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PAK4 inhibition significantly potentiates Gemcitabine activity in PDAC cells via inhibition of Wnt/β-catenin, p-ERK/MAPK and p-AKT/PI3K pathways

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ABSTRACT

Pancreatic Ductal Adenocarcinoma (PDAC) remains one of the most difficult to treat cancers. Gemcitabine is still the standard of care treatment for PDAC but with modest survival benefit and well reported resistance. Here we explored potential of inhibiting p21 activated kinase 4 (PAK4), a downstream protein of KRAS oncogenic pathway, in combination with Gemcitabine in PDAC cells. PAK4 inhibition by KPT-9274 led to significant potentiation of Gemcitabine activity in PDAC cells, with an increase in apoptosis, DNA damage and cell cycle arrest. At molecular level, PAK4 inhibition dose dependently inhibited Gemcitabine-induced β-catenin, c-JUN and Ribonucleotide Reductase subunit 2 (RRM2) levels. PAK4 inhibition further inhibited levels of phosphorylated ERK (p-ERK); Gemcitabine-induced phosphorylated AKT (p-AKT), phosphorylated and total c-Myc. These results suggest possible role of β-catenin, p-ERK and p-AKT, key effector proteins of Wnt/β-catenin, MAPK and PI3K pathways respectively, in sensitisation of Gemcitabine activity with PAK4 inhibition. Our data unravel probable molecular mechanisms behind combination of PAK4 inhibition with Gemcitabine to counter PDAC, which may be unequivocally proved further with knock down of PAK4. Our findings provide a strong rationale to exploit the combination therapy of Gemcitabine and PAK4 inhibitor for PDAC at pre-clinical and clinical levels.

1. Introduction

Pancreatic Ductal Adenocarcinoma (PDAC) is one of the leading causes of cancer-related deaths in humans with a five-year survival rate of 9%, lowest among all types of cancers [1]. PDAC is usually detected only at the later stages when the tumour has already metastasized to distant organs [2]. Gemcitabine is still used as a standard chemotherapy agent for PDAC. Although used widely as a monotherapy or in combination with other chemotherapeutic agents, the survival benefit obtained with Gemcitabine remains modest for PDAC. Patients also develop resistance over the course of treatment [3]. In spite of the fact that clinical pieces of evidence of Gemcitabine resistance are documented, the underlying mechanism of resistance is still not well understood.

KRAS oncogenic pathway remains pivotal in PDAC progression as more than 90% of all PDACs express mutant KRAS [4]. PAK4 (p21-activated Kinase-4) lies in the signalling cascade of KRAS and is activated by small GTPases like Rac and Cdc42. PAK4 belongs to the family of p21-activated kinases, which consists of six isoforms [5]. PAK4, being at the nexus of many oncogenic pathways of PDAC, activates downstream targets such as Raf-1, β-catenin and NFkB. These substrates have a crucial role in controlling cancer hallmarks like proliferation, apoptosis, invasion and metastasis, suggesting key role of PAK4 in PDAC [6].

PAK4 is minimally expressed in normal pancreas but overexpressed in PDAC cell lines and patient samples with higher kinase activity [7,8]. PAK4 has been explored as a biomarker of Gemcitabine resistance as knocking down PAK4 increased sensitivity of PDAC cells to Gemcitabine [9]. Pharmacological inhibition of PAK4 with PF-3758309 increased the sensitivity of Gemcitabine and also maximally inhibited tumor growth of patient-derived cell line [10,11]. Similarly, another PAK4 inhibitor KPT-9274 inhibited the proliferation of multiple PDAC cell lines and

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exhibited synergistic activity with Gemcitabine [12,13]. Recently a novel PAK4 inhibitor, Pakib, has also been shown to increase gemcitabine sensitivity in PDAC cells [14]. So PAK4 is a potential therapeutic target in PDAC, especially in combination with Gemcitabine. However, a significant research gap still exists in understanding the molecular mechanisms behind PAK4 inhibition-mediated potentiation of Gemcitabine activity. The contribution of PAK4 downstream pathways such as PI3K/AKT, MAPK and Wnt/β-catenin, in PAK4 inhibitor-mediated sensitisation of Gemcitabine, also remains unexplored. A recent report suggests that MAPK and Wnt/β-catenin pathways have a pivotal role as simultaneous inhibition of both led to suppression of invasion and metastatic capacity of Gemcitabine-resistant PDAC cells [15]. Similarly, AKT inhibitor MK-2206 enhanced cytotoxicity of Gemcitabine indicating a role of PI3K/AKT pathway [16]. β-catenin, RAF and AKT, being direct targets of PAK4, suggest their possible role in the potentiation of Gemcitabine activity through PAK4 inhibition. However, it warrants a deeper analysis to elucidate the therapeutic benefit of this combination regimen. In this study, we discovered a novel mechanism by which PAK4 inhibition leads to the improvement of Gemcitabine sensitivity. Our findings strongly indicate a role of Wnt/β-catenin, p-ERK/MAPK and PI3K/p-AKT pathways in this phenomenon. Additionally, our findings also have a translational implication in identifying patients that might benefit from such a combination based on Gemcitabine-induced changes in key cellular biomarkers.

2. Materials and methods

2.1. Cell lines, culture conditions and research reagents

SW-1990, PSN-1, BxPC3 and PANC-1 cell lines were purchased from ATCC. MIA PaCa-2 and KP4 were purchased from Sigma-Aldrich and Accegen, respectively. MIA PaCa-2, PANC-1, SW-1990 were cultured in DMEM (Sigma-Aldrich) whereas PSN-1, BxPC3 and KP4 were cultured in RPMI1640 (Sigma-Aldrich). Both the Medias were supplemented with 10% Fetal Bovine Serum (Gibco) and 5% Penicillin-Streptomycin (Sigma-Aldrich). Cell lines were maintained at 37 °C with 5% CO2 in a cell culture incubator. Gemcitabine Hydrochloride was procured from Sigma Aldrich (catalogue number: G6423). PAK4 inhibitors (KPT-9274, catalogue number: S8444 and PF-3758309, catalogue number: S7094) were procured from Selleckchem.

2.2. Colony formation assay

MIA PaCa-2, PSN-1 and KP4 were seeded at a cell density of 5×10^2 cells/well and treated with Gemcitabine/KPT-9274, alone or in combination (final DMSO concentration: 0.1%). After 7 days of treatment, colonies were stained with crystal violet (0.5% crystal violet, 1% methanol and 1% formaldehyde in water) and de-stained with 10% acetic acid. Absorbance was read at 592 nm using Synergy Neo™ reader (Agilent, CA, USA). Average optical density of vehicle control was considered as 100% growth and percent inhibition was determined.

2.3. Anti-proliferation assay

MIA PaCa-2, PSN-1 and KP4 were seeded at a cell density of 1×10^3 cells/well. Treatment was carried out for 7 days in cell culture incubator. Assay was terminated with CellTiterGlo® (Promega) and luminescence was then read using Synergy Neo2™ reader. Cell viability is directly proportional to the amount of ATP. Average Relative Light units (RLUs) of vehicle control were considered as 100% growth, and percent inhibition was determined.

2.4. Cell cycle analysis

MIA PaCa-2 cells were seeded at a cell density of 1×10^5 cells/well and treatment was carried out for 48 h in cell culture incubator. Cells were harvested by trypsinisation, washed with PBS and fixed using 70% ethanol solution. Cells were stained in 50 μg/ml Propidium Iodide (Sigma-Aldrich) solution, containing 1% Triton X-100 (Sigma-Aldrich) and 0.1 mg/ml RNAse (Roche). Stained cells were washed with PBS and acquired using BD FACS Canto-II Flow Cytometer (BD Biosciences, USA). Analysis was done with BD FACSDIVA™ software, BD Biosciences. In brief, cells were acquired in area against width (PE) plot to get rid of aggregates. DNA content (PE stained) was plotted on a linear scale against cell count from gated cell population to obtain histograms. Demarcation of different stages of cell cycle was then carried out and % of cells in G0/G1 phase was considered for cell cycle arrest calculations.

2.5. Western blot and antibodies

MIA PaCa-2 and PSN-1 cells were seeded at a cell density of 1×10^5/well. After 48 h of treatment, cells were harvested by trypsinisation and lysed in 1× cell lysis buffer (Cell Signalling Technology) containing 1× protease and phosphatase inhibitor cocktails (Sigma-Aldrich). For proteasome inhibition, cells were treated with 10 μM of MG-132 (Sigma-Aldrich) for 4 h prior to harvesting (24/48 h post-treatment). Protein estimation was done with bicinchoninic acid method and 40 μg of protein was loaded. SDS-PAGE was carried out and protein was transferred on nitrocellulose membrane by wet transfer at 100 V for 1 h. The membrane was blocked in blocking buffer (5% BSA in TRIS Buffer Saline with 0.1% Tween-20). Incubation in primary antibody (overnight) and secondary antibody (2 h) solution was carried out with washing of membrane in between. The membrane was then developed with SuperSignal™ West Femto (Thermo Scientific) substrate using Bio-Rad Chemidoc™. Primary antibodies (phospho-PAK4 cat. no. 3241, PAK4 cat. no. 62690, cyclin E1 cat. no. 20808, CDK2 cat. no. 2546, CDK4 cat. no. 12790, cleaved PARP1 cat. no. 5625, cleaved caspase-3 cat. no. 9661, cleaved caspase-7 cat. no. 8438, Bax cat. no. 2772, Bcl-xl cat. no. 2764, p-H2AX cat. no. 2577, phospho-cdc2 cat. no. 9111, cdc2 cat. no. 77055, E2f-1 cat. no. 3742, cyclin A2 cat. no. 91500, β-catenin cat. no. 8480, phospho-β-catenin cat. no. 4176, c-JUN cat. no. 9165, RRM2 cat. no. 65939, phospho-GSK-3β cat. no. 9336, GSK-3β cat. no. 9315, phospho-ERK cat. no. 9101, ERK cat. no. 9102, phospho-S6 cat. no. 2211, S6 cat. no. 2217, phospho-AKT cat. no.4060, AKT cat. no.9272, phospho-c-Myc serine 62 cat. no. 13748, phospho-c-Myc Threonine 58 cat. no. 46650 and c-Myc cat. no. 5605) procured from Cell Signalling Technology and (cyclin D1 cat. no. 134175) from Abcam, were used at 1:1000 dilutions. β-Actin antibody (cat. no. A1978, Sigma-Aldrich) was used at 1:5000 dilution. Secondary antibodies (Anti-Rabbit IgG, HRP-linked cat. no. 7074 and Anti-Mouse IgG, HRP-linked cat. no. 7076) procured from Cell Signalling Technology were used at 1:5000 dilutions.

2.6. Densitometry analysis for Western blot

Densitometry analysis was done with ImageJ software. Ratio of total protein to β-actin for each sample was calculated for estimation of fold change with respect to vehicle control.

2.7. Synergy score calculation

Bliss and Highest Single Agent (HSA) synergy scores were calculated with Synergy Finder software (https://synergyfinder.org).

2.8. Statistical Analysis

IC_{50} values were generated utilizing non-linear regression analysis (Four Parameter Logistic curve fit) with the Graph Pad Prism software version 9.0 (Graph Pad Software, Inc., La Jolla, CA 92037, U.S.A.). IC_{50} values were represented as Mean ± S.E.M (Standard Error of Mean). Statistical analysis (One-way ANOVA *post hoc* test) was performed with the Graph Pad Prism. $P < 0.05$ was considered statistically significant.

3. Results

3.1. PDAC cell lines express phospho-PAK4 and total PAK4

PAK4, which belongs to class II of the PAK family, is constitutively phosphorylated at Serine 474, however, remains in an inactive state. Binding to upstream GTPases activates PAK4 [17]. We first assessed expression of phospho-PAK4 (Serine 474) and PAK4 on a panel of PDAC cell lines by Western blot. We observed that all the PDAC cell lines show expression of both phospho-PAK4 and PAK4 [Supplementary Fig. S1].

3.2. PAK4 inhibitor KPT-9274 in combination with Gemcitabine synergistically inhibits clonogenicity and proliferation of PDAC cells

We evaluated the combination effect of KPT-9274 with Gemcitabine in colony formation assay (CFA). In MIA PaCa-2, PSN-1 and KP4 cell lines, KPT-9274 showed robust combination effect with 2–3 fold shift in Gemcitabine IC_{50} (nM), suggesting potentiation of Gemcitabine's activity [Fig. 1A and B]. The combination hints towards synergism as suggested by Bliss and HSA synergy scores [Fig. 1B and Supplementary Fig. S2]. In anti-proliferation assay also, KPT-9274 significantly increased Gemcitabine sensitivity in all 3 tested PDAC cell lines [Fig. 1C].

3.3. KPT-9274 in combination with Gemcitabine enhances apoptosis and DNA damage in PDAC cells

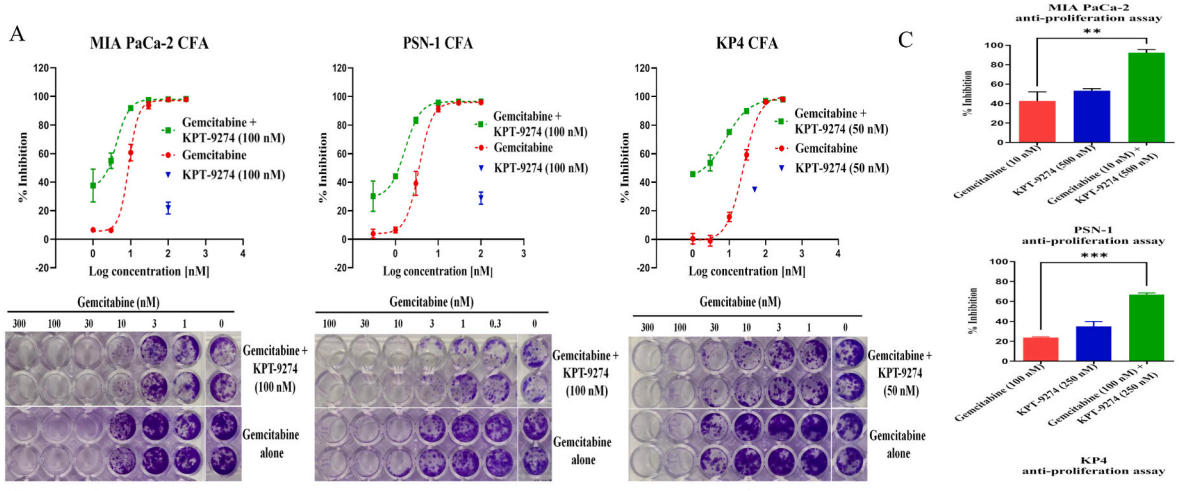
We assessed the effect of combination on apoptosis and DNA damage markers in MIA PaCa-2 and PSN-1 cells by Western blot. Gemcitabine alone exhibited a moderate increase in apoptosis markers (cleaved PARP1, cleaved caspase 3 and 7). However, combination with KPT-9274 significantly enhanced their expression, indicating an increase in apoptosis [Fig. 2A]. The combination regimen enhanced pro-apoptotic marker Bax and reduced anti-apoptotic marker Bcl-xl [Fig. 2A]. Further, KPT-9274 also enhanced DNA damage induced by Gemcitabine, as evident by the expression of p-H2AX [Fig. 2A]. Notably, KPT-9274 alone did not show an appreciable effect on apoptosis and DNA damage markers, underlining the role of PAK4 in combination.

3.4. KPT-9274 significantly increases Gemcitabine induced G0/G1 phase cell cycle arrest in PDAC cells

We investigated the effect of combination on cell cycle in MIA PaCa-2 cells by flow cytometry. Gemcitabine alone induced G0/G1 phase cell cycle arrest, evident by an increase in G0/G1 population ($P < 0.0001$). When combined with 10 μM of KPT-9274, the % of cells in G0/G1 phase significantly increased further ($P < 0.001$). As the proportion of cells in the G0/G1 phase increased, a subsequent decrease in G2/M phase was observed. This indicates accumulation of cells at G0/G1 phase, suggesting enhanced G0/G1 phase arrest in combination [Fig. 2B and C]. To further confirm this effect, we evaluated markers of cell cycle arrest by Western blot. Expression of cyclin D1 with its binding partner CDK4 was significantly reduced in the combination regimen. Correspondingly, expression of cyclin E1 and CDK2 was also inhibited [Fig. 2D]. KPT-9274 also modulated levels of other markers like E2f1, phospho-cdc2 and cyclin A2 when combined with Gemcitabine, further confirming the findings [Supplementary Fig. S3].

3.5. KPT-9274 in combination with Gemcitabine significantly inhibits Gemcitabine induced β-catenin and RRM2 in PDAC cells

Earlier reports suggest a possible link between Wnt/β-catenin pathway and Gemcitabine resistance in PDAC [18]. PAK4 intervenes in Wnt/β-catenin pathway, phosphorylating β-catenin at serine 675 and affecting its stability [19]. RRM2 acts as a primary regulator of RR enzyme complex activity. RRM2 is a Gemcitabine resistance marker and inhibition of RRM2 significantly modulates Gemcitabine sensitivity in PDAC cells [20,21]. c-JUN, a target gene of β-catenin, also acts as a transcription factor of RRM2, by binding to its promoter region [22]. So to understand the possible role of Wnt/β-catenin pathway in



B

Cell Line	Gemcitabine $IC_{50} \pm$ S.E.M. (nM)	Gemcitabine + KPT-9274 $IC_{50} \pm$ S.E.M. (nM)	BLISS Synergy score	HSA Synergy score
MIA PaCa-2	8.866 ± 0.595	4.137 ± 0.902	10.53	14.02
PSN-1	3.549 ± 0.049	1.675 ± 0.084	6.15	11.11
KP4	24.190 ± 1.329	7.920 ± 1.208	12.52	16.86

Fig. 1. *Combination effect of Gemcitabine and PAK4 inhibitor on clonogenicity and proliferation of PDAC cells.* KPT-9274 synergistically potentiates Gemcitabine activity. [A]: Concentration-response of Gemcitabine alone and combination regimen in CFA. KPT-9274 potentiates Gemcitabine activity in all 3 PDAC cells as observed by leftward shift of Dose Response Curve (DRC), *all data is n = 3.* [B]: Mean $IC_{50} \pm$ S.E.M (nM) and Bliss, HSA synergy scores for CFA combination. Combination scores suggest synergism, *> 10: synergistic* [C]: In anti-proliferation assay, combination showed significant improvement of Gemcitabine activity (**P < 0.01, ***P < 0.001 vs. Gemcitabine treated, One-way ANOVA *post hoc* test), *all data is n = 3.*

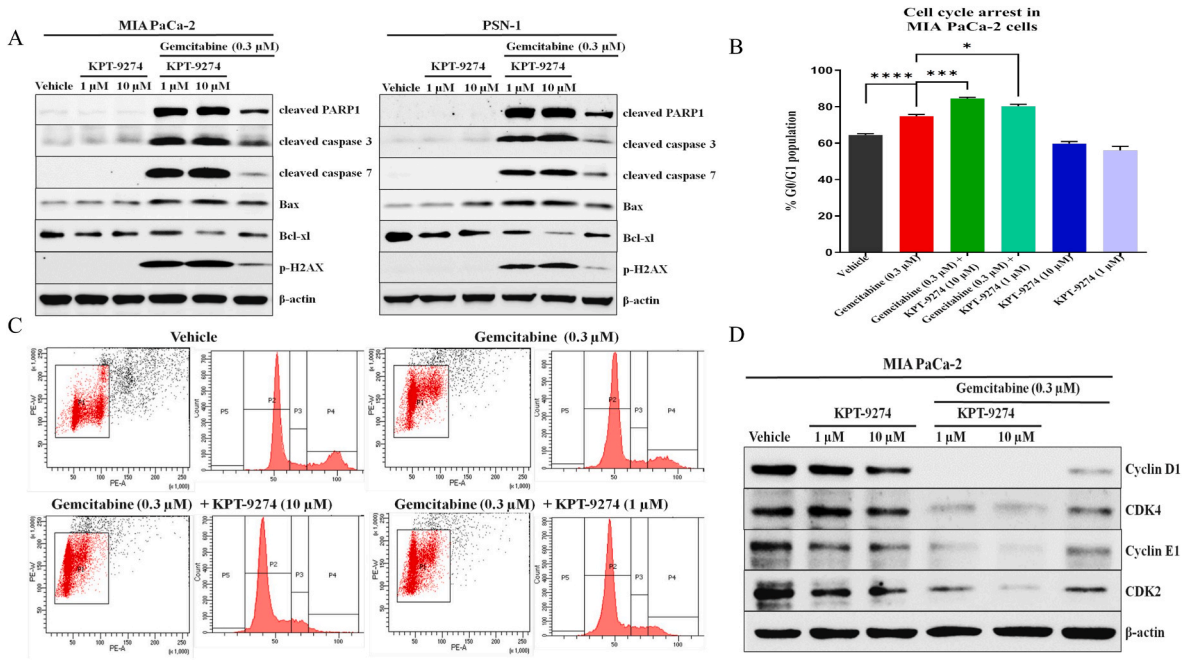


Fig. 2. *Combination effect of Gemcitabine and PAK4 inhibitor on apoptosis, DNA damage and cell cycle arrest in PDAC cells.* KPT-9274 significantly increases Gemcitabine-induced apoptosis, DNA damage and cell cycle arrest in MIA PaCa-2 and PSN-1 cells. [A]: Western blot analysis showed enhanced apoptosis (cleaved caspase 3, 7, cleaved PARP1, Bax and Bcl-xl) and DNA damage (p-H2AX) by combination of Gemcitabine and KPT-9274 [B, C, D]: Combination significantly augmented Gemcitabine induced G0/G1 cell cycle arrest (*P < 0.05, ***P < 0.001 vs. Gemcitabine treated, One-way ANOVA *post hoc* test) as observed with flow cytometry analysis of PI staining and Western blot for cyclin D1, CDK4, cyclin E1 and CDK2. C: P2: G0/G1, P3: S, P4: G2/M, P5: Sub-G1 cell population.

potentiation of Gemcitabine, we estimated the levels of β-catenin, c-JUN and RRM2 in the combination regimen.

Gemcitabine alone led to a concentration-dependent increase in the expression of β-catenin and c-JUN in MIA PaCa-2 and PSN-1 cells. RRM2, which had minimal basal expression in both the cell lines, was also elevated by Gemcitabine. Elevated levels of p-H2AX confirmed the DNA damage induced by Gemcitabine in these cells [Supplementary Fig. S4]. KPT-9274, alone as well as in combination inhibited levels of phospho-PAK4 and PAK4, confirming target engagement. Expression of phospho-β-catenin (serine 675) was also reduced [Fig. 3A]. These results

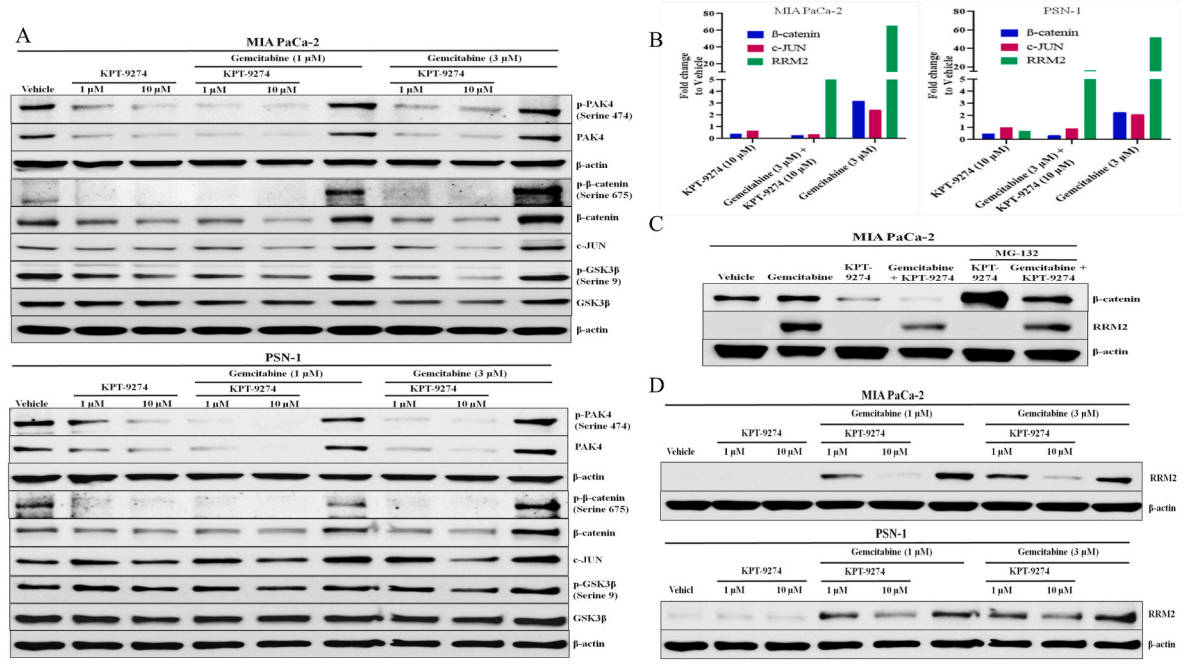


Fig. 3. *Combination effect of Gemcitabine and PAK4 inhibitor KPT-9274 on Wnt/β-catenin pathway in PDAC cells.* KPT-9274 inhibits Gemcitabine-induced β-catenin, c-JUN and RRM2 levels. [A]: KPT-9274 inhibited p-PAK4, PAK4 and p-β-catenin in MIA PaCa-2 and PSN-1 cells. Gemcitabine (3 and 1 μM) enhanced β-catenin and c-JUN expression. KPT-9274 in combination with Gemcitabine reduced both the proteins dose-dependently. Combination also inhibited p-GSK3β. [B]: Densitometry analysis of β-catenin, c-JUN and RRM2 levels in MIA PaCa-2 cells [C]: MG-132 treatment rescued β-catenin and RRM2 levels (Gemcitabine: 3 μM, KPT-9274: 1 μM). [D]: Gemcitabine alone enhanced while combination with KPT-9274 reduced RRM2 expression in MIA PaCa-2 cells.

confirmed the inhibition of PAK4 activity by KPT-9274. Interestingly, KPT-9274 treatment led to significant inhibition of Gemcitabine-induced β-catenin. Remarkably the reduction of β-catenin observed with combination was lesser than basal expression [Fig. 3A and B]. Gemcitabine-induced c-JUN expression was also inhibited in combination regimen [Fig. 3A and B]. To further elucidate this phenomenon, we co-treated proteasomal inhibitor MG-132 in MIA PaCa-2 cells. MG-132 treatment rescued β-catenin levels in both KPT-9274 alone and combination controls. This effect was sustained at both 48 h [Fig. 3C] and 24 h treatment sets [Supplementary Fig. S5], confirming PAK4 inhibitor (PAK4i)-mediated degradation of β-catenin. Interestingly, Gemcitabine-induced RRM2 levels were significantly inhibited with combination of KPT-9274 and MG-132 treatment partially impaired RRM2 inhibition [Fig. 3B, C, 3D, Supplementary Fig. S5]. Combination treatment also reduced phospho-GSK3β (Serine 9) suggesting inhibition of Wnt/β-catenin pathway (Fig. 3A). Thus, our results indicate a significant role of Wnt/β-catenin pathway in sensitisation of PDAC cells to gemcitabine with PAK4i.

3.6. KPT-9274 in combination with Gemcitabine inhibits p-ERK and Gemcitabine-induced p-AKT in PDAC cells

Previous reports suggest the involvement of MAPK pathway in Gemcitabine resistance of PDAC cells. Inhibition of the MAPK pathway has been shown to improve Gemcitabine sensitivity of PDAC through inhibition of p-ERK [23,24]. PAK4 has a significant role to play in MAPK pathway as it phosphorylates both cRAF and MEK, and knockdown of PAK4 is shown to reduce p-ERK levels in PDAC cells [6,7]. Given the direct intervention of PAK4 in MAPK pathway, we wanted to understand the possible role of p-ERK/MAPK pathway in the potentiation of Gemcitabine sensitivity in PDAC cells with PAK4i. We assessed this hypothesis by evaluating levels of p-ERK and its downstream protein phosphorylated S6 (Serine 235/236) in combination regimen. Gemcitabine alone did not show an appreciable effect on p-ERK [Supplementary Fig. S6]. KPT-9274 in combination with Gemcitabine significantly and maximally inhibited p-ERK in both the PDAC cell lines [Fig. 4A]. The effect on p-ERK was further corroborated by the inhibition of p-S6 (Serine 235/236) in combination regimen [Fig. 4A].

PI3K/AKT pathway also remains crucial in PDAC progression and has been a focus of research in drug discovery [25]. Activated AKT (p-AKT) is known to promote chemo-resistance of PDAC and AKT inhibitors have shown promise for combination therapy with Gemcitabine [16,26]. PAK4 directly interacts with p85 alpha subunit of PI3K and depletion of PAK4 inhibits p-AKT (Serine 473) levels in PDAC cells [27]. PAK4 knockdown also led to inhibition of p-AKT further confirming the role of PAK4 in PI3K/AKT pathway [7]. So we assessed the effect of PAK4 inhibition on p-AKT levels, especially in combination with Gemcitabine. We observed that Gemcitabine dose-dependently increased AKT activation in PDAC cells, as evident by p-AKT (Serine 473) levels [Supplementary Fig. S7]. Combination with KPT-9274 significantly reduced Gemcitabine induced p-AKT levels [Fig. 4B]. The combination also inhibited phospho-GSK3β (Serine 9), a substrate of AKT, further confirming the inhibition of AKT activity [Fig. 3A]. Our results thus indicate a potential role of both MAPK and AKT pathways (via inhibition of p-ERK and p-AKT respectively) in PAK4i-mediated potentiation of Gemcitabine activity in PDAC cells.

3.7. KPT-9274 in combination with Gemcitabine inhibits c-Myc levels PDAC cells

p-ERK and p-AKT (via GSK3β) are known to modulate c-Myc levels in cancer cells. p-ERK phosphorylates c-Myc at serine 62, leading to c-Myc stability. The second phosphorylation event on c-Myc at Threonine 58 is carried out by GSK3β, a target of p-AKT. Both phosphorylation sites are vital in Myc being subjected to proteasomal degradation [28]. Since we observed inhibition of both p-ERK and p-AKT-p-GSK3β, we assessed its implications on levels of phosphorylated c-Myc and total c-Myc. The combination showed significant inhibition of both p-c-Myc (Serine 62) and total c-Myc in PDAC cells [Fig. 4A and C]. We also observed inhibition of p-c-Myc (Threonine 58) levels [Supplementary Fig. S8]. MG-132 rescued levels of c-Myc, confirming degradation of c-Myc with combination of Gemcitabine and KPT-9274 [Fig. 4D, Supplementary

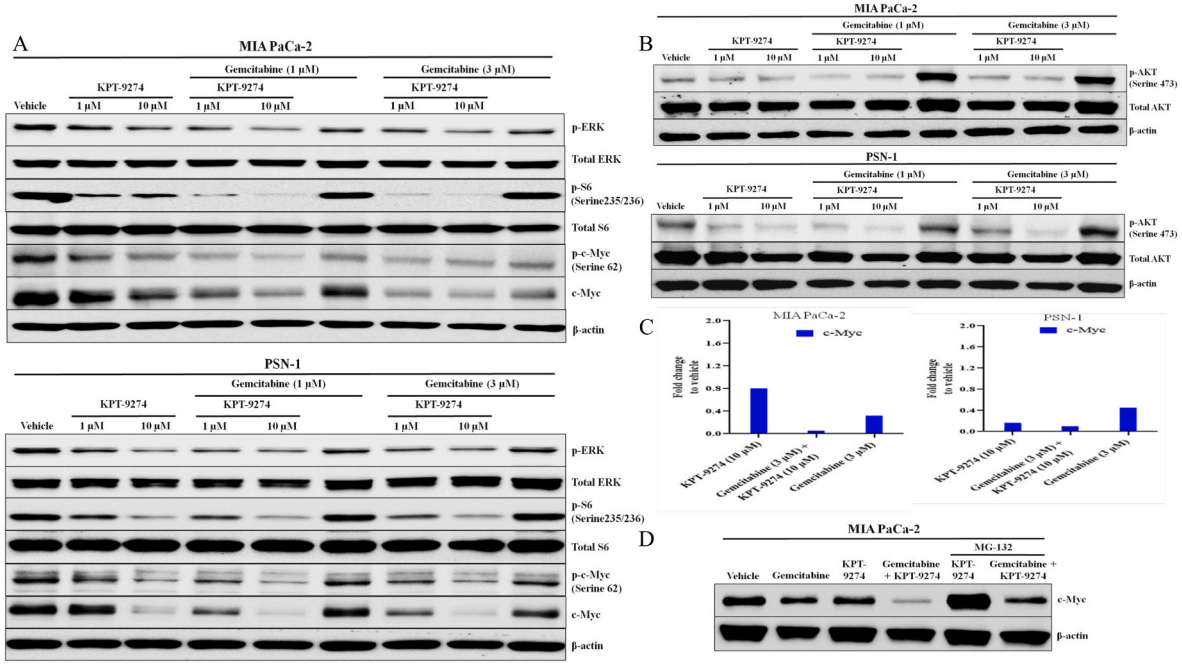


Fig. 4. *Combination effect of Gemcitabine and PAK4 inhibitor on p-ERK/MAPK and PI3K/p-AKT pathway in PDAC cells*. KPT-9274 inhibits p-ERK and Gemcitabine-induced p-AKT levels. [A]: Combination regimen exhibited robust inhibition of p-ERK, p-S6, p-c-Myc (serine 62) and c-Myc [B]: KPT-9274 dose-dependently decreased Gemcitabine-induced p-AKT levels. [C]: Densitometry analysis of c-Myc levels in MIA PaCa-2 cells [D]: MG-132 treatment rescued c-Myc levels in MIA PaCa-2 cells (Gemcitabine: 3 μM, KPT-9274: 1 μM).

Fig. S9].

3.8. Inhibitory effects on β-catenin, RRM2 and c-Myc were sustained with combination of PF-3758309 and Gemcitabine in PDAC cells

So far, our results indicate a possible role of Wnt/β-catenin, p-ERK/MAPK and PI3K/p-AKT pathways in the potentiation of Gemcitabine activity by PAK4i. However, KPT-9274 along with PAK4 also inhibits enzyme Nicotinamide Phosphoribosyl Transferase (NAMPT) [29]. Thus we wanted to confirm the specific role of PAK4 in mediating the Gemcitabine sensitivity in PDAC cells. We evaluated another PAK4 inhibitor PF-3758309 for its effect on β-catenin, RRM2 and c-Myc levels. Levels of phospho-PAK4 were inhibited in presence of PF-3758309 in both the PDAC cells [Fig. 5A]. As observed with KPT-9274, PF-3758309 also inhibited Gemcitabine-induced β-catenin (and p-β-catenin), c-JUN levels [Fig. 5A]. Gemcitabine-induced RRM2 levels were also inhibited [Fig. 5A]. Further, combination regimen showed robust inhibition of both p-c-Myc (Serine 62) and c-Myc levels **[**Fig. 5B**]**. These findings corroborate the results produced with KPT-9274, further confirming the role of PAK4 in potentiation of Gemcitabine activity.

4. Discussion

Gemcitabine remains the first line of treatment for PDAC including advanced pancreatic cancers [3]. However, intrinsic as well as acquired resistance against Gemcitabine significantly limits its effectiveness [20,30]. Thus, identification of molecular markers to predict gemcitabine resistance is of utmost importance and is a subject of extensive research. RRM2 has been shown to be one of the important markers for Gemcitabine sensitivity as patients which showed poor prognosis had higher levels of RRM2 [20,31]. Similarly, PAK4 was evaluated as a predictive marker for gemcitabine sensitivity in PDAC cells and its down-regulation enhanced human Equilibrative Nucleoside Transporter 1 (hENT1) expression [9]. This inverse correlation between PAK4 and hENT1 makes PAK4 a potential target for co-treatment with Gemcitabine. Our work has first time revealed a correlation between PAK4 and RRM2. Most importantly we observed inhibition of Gemcitabine induced RRM2 with PAK4 inhibition.

PAK4 lies downstream of KRAS oncogenic signalling pathway and has serious repercussions on mutant KRAS-mediated cancer cell growth [32]. KRAS remains a crucial driver of cancer proliferation, especially of PDAC, and several attempts have been made to target inhibitors against effector molecules in this pathway, including the RAC1 Small GTPases effector like PAK4 [33]. All this has culminated in PAK4 being explored as a therapeutic target for PDAC management. Remarkably PF-3758309, KPT-9274 and pakib showed promise as a combination partner with Gemcitabine [10–14]. So PAK4 inhibitor can be utilised as a combination therapy partner with Gemcitabine for PDAC management. However, molecular insights are needed to further exploit this phenomenon in order to come up with potential PDAC therapy. Our research primarily focussed on this aspect of the combination treatment of Gemcitabine and PAK4i. Here we tried to unravel the molecular mechanisms of combination effect in PDAC cells.

PAK4 remains at the core of many molecular pathways involved in multiple cellular processes like Wnt/β-catenin, MAPK and PI3K/AKT [34]. We hypothesised the involvement of these PAK4 downstream pathways in PAK4i-mediated potentiation of gemcitabine activity in PDAC cells. We observed a dose-dependent increase in β-catenin and c-JUN levels with Gemcitabine in PDAC cells. Wnt/β-catenin is one of the crucial pathways in pancreatic carcinogenesis, involved in survival and imparting drug resistance [35]. So increased β-catenin expression possibly indicates a survival mechanism initiated by PDAC cells to counter genomic stress of Gemcitabine. Not only did Gemcitabine increase β-catenin expression but it also causes PDAC cells to dose-dependently induce expression of RRM2. It is possible that induced RRM2 by Gemcitabine is a result of transcriptional activity of induced c-JUN as described earlier [22]. In fact, RRM2 levels are induced with Gemcitabine in PDAC cells and highly up-regulated in case of Gemcitabine resistant PDAC cells [36].

In our studies, when KPT-9274 was evaluated in combination with Gemcitabine, it directly affected the survival of PDAC cells as indicated by enhanced anti-proliferation and anti-clonogenicity. The combination significantly elevated apoptosis and G0/G1 cell cycle arrest induced by Gemcitabine. When we tried to elucidate the molecular mechanisms behind this potentiation, we observed a marked decrease in Gemcitabine-induced β-catenin and RRM2 levels in combination with KPT-9274. Similar effects were seen with other PAK4i (PF-3758309), showing that the effect was specific to the inhibition of PAK4 activity. Further experiments in PDAC cells with PAK4 knockdown can validate this phenomenon. However, this is still a significant finding and

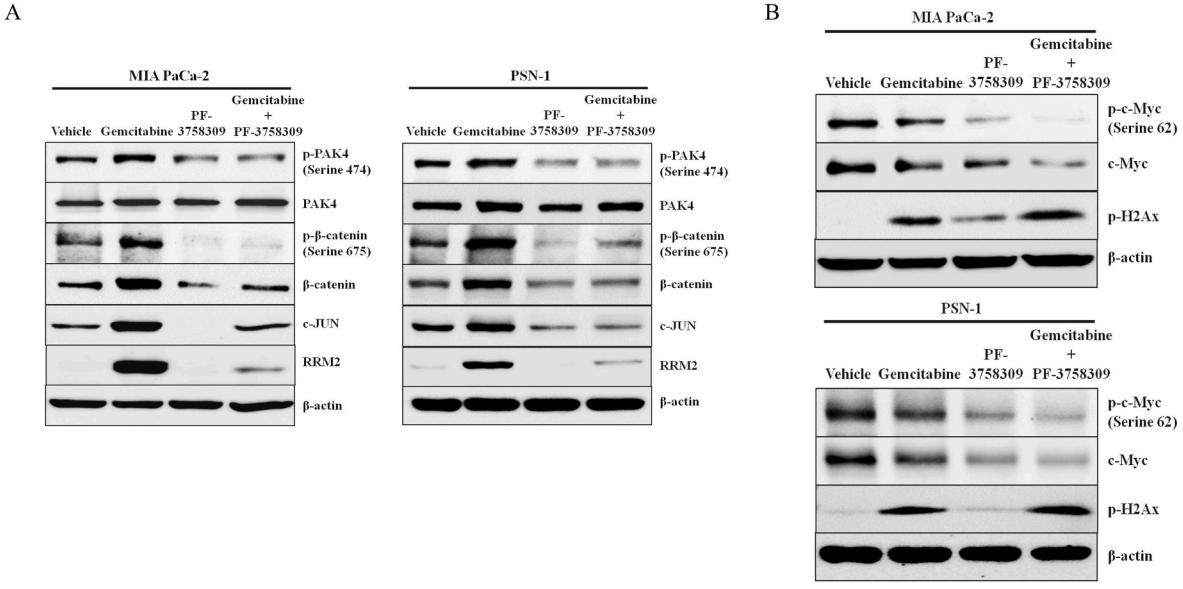


Fig. 5. *Combination effect of PAK4 inhibitor with Gemcitabine was sustained with PF-3758309.* PF-3758309 (0.1 μM) also in combination with Gemcitabine (3 μM) inhibits PAK4 downstream pathways in MIA PaCa-2 cells. [A]: Combination regimen showed significant inhibition of p-PAK4, p-β-catenin and Gemcitabine-induced β-catenin, c-JUN, RRM2 levels. [B]: Robust inhibition of p-c-Myc (serine 62) and c-Myc was observed in combination treatment.

highlights the strong combination potential of PAK4i and Gemcitabine. As RRM2 remains a crucial clinical marker, especially in case of Gemcitabine-resistant PDAC, this result bolsters confidence in combining PAK4 inhibitor with Gemcitabine. RRM2 expression can be regulated at gene, protein as well as by post-translational modification [37]. We observed moderate rescue of RRM2 levels with proteasome inhibitor co-treatment, hinting towards RRM2 degradation in combination treatment. However, the effect on RRM2 gene expression via c-JUN inhibition cannot be ruled out.

Besides the Wnt/β-catenin pathway, our research also unravelled the involvement of two other crucial pathways in PAK4i-mediated Gemcitabine sensitisation of PDAC cells. ERK has been manifested as a crucial culprit of PDAC progression and inhibition of ERK reversed the acquired resistance to Gemcitabine [23]. Similarly, p-AKT has been investigated as a prognostic biomarker for Gemcitabine resistance and AKT inhibitors exhibited potential for co-treatment with gemcitabine [16,38]. KPT-9274 was able to inhibit p-ERK levels in PDAC cells. Combination with Gemcitabine strikingly reduced p-ERK and p-S6 levels further. Surprisingly, KPT-9274 and Gemcitabine co-treatment had a marked effect on p-S6 levels as compared to either of them alone. Combination was also able to inhibit Gemcitabine induced p-AKT levels in PDAC cells. Simultaneous inhibition of p-ERK and p-AKT pathways with PAK4i (in combination with Gemcitabine) highlights the potential of our findings; however additional investigation needs to be carried out with PAK4 knockout to corroborate. Our data lays path forward to further investigate this mechanism in order to come up with better targeting strategies.

When we assessed this further, we observed significant inhibition of c-Myc levels in combination regimen. This is a pivotal finding of our research as c-Myc is involved in multiple pro-tumorogenic pathways, including drug resistance [39]. We detected a pronounced reduction of both p-c-Myc and c-Myc in combination treatment. Reduced p-ERK-c--Myc levels have been shown to cause detrimental effects on Gemcitabine-treated PDAC cells [40]. Since c-Myc is controlled by both p-ERK/MAPK and PI3K/AKT pathways, we propose this modulation of c-Myc is a result of inhibition of both p-ERK and p-AKT. Proteasomal inhibitor rescued c-Myc levels further crediting our hypothesis that concomitant treatment of PAK4i and Gemcitabine induced c-Myc degradation. Our results indicate that inhibition p-ERK-p-AKT-c-Myc axis is leading to the potentiation of Gemcitabine sensitivity of PDAC cells with PAK4 inhibition.

5. Conclusion

In summary, our research highlights the potential involvement of Wnt/β-catenin, p-ERK/MAPK and p-AKT/PI3K in PAK4i-mediated potentiation of Gemcitabine activity. Based on our findings, the enhanced anticancer activity of Gemcitabine in combination with PAK4 inhibitor can be attributed to the simultaneous inhibition of these key signalling cascades. These findings highlight further need to investigate the involved molecular pathways in-depth for better understanding of cellular and molecular changes. It also warrants evaluation in xenograft studies to come up with an effective combination regimen in countering Gemcitabine resistance of PDAC. Our findings can be a stepping stone towards further pre-clinical and clinical studies to emerge with successful combination treatment strategies.

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Research data/raw data

Experimental Raw Data is available with the corresponding author. Data will be made available on request.

Credit author statement

Charudatt Samant: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Roles/Writing - original draft, Writing - review & editing. Ramesh Kale: Conceptualization, Data curation, Methodology. Anand Bokare: Formal analysis, Supervision and Writing - review & editing. Mahip Verma: Formal analysis, Statistical Analysis, Supervision and Writing - review & editing. K. Sreedhara Rangnath Pai: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Writing - review & editing. Mandar Bhonde: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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