apoptosis via MARP-, ErbB-, mTOR-signaling pathways. PAKib could also regulate cell junction and focal adhesion via Rho- and Rac-dependent pathways. The involvement of PAKib in virus infection and anti-virus networks suggested a possible role of PAKib in inflammation and immune response.

#### Discussion

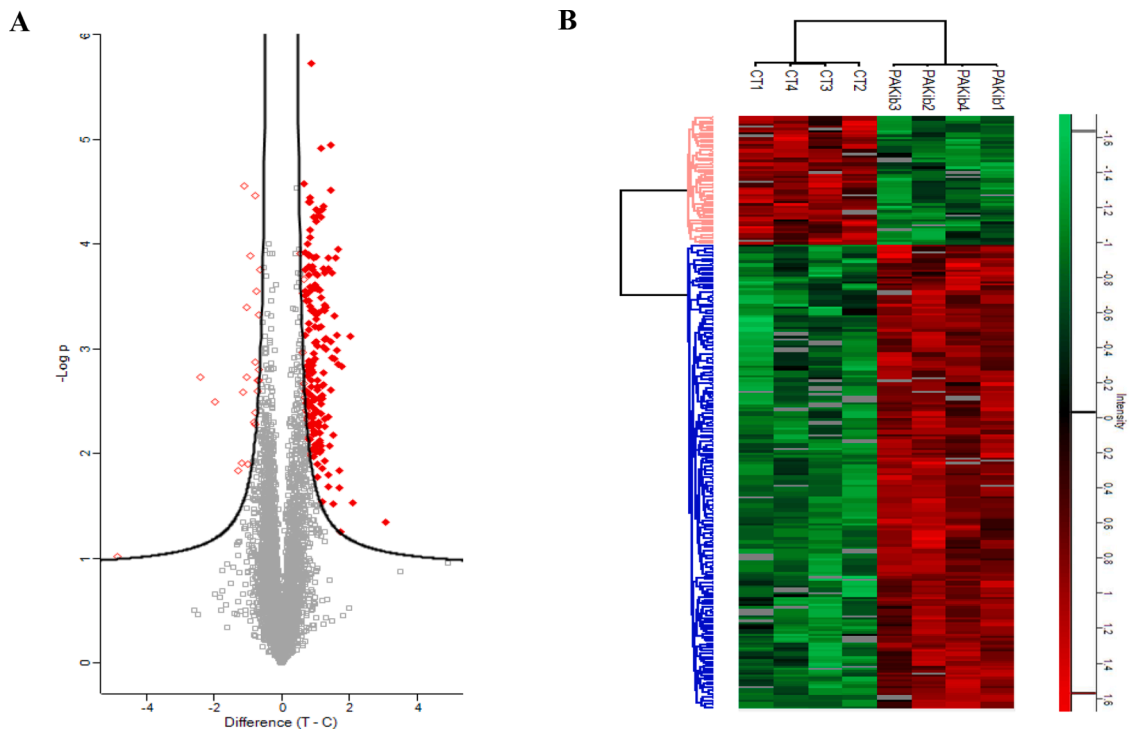
KRas mutations account for over 95% of PDA patients. KRas-driven cellular and animal cancer models have advanced our understanding of the initial and progression of various stages of PDA. However, there is very little progress in the development of KRas-targeted agents for the application in cancer treatment due to the absence of any druggable cavity in KRas protein structure. Recently specific inhibitors for mutant KRas<sup>G12C</sup> (glycine 12 to cysteine) have shown some effects on lung and



**Fig. 3.** PAKib suppressed PC cell growth by inducing cell cycle arrest and cell death. PANC-1 (A) and KPCPAK1WT833 (B) cells were treated with PAKib for 24h before being harvested for propidium iodide (PI) staining and flow cytometry as described in the Materials and Methods. The acquired data were analysed with the FlowJo software. The results were selected and represented from three independent experiments.



**Fig. 4.** PAKib suppressed PC growth *in vivo* and enhanced the inhibitory effect of gemcitabine on PC. KPCPAK1WT833 cells (50,000 cell/100  $\mu\text{l}$  DMEM plus 5% FBS) were subcutaneously injected into the back of the right hind leg of a mouse. The mice were treated with PAKib (40 mg/Kg) by a subcutaneous injection (s.i.) every second day when tumour volume reached above 70  $\text{mm}^3$  in set one (A-D) experiment where the initial average tumour volumes were  $77.51 \pm 12.27 \text{ mm}^3$  and  $70.77 \pm 11.41 \text{ mm}^3$  for control (CT, n=4) and PAKib (n=4) respectively (B). In set two experiment (E-H), when tumour volume reached 100  $\text{mm}^3$ , mice were treated with gemcitabine (Gem, 25 mg/Kg) alone every fourth day by intraperitoneal injection (i.p.) or Gem plus PAKic (40 mg/Kg, s.i. every second day). For set two experiment, the initial tumour volumes were  $106.34 \pm 12.48 \text{ mm}^3$ ,  $113.12 \pm 13.82 \text{ mm}^3$ , and  $114.53 \pm 3.94 \text{ mm}^3$  for CT (n=5), Gem alone (n=4) and Gem plus PAKib (n=4) respectively (F). \*, p<0.05 compared to CT; #, p<0.05 compared to Gem plus PAKib.



**Fig. 5.** PAKib induced changes in phosphosites and the phosphorylated proteins. PANC-1 cells were treated with PAKib (10  $\mu$ M) for 48h before the cells were lysed and the resultant cell lysates were subjected to a proteomic analysis as described in the Material and Methods. 4 repeated samples for control (CT) and PAKib-treated separately were applied in the experiment. The raw data generated through a Mass spectrum were processed using MaxQuant software, and resultant data were input into a Perseus software (version 1.6.14.0) where the changes in phosphosites and related phosphorylated proteins were identified. Statistically significant phosphosites were calculated and the mapped proteins were shown in the Volcano plot (A). The phosphosites with significant changes were used to generate a heatmap (B). Green represented the phosphorylated proteins decreased by PAKib compared to CT while Red PAKib-increased phosphorylated proteins. T: PAKib-treated; C: control

**Table 2**  
PAKib down-regulated phosphorylated proteins involved in KEGG pathways

Gene names	KEGG pathways
ZAK	MAPK signaling pathway;Tight junction
PML	Acute myeloid leukemia;Endocytosis;Pathways in cancer;Ubiquitin mediated proteolysis
HMGCR	Bile secretion;Terpenoid backbone biosynthesis
PSD3	Endocytosis
LMNA	Arrhythmogenic right ventricular cardiomyopathy (ARVC);Dilated cardiomyopathy;Hypertrophic cardiomyopathy (HCM)
ITGB4	Arrhythmogenic right ventricular cardiomyopathy (ARVC);Dilated cardiomyopathy;ECM-receptor interaction;Focal adhesion; Hypertrophic cardiomyopathy (HCM);Regulation of actin cytoskeleton
ARHGEF11	ko05152;Vascular smooth muscle contraction
STK11	Adipocytokine signaling pathway;mTOR signaling pathway
UBE3A	Ubiquitin mediated proteolysis
ACL1	Carbon fixation pathways in prokaryotes;Citrate cycle (TCA cycle)
NCOR2	Notch signaling pathway
IRS1	Adipocytokine signaling pathway;Aldosterone-regulated sodium reabsorption;Insulin signaling pathway;Neurotrophin signaling pathway;Type II diabetes mellitus
SMC4	Cell cycle - yeast
ACSS2	Carbon fixation pathways in prokaryotes;Glycolysis / Gluconeogenesis;Methane metabolism;Propanoate metabolism; Pyruvate metabolism
LLGL2;	Tight junction
LLGL1	
ABLIM1	Axon guidance

colon cancers [18]. These inhibitors are not suitable for PDA as 90% of PDA carry KRas<sup>G12D</sup> (glycine 12 to aspartic acid) and KRas<sup>G12V</sup> (glycine 12 to valine) mutations [2], which are so far undruggable as the amino acids substituted by mutation are currently chemically unreactable [19].

PAKs, positioned at the junction of several oncogenic pathways [20] are key effectors of Rho family GTPases downstream of Ras. PAK4 is a key downstream effector of CDC42 [21]. PAK4 gene is amplified in PDA and possibly associated with poor prognosis [22]. Amplification of PAK4 is co-existed with oncogenic Ras in pancreatic cancer [23]. PAK4 expression level in PC cell lines is negatively related to gemcitabine sensitivity [24]. PAK4 also mediates pancreatic stellate cell-stimulated proliferation of PC cells by activating PI3K/Akt pathway [25].

We have demonstrated here that a newly developed PAK4 inhibitor, PAKib, suppressed PC growth in cell culture and in a syngeneic mouse model of PC, and that PAKib enhanced the inhibitory effect of gemcitabine on PC *in vitro* and *in vivo*. We have also revealed that PAKib inhibited PC cell growth by inducing cell death and cell cycle arrest, which is confirmed by our data obtained from the proteomic study showing the involvement of PAKib in cell cycle and apoptosis via multiple signaling pathways including MARP, ErbB, and mTOR.

The inhibition by PAKib of PC growth *in vivo* is quite potent, demonstrated by over 50% reduction of both tumour volume and weight by 10 days of treatment (Fig. 4B&C). PAKib also stimulated the inhibitory effect of gemcitabine on PC dramatically when combined with gemcitabine, by further decreasing tumour volume and weight to 21% and 27% of gemcitabine treatment alone respectively. These data provide a solid foundation for further testing this PAK4 inhibitor in clinical settings with PC patients. Except for PC cells, PAKib also suppressed growth of many other types of cancer cells including colorectal, lung, breast, brain, skin, prostate, and cholangiocarcinoma as shown in the supplementary Table 1, implicating the potential application of PAKib in the treatment of other types of cancers.

Recently PAK4 inhibition has been shown to improve PD-1 blockade immunotherapy in a skin cancer model of mice [26], indicating a role of PAK4 in tumour immune response. Most newly developed chemical

**Table 3**  
PAKib up-regulated phosphorylated proteins involved in KEGG pathways

Gene names	KEGG pathways
MARCKS	Fc gamma R-mediated phagocytosis
NUP133	RNA transport
NUP98	RNA transport
RANBP2	RNA transport
NUP35	RNA transport
THOC2	RNA transport;Spliceosome
UFDF1L	Protein processing in endoplasmic reticulum
LMNA	Arrhythmogenic right ventricular cardiomyopathy (ARVC);Dilated cardiomyopathy;Hypertrophic cardiomyopathy (HCM)
PXN	Bacterial invasion of epithelial cells;Chemokine signaling pathway; Focal adhesion;Leukocyte transendothelial migration;Regulation of actin cytoskeleton;VEGF signaling pathway
STMN1	MAPK signaling pathway
HSPD1	ko05152;RNA degradation;Type I diabetes mellitus
ARFGAP1	Endocytosis
ZYX	Focal adhesion
SQSTM1	Osteoclast differentiation
RPL18	Ribosome
UPP1	Drug metabolism - other enzymes;Pyrimidine metabolism
WDR43	Ribosome biogenesis in eukaryotes
GTSE1	p53 signaling pathway
NCK1	ErbB signaling pathway;Pathogenic Escherichia coli infection;T cell receptor signaling pathway
EIF4B	mTOR signaling pathway;RNA transport
COPA	Neuroactive ligand-receptor interaction
THOC1	RNA transport;Spliceosome
FLNB	Focal adhesion;MAPK signaling pathway
PSMA4	Proteasome
ACIN1	mRNA surveillance pathway;RNA transport;Spliceosome
SF3B1	Spliceosome
STAT5B	Acute myeloid leukemia; Chemokine signaling pathway; Chronic myeloid leukemia; ErbB signaling pathway; Jak-STAT signaling pathway;ko05152;Measles;Pathways in cancer

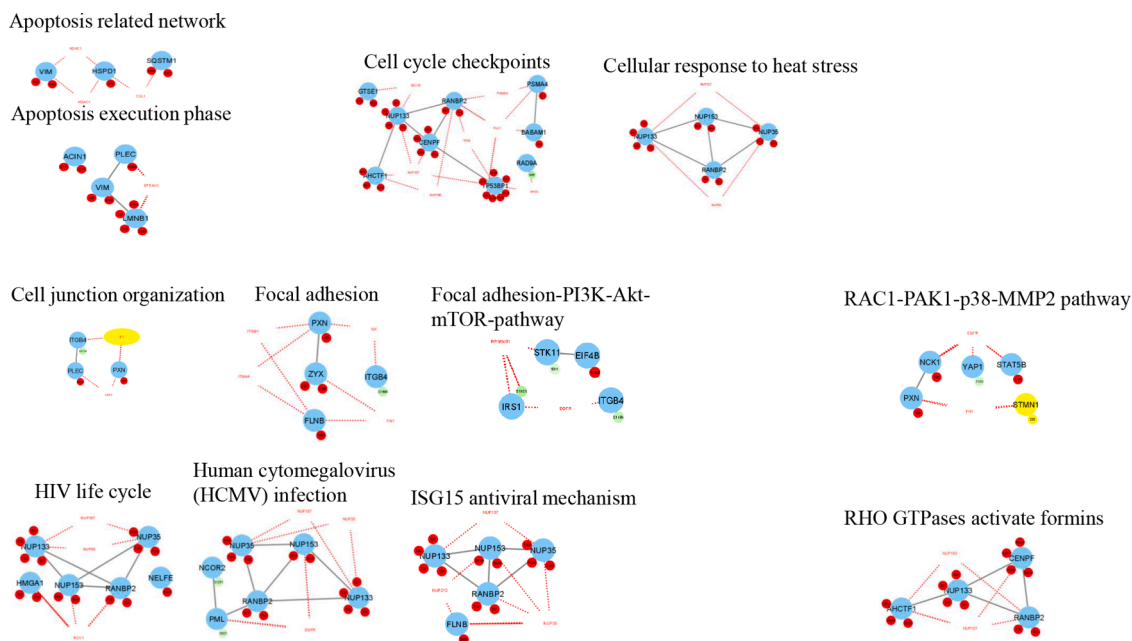
compounds are initially tested in xenografted tumour models in Scid mice which have their immune system compromised. We have tested this novel PAK4 inhibitor, PAKib in a syngeneic mouse model in which the mice used have a fully functioning immune system. This enables us

to explore the potential effect on tumour immune response. Indeed, our preliminary data from a flow cytometry study of tumour tissues dissected from mice treated by gemcitabine alone and gemcitabine plus PAKib, showed that PAKib may affect the infiltration of numbers of immune cells carrying markers of B cell (B220), T cell (CD4, CD8, PD-1) and myeloid cells (CD11b, Ly6c), and that PAKib may also affect the numbers of cells expressing PD-L1 (Supplementary Fig.1). These data implied the effect PAKib on the tumour immune response of PC. We are currently carrying on more extensive studies to investigate the role of PAKib in tumour immune response.

Only limited numbers of PAK4 inhibitors have been tested in human clinical trials although a few PAK4 inhibitors have been developed. PF-3758309, the first developed PAK4 specific inhibitor, failed in a phase I clinical trial (NCT00932126) because of unsatisfied pharmacokinetics (PK) and poor tumour response [27]. KPT-9274, a dual inhibitor targeting PAK4 and nicotin-amid phosphoribosyl transferase (NAMPT) is currently in a phase I clinical trial (NCT02702492) in patients with advanced solid tumours and non-Hodgkin lymphoma and its safety and efficacy are yet to be found out [28]. LCH-7749944, another PAK4 inhibitor showed inhibition of gastric cancer, but its clinical utility has yet to be explored [29]. Depside the preclinical agents developed, there is no product of PAK4-targeted compounds in clinic use. The unsuccessful clinical application of PAK4 inhibitors highlights the unmet clinical challenge of therapeutically targeting PAK4 in cancer treatment, specifically pancreatic cancer. PAKib, a newly developed PAK4 inhibitor suppressed PC in cells and in preclinical mouse models and enhanced the inhibitory effect of gemcitabine on PC *in vitro* and *in vivo*, which demonstrated a great potential of PAKib in clinical settings.

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**Fig. 6.** PAKib affected intracellular pathways. The intracellular pathways affected by PAKib were calculated and identified using Cytoscape software. The data with significant changes in phosphosites generated through Perseus was formatted and entered to Cytoscape software where the related network pathways (solid grey lines) were identified, and the extended network pathways (red dotted lines) were also deduced. The red notes represented PAKib-increased phosphosites in related proteins while the green notes the PAKib-decreased phosphosites in related proteins.

## Author contribution

HH & MN made conceptual design of the study, performed mouse experiments and overall data analysis; HH & CD performed kinase assay and cell-based assays; CD and CA performed the mass spectroscopy; LD did the flow cytometry assay and analyzed the data; JZ was responsible for the design and production of the inhibitor; HH, CA and YM performed the analysis of the data obtained from the mass spectrum.

## Declaration of Competing Interest

MN and JZ are on the Board of Pakinax Pty Ltd that was involved in the manufacture or the PAKib. JZ is current CEO of Pakinax Pty Ltd. HH is a medical advisor to Pakinax Pty Ltd and was involved in the manufacture of PAKib.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101329.

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