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The Dual PI3K/mTOR Inhibitor NVP-BEZ235 Enhances the Antitumoral Activity of Gemcitabine in Human Pancreatic Cancer Cell Lines

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Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is one of the most common malignant tumors still associated with poor prognosis in advanced stages. Gemcitabine is one of the standard agents for the treatment of PDAC, without having a major impact on the clinical outcome. Combining two compounds acting via different ways of action may result in a better efficacy.

Methods: We investigated the effects of gemcitabine in combination with the dual PI3k/mTOR inhibitor BEZ235 in four human pancreatic cancer cell lines (Panc-1, BxPC-3, MiaPaCa-2 and AsPC-1). The cells were analysed with MTT assay for cell viability, FACS-analysis for cell cycle distribution. Real-time RT-PCR and Western blot for survivin/BIRC5, STAT3, BCl-xL and WNT16 mRNA and protein expression and γH2AX.

Results: Application of NVP-BEZ235 or gemcitabine inhibited cell viability of AsPC-1 and BxPC-3 cells while Panc-1 and MiaPaCa-2 remained nearly unaffected. Combined treatment of gemcitabine and BEZ235, however, enhanced the inhibitory effect on cell viability of Panc-1 and MiaPaCa-2 cells of about 80% compared to control cells. This effect was boosted by time-delayed application of the two compounds. The biggest impact on cell growth, viability and downstream gene regulations were achieved by a sequential incubation with gemcitabine followed by BEZ235 24 hours later.

Conclusions: Combining gemcitabine with dual PI3K/mTOR inhibitors like NVP-BEZ2235 improved the efficacy on growth inhibition in human pancreatic cell lines especially by sequential application of both agents.

Keywords: Pancreatic cancer; NVP-BEZ235; Gemcitabine; PI3K/AKT/mTOR pathway

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most common malignant tumors with poor prognosis and a disappointing 5-year overall survival rate of about only 15% in advanced stages [1-3].

Pancreatic cancer is a result of multiple genetic alterations for example activation of the K-RAS or BRAF oncogenes, as well as inactivation of the tumor-suppressor genes DPC4, CDKN2A and TP53 [4,5]. Additionally, downregulation of STAT3 signaling has been shown to induce apoptosis but also to promote anti-apoptotic gene expression in human pancreatic cancer cells [6-8]. Less frequently altered genes in PDAC are e. g. amplification of the epidermal growth factor receptor (EGFR), Akt2 and HER2/neu [9]. Moreover, an increased activation of the PI3K/AKT-pathway has been detected in about half of pancreatic cancers [9,10] possibly activated by oncogenic K-RAS expression [11]. Mammalian target of rapamycin (mTOR), another downstream effector in the PI3K pathway, is also activated in many PDACs and the inhibition of mTOR decreases growth of several PDAC cell lines [12,13]. Additionally, the deletion of PTEN promotes cancer progression and invasion [14].

Survivin, the smallest member of IAP (inhibitor of apoptosis) family, is a dual functional protein acting as a critical apoptosis inhibitor and key cell cycle regulator. Aberrant activation of receptor tyrosine kinases (RTKs) and the downstream signaling, such as PI-3K/Akt, MEK/MAPK, mTOR, and STAT pathways, have frequently been shown to upregulate survivin expression, leading to an increase of tumor growth [15].

Gemcitabine (difluorodeoxycytidine: 2`,2`-dFdC, GEM) has been the standard in first-line therapy for decades although it has a response rate of less than 20%. As a deoxycytidine analogue, gemcitabine is phosphorylated intracellular by deoxycytidine kinase (dCK) to produce active diphosphate (dFdCDP) and triphosphate (DFdCTP). This tends to result in inhibiting ribonucleoside reductase (RR) and blocking DNA replication [16]. The main back bone agent in PDAC is still gemcitabine, but meanwhile the combination FOLFIRINOX demonstrated some superiority to gemcitabine alone [17]. Additionally, the combination of gemcitabine with nab-paclitaxel improved overall survival (OS) from 6.7 to 8.5 months [18]. Combinations of gemcitabine with VEGFR directed tyrosine-kinase inhibitors (TKI) as sunitinib or axitinib were disappointing [19,20]. So, there is still a medical need for new more effective therapeutic options.

NVP-BEZ235 is a novel dual PI3K/mammalian target of rapamycin (PI3K/mTOR) inhibitor, undergoing phase I/II in human clinical trials [21]. The inhibitory effect of this imidazole [4,5-*c*]quinoline derivative is based on its competing activity to the ATP-binding cleft of these enzymes [22]. NVP-BEZ235 or the dual PI3K/mTOR inhibitor NVP-BGT226 mainly induce G0/G1 arrest in various tumor cell lines [23] rather than apoptosis and regulate survivin gene expression in human pancreatic cancer cells [24,25]. Recently, Awasthi et al. [26] reported about an enhancing effect of NVP-BEZ235 on chemotherapy and antiangiogenic response in pancreatic cancer. In regard to the role of

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PI3k/mTOR in PDAC and the mode of action of NVP-BEZ235, the combination of gemcitabine with the dual PI3k/mTOR inhibitors seems to be an interesting approach.

Therefore, we examined whether the dual PI3K/mTOR-inhibitor NVP-BEZ235 may improve the cytotoxic effect of the standard drug gemcitabine and whether the sequencing of both agents might be of importance. Additionally, we analysed the molecular mechanisms of NVP-BEZ235 besides PI3K/mTOR inhibition resulting in enhancing antitumor effects when combined with gemcitabine.

Materials and Methods

Cell lines and treatments

The pancreatic cancer cell lines MiaPaCa-2[1], Panc-1[2], AsPC-1[2] and BxPC-3[3] ([1] kindly provided by Prof. F. Gansauge, Ulm; [2] purchased from CLS, Heidelberg, Germany; [3] purchased from ATCC, USA) were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100U/mL Penicillin/0.1 g/L, Streptomycin and 4mM L-Glutamin. The cell lines were tested negative for mycoplasma using the PCR-mycoplasma diagnostic-kit VenorGeM (Minerva biolabs, Berlin, Germany). Fetal calf serum (FCS) and Penicillin/Streptomycin were purchased from PAA Laboratories (Coelbe, Germany). RPMI 1640, phosphate-buffered saline (PBS) and glutamine were obtained from Invitrogen (Karlsruhe, Germany). The dual PI3K/mTOR-Inhibitor NVP-BEZ235 (kindly provided by Novartis Pharma, Basel, Switzerland) was dissolved in DMSO (Sigma-Aldrich, Deisenhofen, Germany) as 10mM stock solution and kept frozen (-20°C) until use. Gemcitabine (Fresenius Kabi, Bad Homburg, Germany) was dissolved in PBS as 10mM stock solution and used immediately.

Proliferation assay

Cell proliferation was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich, Deisenhofen, Germany). Cells, incubated with different concentrations of NVP-BEZ235 (500nM-5 µM), gemcitabine (20-100 µM) or in combination with NVP-BEZ235/gemcitabine and control cells (DMSO-treated) were analysed after 24, 48 and 72 hours. All assays were performed in triplicate. A solution of MTT (5 mg/mL in phosphate-buffered saline) was added to each flask to a final concentration of 0.5 mg/mL. After 3 h incubation dark blue formazan was solubilized with isopropanole/0.04N HCl. Absorbance was measured at 590 nm (Ultrospec III, Pharmacia Biosystems, Freiburg, Germany).

Transfection (siRNA)

To compare the effect of the dual PI3k/mTOR inhibitor NVP-BEZ235 with the specific inhibition of PI3k and mTOR pathway, both pathways were blocked in mRNA level with specific siRNA (PI3k siRNA #sc-270137 Santa Cruz, California, USA), mTOR siRNA Signal Silence II (New England Biolabs, Frankfurt, FRG). For this, the cells were replaced in serum-free medium and transfected with 2 µl siRNA + 10 µl Lipofectamine (Invitrogen 11668-019 by Life Technologies) in 88 µl of Opti-MEM ® I (reduced serum medium by Invitrogen Life Technologies) with siRNA alone or in combination with both siRNA and/or gemcitabine. After incubation for 3h, Opti-MEM ® was removed and 5 ml of new medium was added to the cells.

Determination of the number of cells

To determine the number of vital cells these were mixed with trypan blue (Trypan blue solution: 0.5% (w / v) trypan blue in PBS). While viable cells in the layer appear under the microscope light, the dead cells are stained in dark blue. Cells were counted in the Neubauer chamber.

RNA preparation and RT-PCR

Cells were harvested, centrifuged and washed with PBS. Total RNA was isolated with RNeasy mini kit (Qiagen, Hilden, Germany). All cDNA products were purchased from Invitrogen (Karlsruhe, Germany). The cDNA was synthesized from 2 µg total RNA and 1 µL (200 U) Superscript II RT, 4 µL 5 x first strand buffer, 1 µL random primers, 2 µL DTT (0.1 mM), 0.2 µL dNTP-mix (100 mM) in 20 µL reaction volume. Reaction conditions were 25°C 10', 42°C 50' and 70°C 15'.

Real-time RT-PCR

The cDNA was subjected to Real-time RT-PCR analyses targeting survivin/BIRC5, BCL-xL, STAT3, WNT16, mTOR, PI3k p110 and GAPDH as control. Analyses were performed using StepOne Real-time PCR System (Applied Biosystems, Darmstadt, Germany). Relative gene expression values were determined by the ΔCt method using the StepOne v2.1 software (Applied Biosystems, Darmstadt, Germany). Data are presented as the fold difference in expression normalized to the housekeeping gene GAPDH as endogenous reference and relative to control cells.

The following primers were used:

STAT3	Forward primer: GCACAGATTGCCTGCATTG
	Reverse Primer: CTGCTAATGACGTTATCCAGT
Survivin/BIRC5	Forward Primer: TTTCTCAAGGACCACCGCAT
	Reverse Primer: ATGAAGCCAGCCTCGGCCAT
BCL-xL	Forward Primer: CTCCCGACCTGTGATAC
	Reverse Primer: CCAAAGCCAAGATAAGATTCTG
WNT 16	Forward Primer: TGCCTGTAAGAGTGTTCCCAGA
	Reverse primer: CAATCCTCTGGATCAGCTTGTG
mTOR	Forward primer: GGC CGA CTC AGT AGC AT
	Reverse Primer: CGG GCA CTC TGC TCT TT
PI3K p110	Forward primer: CTGTGTGGGACTTATTGAGGT GGTGC
	Reverse Primer : GGCATGCTGTCGAATAGCTAGA TAAGC
GAPDH	Forward Primer: GCCTCAAGATCATCAGCAATG
	Reverse Primer: CTTCCACGATACCAAAGTTGTC

Amplification mixes (20 µL) contained 1 µL of Taq Man Gene Expression Assay (20X; Applied Biosystems, Darmstadt, Germany), 2 µL cDNA template, 10 µL Fast Advanced Master Mix (2X) (Applied Biosystems, Darmstadt, Germany) and 7µL Nuclease-Free Water (Ambion, Applied Biosystems, Darmstadt, Germany). Thermal cycling consisted of 2 min at 50°C, 20 sec 95°C, followed by 40 cycles at 95°C for 1 sec and 60°C for 20 sec using the StepOne System. All tests were carried out in triplicate.

Western blot analysis

For Western blot analysis the cells were plated in T-25 flasks and grown to 40-50% confluency. The cells were treated with NVP-BEZ235 (1 µM) and gemcitabine (30,50 µM) for 72 hours. Control cells were

incubated with DMSO alone. The cells were harvested in lysis buffer (50 mM Tris pH 8.0, 2% SDS, 1 mM EDTA, 150 mM NaCl). Viscosity was reduced by incubation with benzonase (Merck, Darmstadt, Germany). The homogenate was centrifuged for 10 min at 14,000 × g and the supernatant was used for protein determination. The protein amount was measured by the Lowry method (Bio-Rad DC Protein Assay, Bio-Rad, Germany). Cell lysate proteins were separated by SDS-PAGE gel, blotted to Trans-Blot Transfer Medium Membrane (Bio-Rad, Hercules, USA) and probed with anti-survivin/BIRC5 (sc-17779), mTOR Antibody(#2972cell Signaling), Phospho-mTOR Ser2448 Antibody (#2971 cell Signaling), Akt pan (#2920S cell Signaling), Anti-AKT2 antibody (ab13991), Anti-AKT2 phospho S474 (#ab38513), anti-BCL-xL antibody (sc-8392), anti-PARP-1 antibody (sc-8007) and anti-a-Tubulin (sc-8035) from Santa Cruz Biotechnology (California, USA). All Western blots were developed using the ECL technique (Amersham Biosciences, UK).

Cell cycle analysis

For cell cycle analysis, cells were plated in T-25 flasks and grown to 40-50% confluence. The cells were treated with NVP-BEZ235 (1 μM) and gemcitabin (30 μM) for 24-72 hours. The cells were harvested and washed with PBS and fixed in 70% cold ethanol over night at -20°C, additionally treated with 1 mg/mL of RNase A (Sigma-Aldrich) for 30 min and stained with 100 μg/mL of 7-AAD. The DNA content of 10,000 cells was determined.

Apoptosis assay/FACS analysis

PE Annexin V Apoptosis Detection Kit I (#559763, BD Pharmingen).Cells were plated in T-25 flasks and grown to 40-50% confluence. The cells were incubated with NVP-BEZ235 (1 μM) and gemcitabine (30 μM) for 24-72 hours. Control cells were incubated with DMSO alone. Cells were washed with PBS and resuspended in 100 μl incubation buffer. Add 5 μl PE Annexin V and 7-AAD and incubated for 15 min at room temperature in the dark. 400 μl of 1 x binding buffer to each sample was added and at least 10,000 cells were analysed by flow cytometry.

Flow-cytometry measurement for γH2AX

In brief, cells were washed twice with 1x PBS and incubated with trypsin. The cell pellet was suspended in 500 μl ice cold PBS + 10% FCS+ 1% sodium azide. The cells were incubated with γH2AX primary antibody (1:500) (Abcam rabbit polyclonal γH2AX phospho S139 ab11174) for 30 min at 4°C and washed 3x with cold PBS + 10% FCS+1% sodium azid. The cells were 20-30 min incubated with secondary polyclonal goat anti rabbit IgG-Fc (FITC) antibody (1:250) (Abcam ab97199) in 3% PBS/BSA+1% sodium azide at 4°C in the dark. Washed three times with 3% PBS/BSA+1% sodium azide and suspended in 1 mL PBS.

Statistical analysis

All data expressed are the mean ± SEM from at lStatistical diexpressed as mean with standard error of mean. The statistical significance of difference was analyzed by two-way analysis of variance (ANOVA) and/or Student's t-test using GraphPadPrism 6.0 software. A value of $P<0.05$ was considered significant.

Results

The effect of NVP-BEZ235 and gemcitabine on cell viability and cell growth

Our results show that application of NVP-BEZ235 (1 μM) and gemcitabine (30 μM) influences cell viability in BxPC-3 and AsPC-1 cells more pronounced than in Panc-1 and MiaPaCa-2 cells. Both dosages are based on diluting experiments choosing the optimal range (data not shown). Combined treatment of gemcitabine and NVP-BEZ235 lead to a greater inhibitory effect than single agents on cell viability in Panc-1 and MiaPaCa-2 cells, which are both gemcitabine resistant, but not in BxPC-3 and AsPC-1 cells. This effect is amplified by time-delayed application of these compounds in two of the cell lines ($p<0.001$). The biggest effects on cell growth inhibition are achieved by a pretreatment with gemcitabine followed by application of NVP-BEZ235 24 hours later (Figure 1).

These results are also reflected by the growth curve of the four cell lines treated with both agents alone or in combination using different sequences showing the sequence GEMàBEZ to be the most effective one in inhibiting cell growth (Figure 1; $p<0.001$).

Real-time RT-PCR analysis of Survivin, STAT3, BCL-xL, and WNT16 mRNA expression

An increased gene expression of survivin/BIRC5 mRNA can be observed after treatment with gemcitabine, while treatment with NVP-BEZ235 decreased the expression of survivin/BIRC5 mRNA in three cell lines.

The cell lines MiaPaCa-2, AsPC-1 and BxPC-3 show a decreased expression level of survivin/BIRC5 mRNA after NVP-BEZ235 alone or simultaneous and time-delayed treatment with gemcitabine ($p<0.001$). Interestingly, the simultaneous treatment in PANC-1 cells did not clearly reduce the survivin mRNA expression compared to controls or gemcitabine alone.

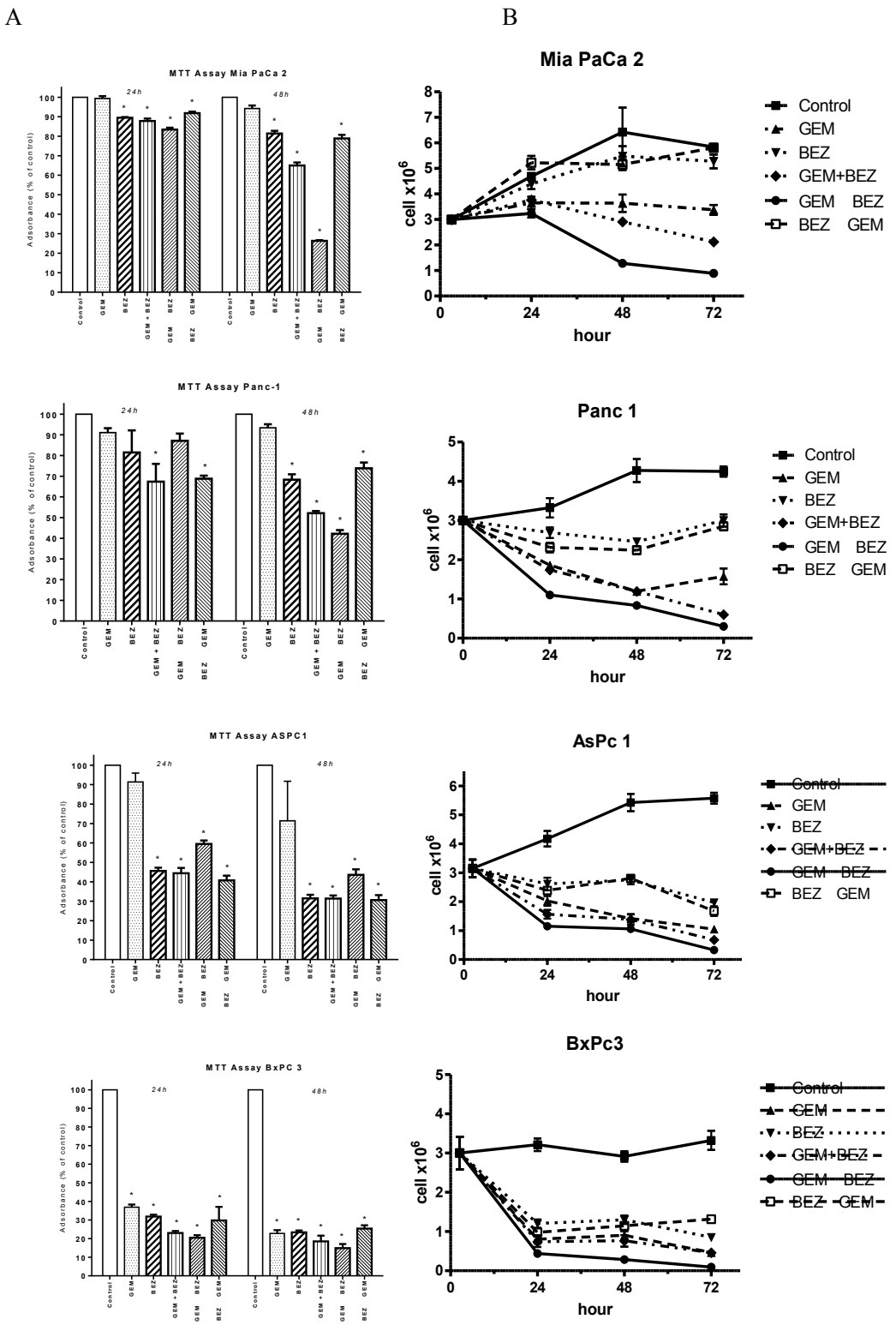
Analogue to survivin/BIRC5, STAT3 gene expression is increased after gemcitabine administration, while treatment with NVP-BEZ235 alone or in combination reduces the expression of STAT3-mRNA in three of four cell lines ($p<0.001$).

Similarly, the expression of the anti-apoptotic protein BCL-xL is decreased after NVP-BEZ235 treatment, while gemcitabine treatment leads to elevated expression levels of BCL-xL. Applying the time-delayed administration regime a decreased gene expression of STAT3 and BCL-xL-mRNA is detected in MiaPaCa-2, Aspc-1 and BxPC-3 but not in Panc-1, while the simultaneous treatment results in decreased levels only in MiaPaCa-2 and Aspc-1. Interestingly, the mRNA expression of STAT3 is increasing after simultaneous treatment in PANC-1 cells (Figure 2). The gene expression of WNT16 is significantly enhanced by BEZ.

The combination of GEM with BEZ shows a heterogeneous pattern on WNT16 in the different cell lines. Whereas in MiaPaCa-2 and PANC-1 cells GEM reduced the increase of WNT16 expression, it has no or even a boosting effect on WNT16 expression. However, the data demonstrate that NVP-BEZ235 enhances the expression of Wnt16, when it is administered alone or before gemcitabine (Figure 2).

Influence of NVP-BEZ235 and gemcitabine application on cell cycle distribution

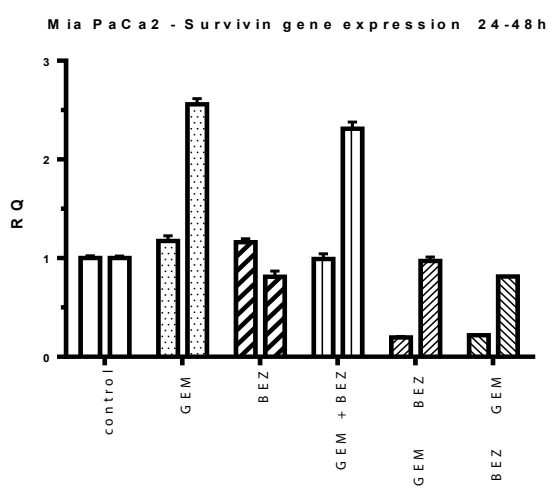
Cell cycle analysis revealed that treatment with NVP-BEZ235 leads to G0/G1 arrest in all cell lines. In contrast to this, gemcitabine induced an S-Phase arrest. Simultaneous treatment with both compounds or the sequence GEM◊NVP-BEZ235 led to an increased S-Phase arrest of the cells as well except in PANC-1 cells. A pre-treatment with NVP-BEZ235 followed by 24 hours later administration of gemcitabine lead to a decreased number of cells in the S-Phase and an increase of cells undergoing G0/G1 arrest (Figure 3).

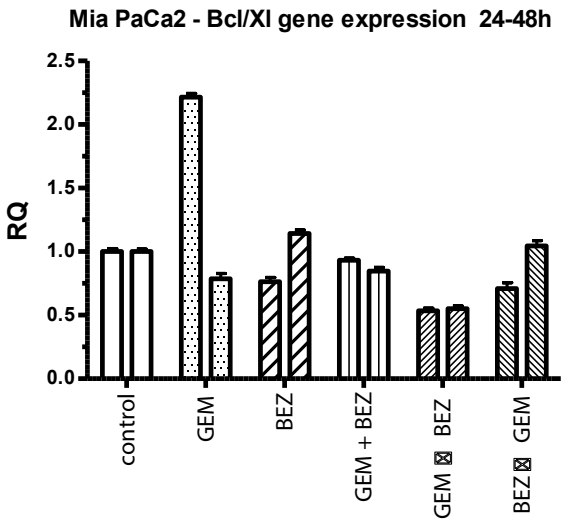


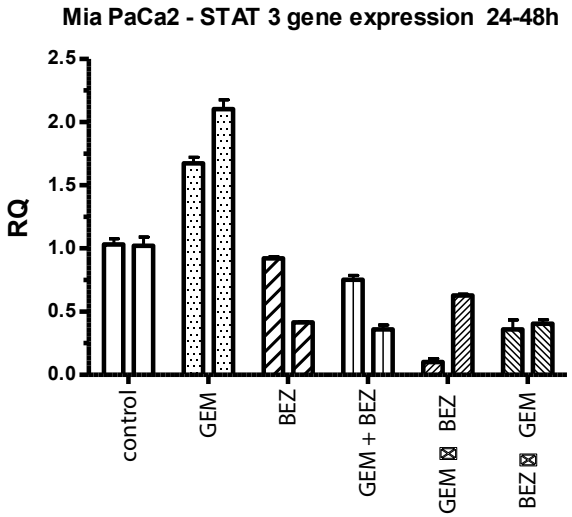
Gemcitabine (GEM) and NVP-BEZ235 (BEZ) application as stand-alone influences cell viability in a concentration-independent manner (data not shown). (A) Combined treatment with gemcitabine and NVP-BEZ235 (GEM+BEZ) leads to an enhanced decrease of cell viability especially in MiaPaCa-2 nd PANC-1 cells. This effect is partially enforced by 24h time-delayed usage of these compounds, especially if gemcitabine is added first (GEM+BEZ). The date are presented as relative absorbance in comparison to that of the control (=100%) (A) (*p<0.0001).

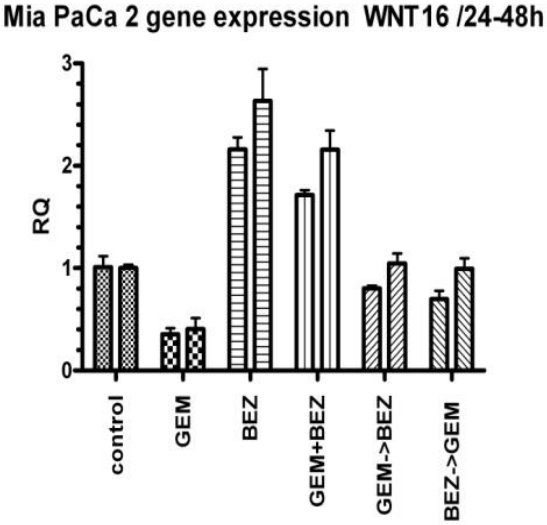
(B) The four cell lines were incubated in triplets with gemcitabine and NVP-BEZ235 as single agents or in combination using different sequences. The cells were counted every day using the Neubauer chamber. The benchmark was 3x106 cells/ml.

Figure 1: Cell viability assay (MTT-Assay) after NVP-BEZ235 and gemcitabine application and growing curves

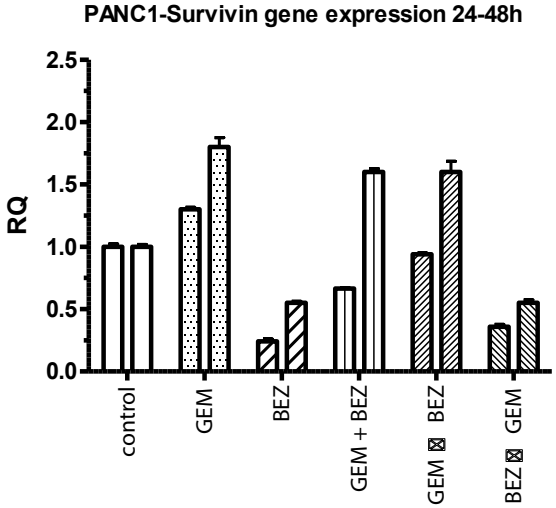

Mia PaCa2 - Survivin gene expression 24-48h
RQ
control
GEM
BEZ
GEM + BEZ
GEM ⊠ BEZ
BEZ ⊠ GEM

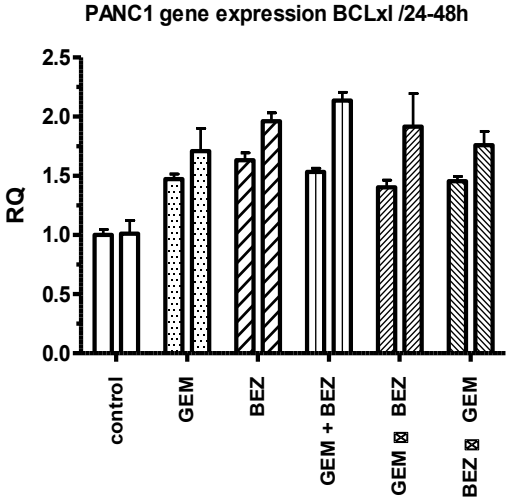

Mia PaCa2 - Bcl/XI gene expression 24-48h
RQ
control
GEM
BEZ
GEM + BEZ
GEM ⊠ BEZ
BEZ ⊠ GEM

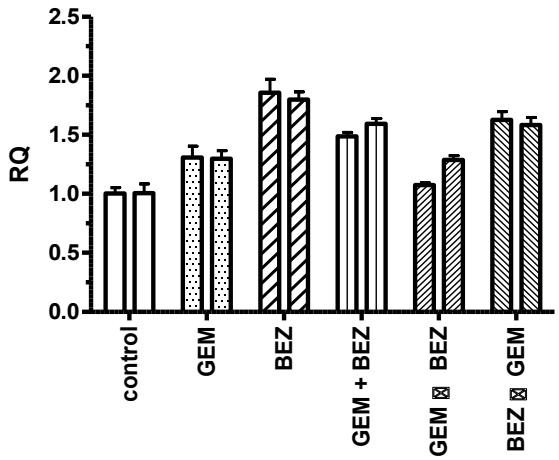

Mia PaCa2 - STAT 3 gene expression 24-48h
RQ
control
GEM
BEZ
GEM + BEZ
GEM ⊠ BEZ
BEZ ⊠ GEM

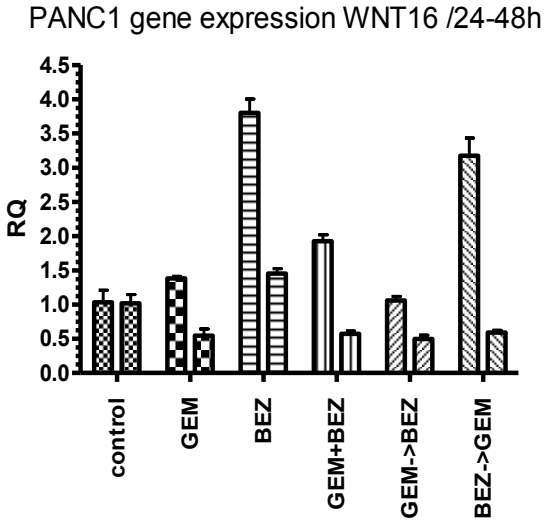

Mia PaCa 2 gene expression WNT16 /24-48h
RQ
control
GEM
BEZ
GEM+BEZ
GEM->BEZ
BEZ->GEM

B) Panc-1

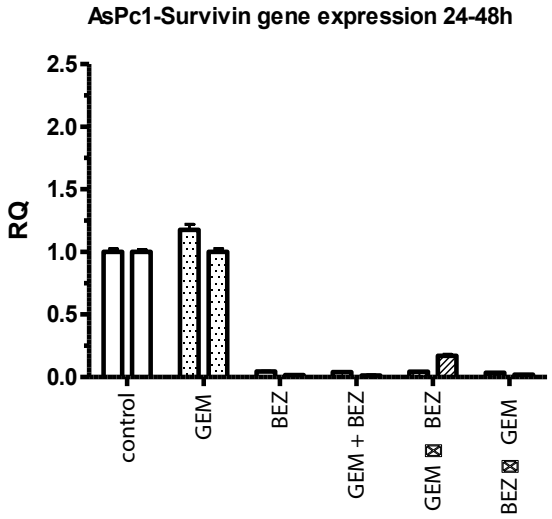

PANC1-Survivin gene expression 24-48h
RQ
control
GEM
BEZ
GEM + BEZ
GEM ⊠ BEZ
BEZ ⊠ GEM

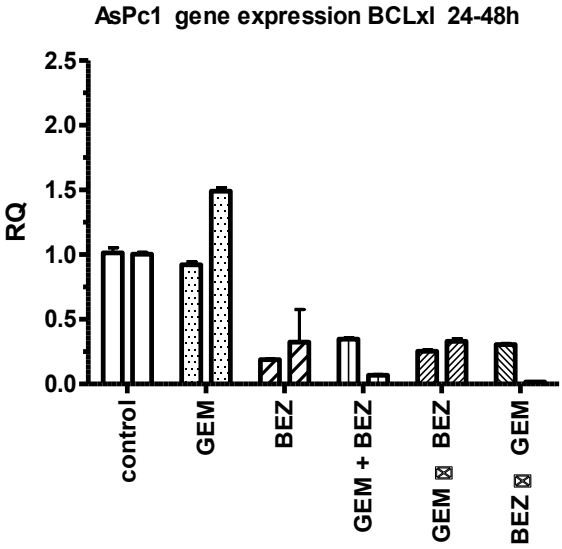

PANC1 gene expression BCLxl /24-48h
RQ
control
GEM
BEZ
GEM + BEZ
GEM ⊠ BEZ
BEZ ⊠ GEM

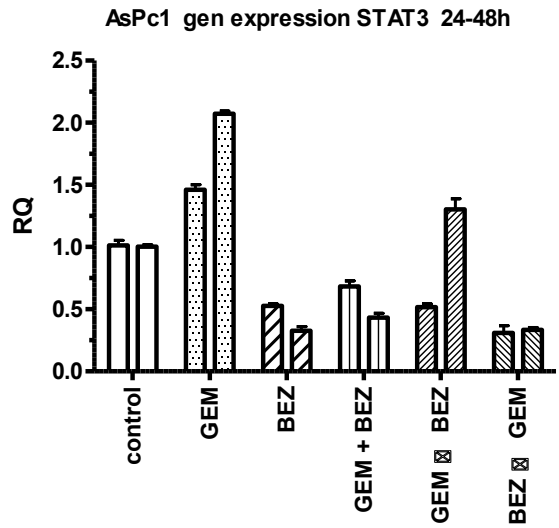

PANC1 gene expression STAT3 24-48h
RQ
2.5
2.0
1.5
1.0
0.5
0.0
control
GEM
BEZ
GEM + BEZ
GEM ⊠ BEZ
BEZ ⊠ GEM

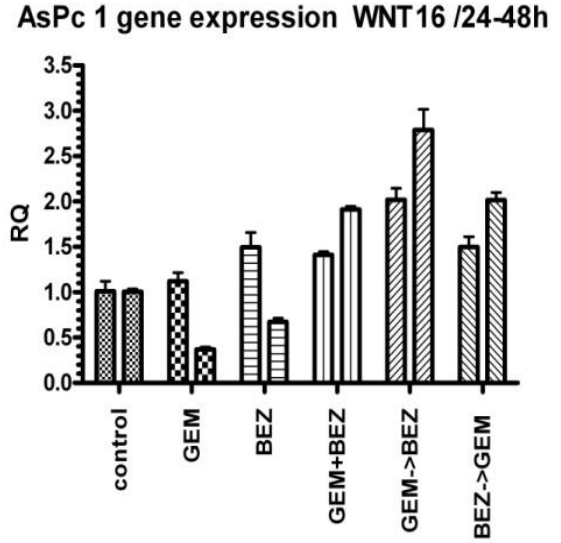

PANC1 gene expression WNT16 /24-48h
RQ
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
GEM
BEZ
GEM+BEZ
GEM->BEZ
BEZ->GEM

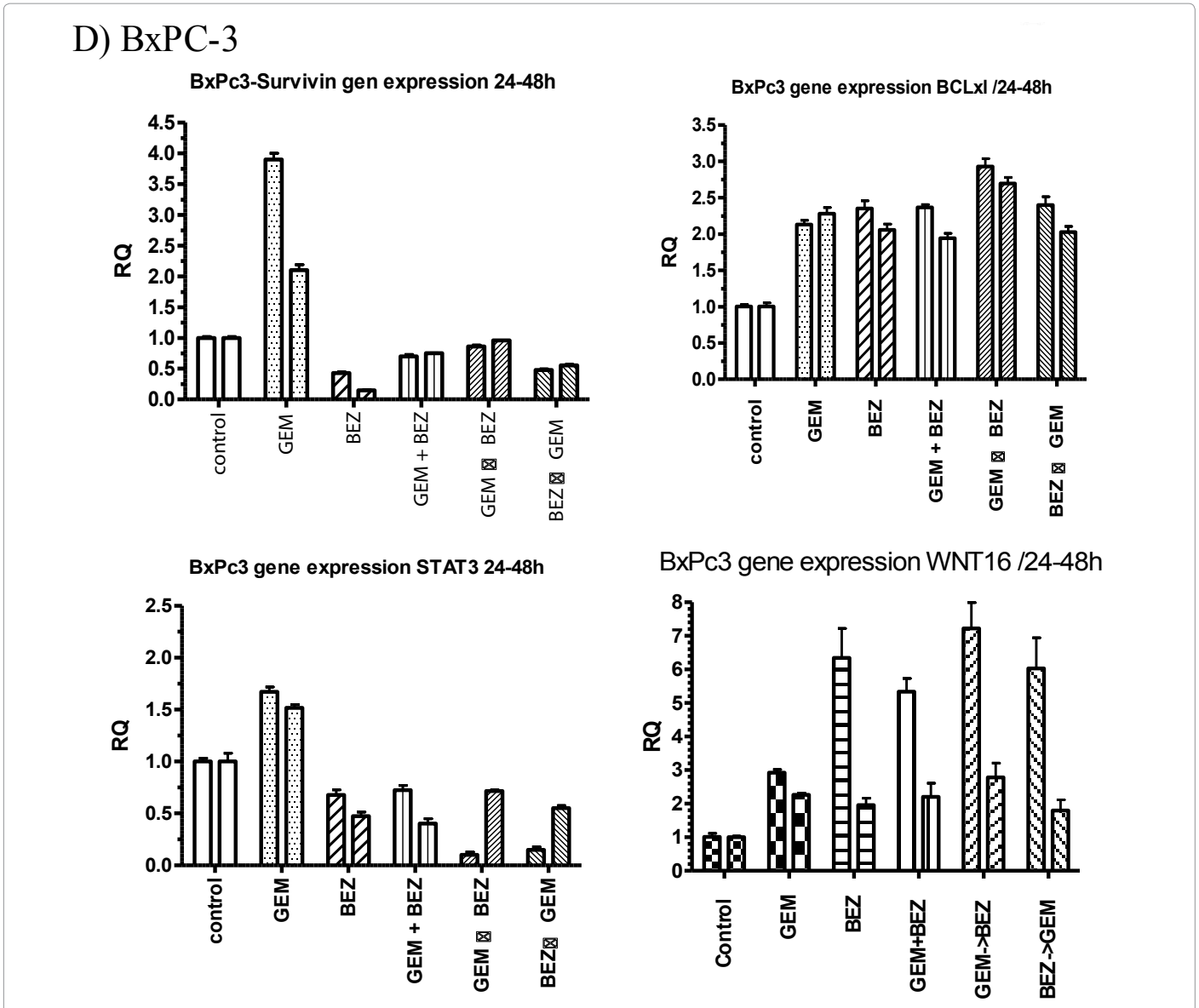
C) AsPC-1


AsPc1-Survivin gene expression 24-48h
RQ
2.5
2.0
1.5
1.0
0.5
0.0
control
GEM
BEZ
GEM + BEZ
GEM ⊠ BEZ
BEZ ⊠ GEM


AsPc1 gene expression BCLxl 24-48h
RQ
2.5
2.0
1.5
1.0
0.5
0.0
control
GEM
BEZ
GEM + BEZ
GEM ⊠ BEZ
BEZ ⊠ GEM


AsPc1 gen expression STAT3 24-48h
RQ
2.5
2.0
1.5
1.0
0.5
0.0
control
GEM
BEZ
GEM + BEZ
GEM ⊠ BEZ
BEZ ⊠ GEM


AsPc 1 gene expression WNT16 /24-48h
RQ
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
GEM
BEZ
GEM+BEZ
GEM->BEZ
BEZ->GEM



The level of Survivin/BIRC5-mRNA is increasing after treatment with gemcitabine (GEM), while the application of NVP-BEZ235 (BEZ) decreases the expression of Survivin/BIRC5 mRNA. Simultaneous treatment with NVP-BEZ235 and gemcitabine shows an increased Survivin/BIRC5 mRNA expression, while the 24h time-delayed administrations (GEM◊BEZ, BEZ◊GEM) results in decreased expression levels. Increased STAT3 gene expressions can be detected after gemcitabine administration, while treatment with NVP-BEZ235 alone reduced the expression of STAT3 mRNA.

Similar to this, the expression of BCL-xL is decreasing after NVP-BEZ235 treatment, while gemcitabine treatment leads to elevated expression levels of BCL-xL. Using the 24h time-delayed administration regime a decreased gene expression of STAT3 and BCL-xL mRNA is detected. The mRNA expression of both genes, STAT3 and BCL-xL, is increasing after simultaneous treatment with both compounds.

The gene expression of WNT16 is significantly enhanced by BEZ. The combination of GEM with BEZ shows a heterogeneous pattern on WNT16 in the different cell lines.

Figure 2: Real-Time RT-PCR gene expression analysis of the anti-apoptotic proteins Survivin/BIRC5, STAT3 and BCL-xL and of WNT16.

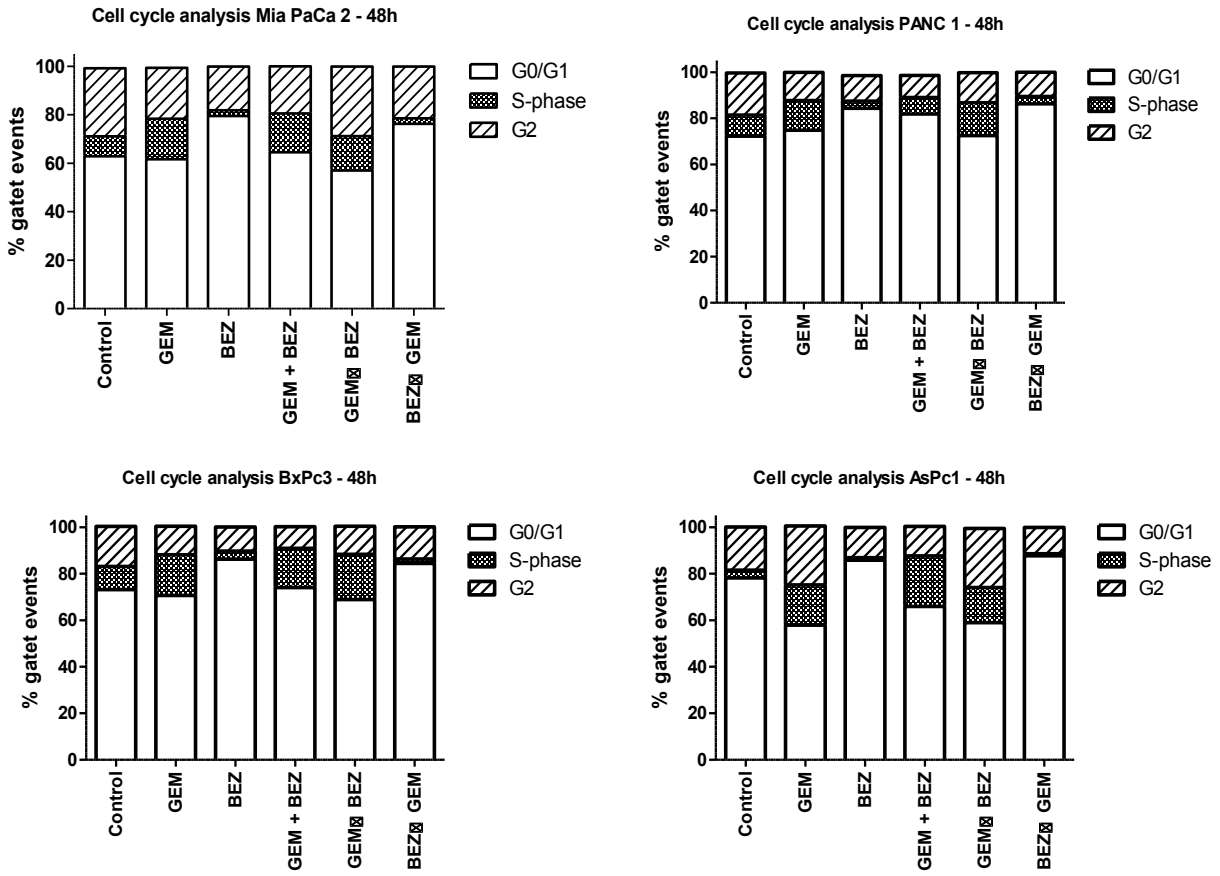
Inhibition of PI3K and mTOR after treatment with siRNA and NVP-BEZ235

To demonstrate the inhibitory effects of ***NVP-BEZ235*** on the PI3k and mTOR pathway, we inhibited both pathways with PI3k and mTOR specific siRNA alone and in combination with gemcitabine using BxPc3 cells. We found that PI3k and mTOR mRNA were upregulated by gemcitabine incubation, but clearly inhibited by specific siRNA. The upregulation of mTOR and PI3k by gemcitabine could be inhibited with the combination of specific siRNA (Figure 4).

Additionally, cells were treated with 1 µM NVP-BEZ235, 50 µM gemcitabine or with a combination of both compounds for 2 and 4 hours. The activated, phosphorylated form of Akt2 (p-Akt) was not affected after treatment with NVP-BEZ235. The activated, phosphorylated form of mTOR (p-mTOR) was remarkable inhibited after treatment with NVP-BEZ235 after 2 and 4 hours (Figure 5a).

Induction of survivin after treatment with gemcitabine

The Western blot analysis shows the expression of survivin/BIRC5 after treatment with 30 µM gemcitabine, 50 µM gemcitabine, 500 nM



Application of NVP-BEZ235 leads to G0/G1 arrest in all four cell lines. Gemcitabine induces S-Phase arrest. By simultaneously treatment with both compounds, cells still arrest in S-Phase. This affect can be elevated by treating the different cell lines 24 hours prior the administration of NVP-BEZ235 with gemcitabine. A pretreatment with NVP-BEZ235 followed by 24 hours later administration of gemcitabine leads to a decreased number of cells in the S-Phase and an increase of cells undergoing G0/G1 arrest.

Figure 3: Cell cycle Analysis after NVP-BEZ235 and gemcitabine treatment.

NVP-BEZ235, 1 µM NVP-BEZ235, 1 µM NVP-BEZ235 and 30/50 µM gemcitabine simultaneously, 30/50 µM gemcitabine 24 hours prior to 1 µM NVP-BEZ235 and 1 µM NVP-BEZ235 24 hours prior to 30/50 µM gemcitabine. It was confirmed, that gemcitabine induced survivin/BIRC5 expression. In combination with NVP-BEZ235 the expression was down-regulated to normal levels (Figure 5b).

Induction of apoptosis analyzed by Annexin V/PE-Assay

Annexin V/PE-Assays demonstrate an increase of apoptotic and necrotic cells in all investigated cell lines after gemcitabine treatment compared to cells treated with NVP-BEZ235 alone. Simultaneous application or (pre-)treatment with gemcitabine results in a high amount of apoptotic and necrotic cells, while pre-treatment with NVP-BEZ235 leads to decreased levels of apoptotic and necrotic cells (Figure 6).

Analysis of double strand breaks with γH2AX

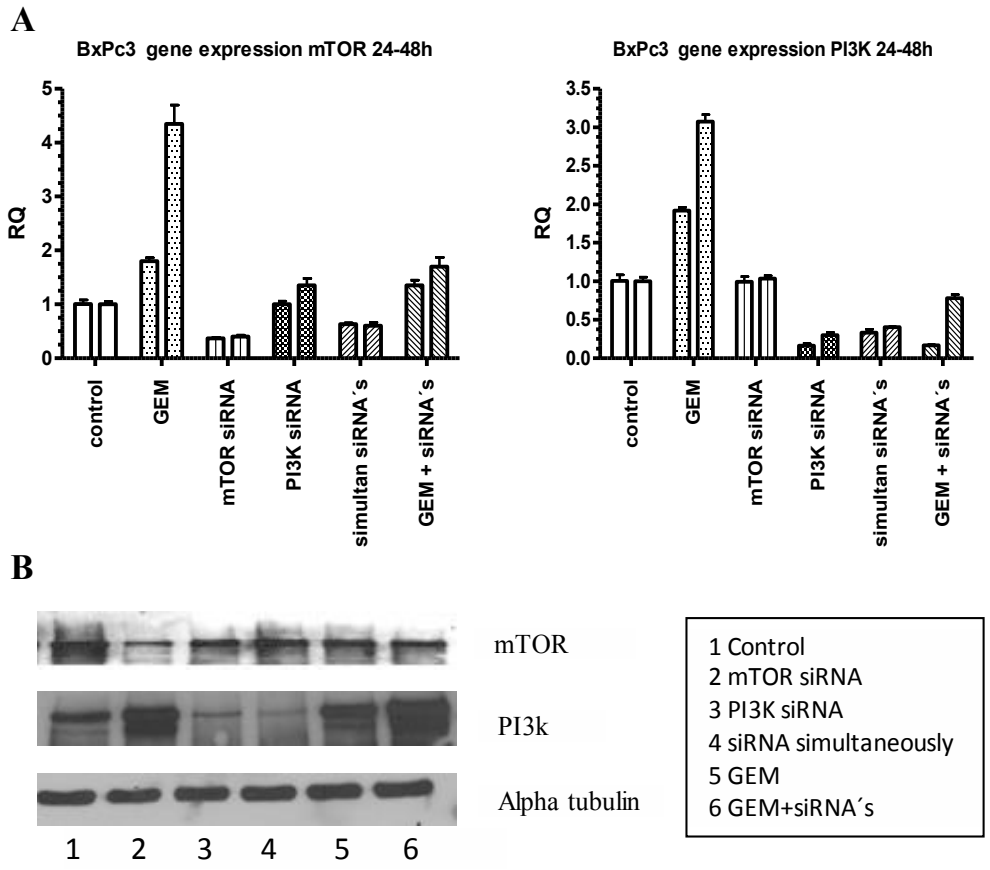
To evaluate the assumption that sequential administration of gemcitabine and NVP-BEZ235 24 hours later induces more DNA strand break events we analysed the amount of double strand breaks with γH2AX by Cantor2.

γH2AX is a variant histone H2A which replaces conventional H2A in a subset of nucleosomes. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries, which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post- translational- modifications of histones also called histone code and nucleosome remodelling. This is required for checkpoint-mediated arrest of cell cycle progression in response to low doses of ionizing radiation and for efficient repair of DNA double strand breaks specifically when modified by C-terminal phosphorylation.

Our results show an enhancement of H2A.X detection after gemcitabine/BEZ235 treatment, confirming an increase in DNA strand break events (Figure 7).

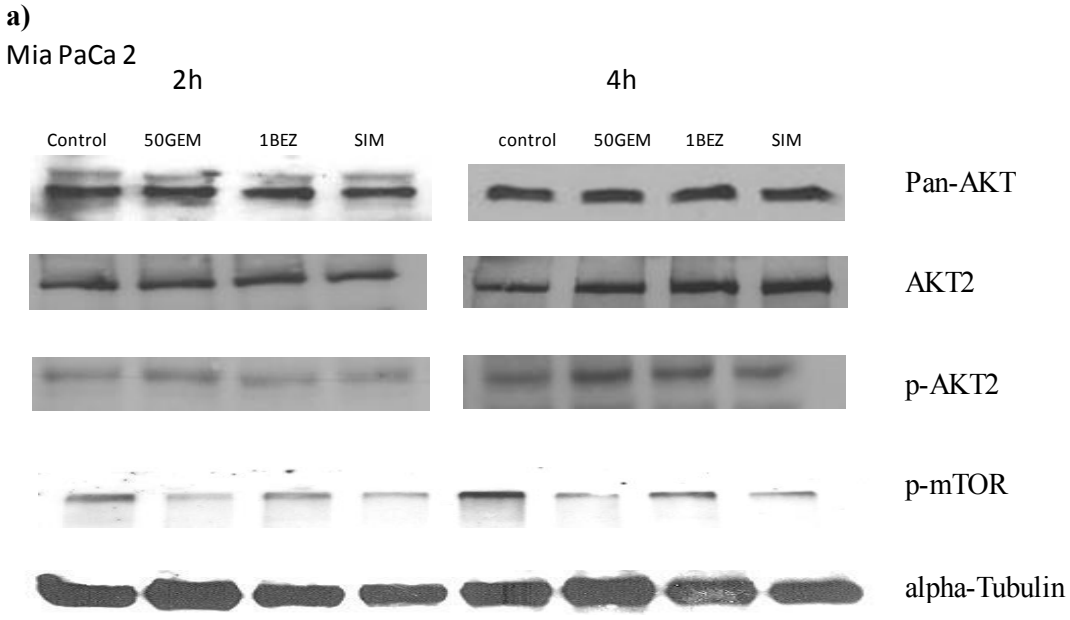
Discussion

The effects of the dual PI3k/mTOR inhibitor NVP-BEZ235 or gemcitabine on the cell viability in all four pancreatic cell lines differed considerably. BxPC-3 was the most sensitive cell line to gemcitabine treatment, which is in agreement to the studies of Duxbury et al., [27] and Arumugam [28]. The cell line BxPC-3 was also the most sensitive cell line to incubation with NVP-BEZ235. Treatment with 30 or 50 µM gemcitabine as well as 1 µM NVP-BEZ235 resulted in an 80% inhibition of cell viability after 72h. In terms of a relationship



mTOR and PI3k mRNA were clearly inhibited by specific siRNA after 24h and 48 h, whereas gemcitabine alone increased the gene expression of both pathways (A). The Western blot shows a reduction of the corresponding protein levels (B). The MTT-Assay reveals a reduction of cell viability by mTOR siRNA treatment but even more after PI3K siRNA treatment and simultaneous siRNA treatment, respectively. But best results were achieved with gemcitabine alone and especially with gemcitabine combined with both siRNAs (C).

Figure 4: Inhibition of the mTOR and PI3k pathway by specific siRNA and NVP-BEZ235 alone and in combination with gemcitabine



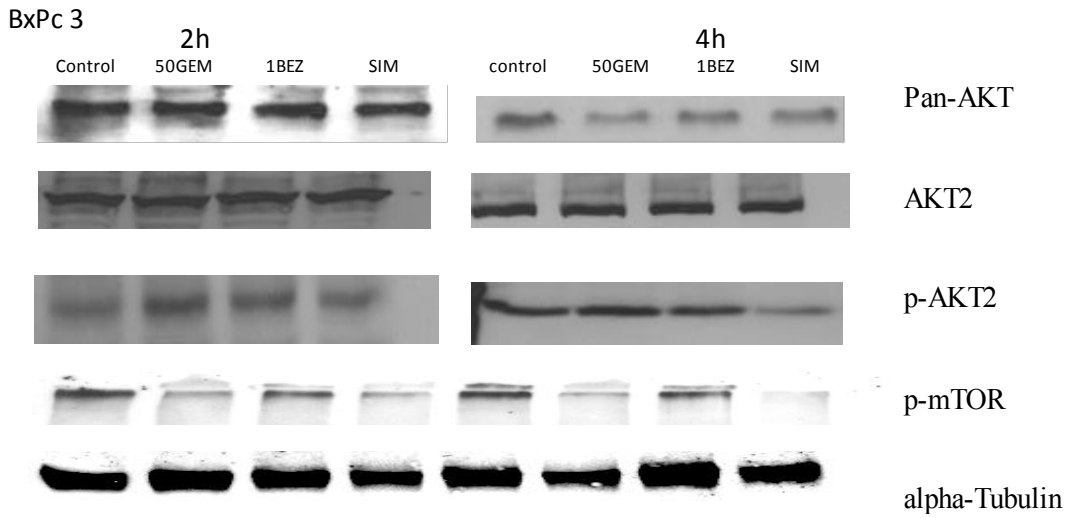


Figure 5a: Western blot analysis of pan-AKT, AKT2, p-Akt2 and p-m-TOR expression after treatment with gemcitabine and NVP-BEZ235.

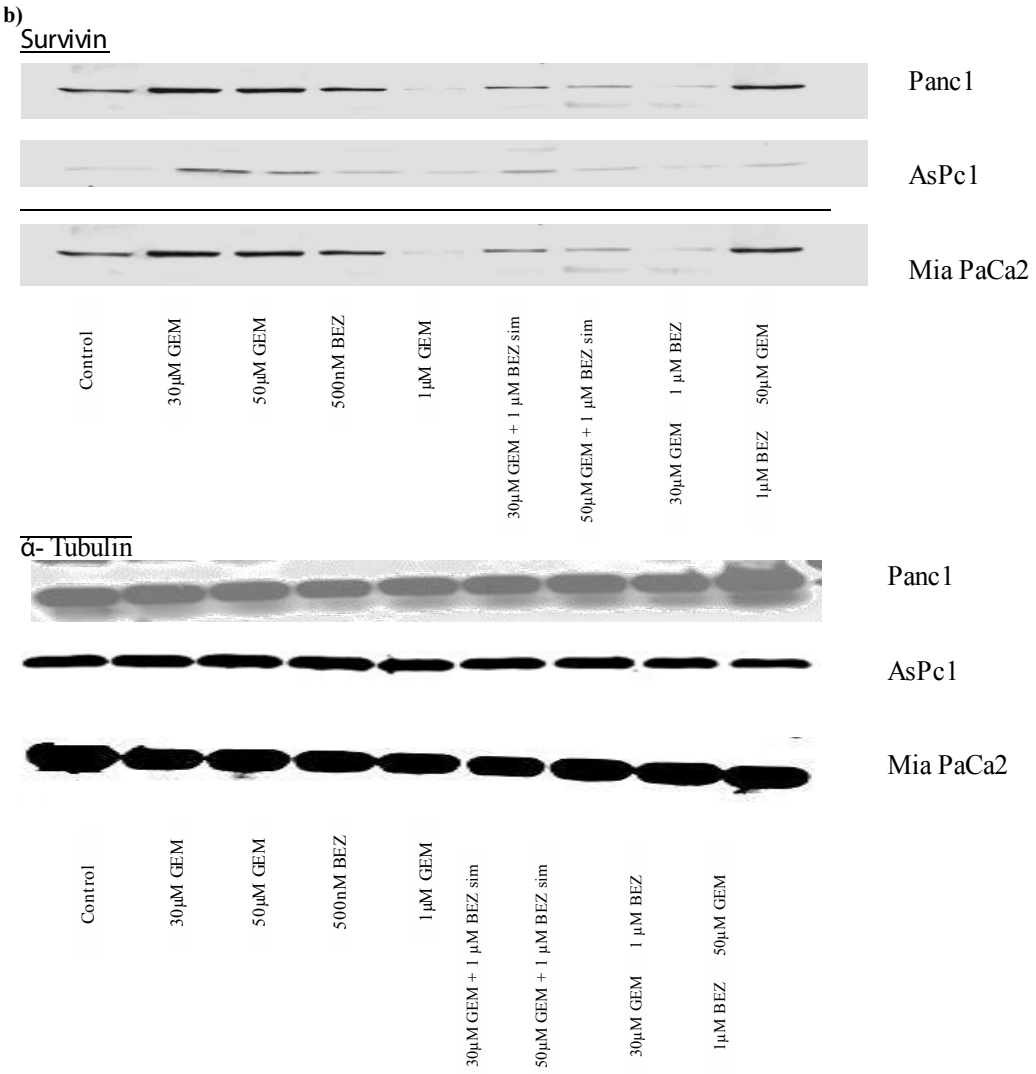
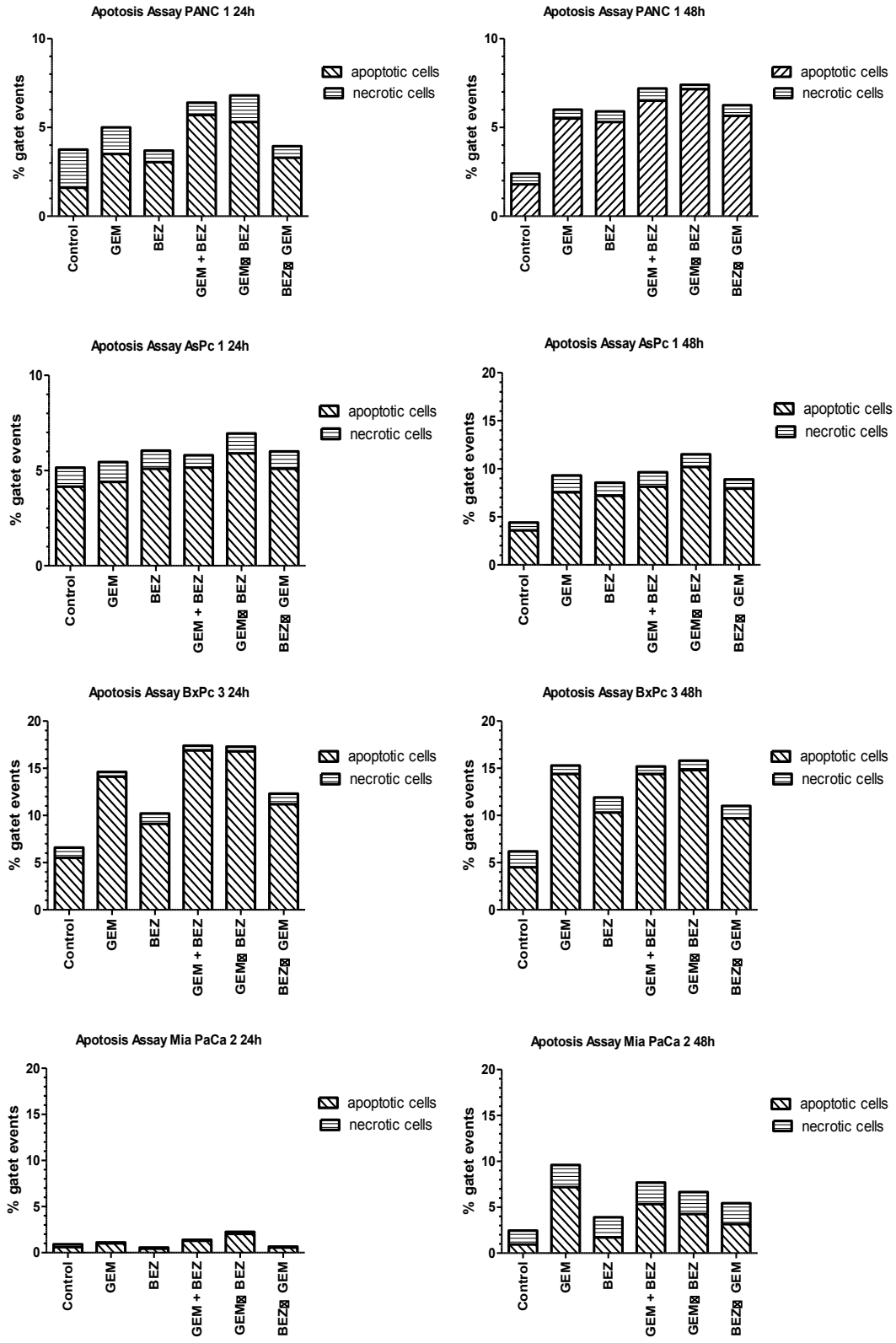
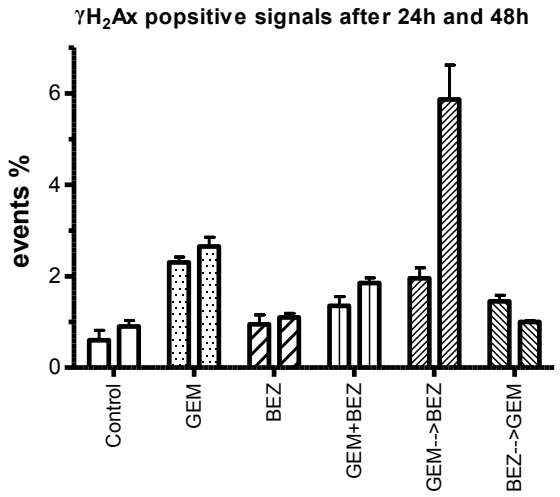


Figure 5b: Western blot analysis of Survivin/BIRC5 and Alpha Tubulin ,in 3 pancreatic cancer cell lines, after treatment with gemcitabine and NVP-BEZ235.



The analyses were performed by annexin V/PE FACS measurement according to the manufacturer's protocol. The cells were treated with gemcitabine (30 µM) and NVP-BEZ235 (1 µM) for 24-48 hours. Control cells were incubated with DMSO alone. The fluorescence of 10,000 cells was determined after subtracting the background fluorescence of control cells.

Figure 6: Examples for induction of apoptosis by gemcitabine, NVP-BEZ235 or by combination of both simultaneously or sequentially after 24 hours.



Flow cytometry analysis of double strand breaks with γH2AX in Mia PaCa2 cells following incubation with 30 µM gemcitabine (GEM), 1 µM NVP-BEZ235 (BEZ) or both simultaneously or sequentially for 24 and 48 hours

The figure shows that the most events could be observed with the sequence GEM→BEZ ($p<0.001$).

Figure 7: Flow cytometry analysis of double strand breaks with γH2AX.

between sensitivity to NVP-BEZ235, the mutation of K-ras might serve as a resistance factor to the dual inhibitor [28] and gemcitabine [29]. The cell line BxPC-3 is K-ras wild-type while the other three cell lines harbour mutated K-ras oncogenes, which are also less sensitive to treatment with gemcitabine [30]. While the incubation of AsPC-1 resulted in a 60% inhibition of cell viability the cell lines MiaPaCa-2 and Panc-1 remained nearly unaffected. NVP-BEZ235 was a little more effective. The reason for the nearly complete resistance of MiaPaCa-2 and Panc-1 against gemcitabine and NVP-BEZ235 needs to be further investigated.

There is a strong inhibition of mTOR compared to Akt in all four cell lines. Despite failure of Akt2 inhibition there was also no Akt2 activation after treatment with NVP-BEZ235. NVP-BEZ235 is predominantly utilized targeting activated Akt by PI3K p110a gene mutations or loss of PTEN. Both alterations are not common in pancreatic cancer. But, in contrast to rapamycin and rapalogs the dual inhibitor NVP-BEZ235 is able to fully suppress both TORC kinases, TORC1 and TORC2. This might be sufficient for a strong anti-proliferative effect in pancreatic cancer cells making PI3K inhibition dispensable [31]. Recently, Soares et al. [32] proposed that the dual PI3K/mTOR NVP-BEZ suppresses a novel negative feedback loop mediated by mTORC2 thereby leading to enhanced MEK/ERK pathway activity in pancreatic cancer cells.

Best results concerning decreased cell viability were achieved by a combined treatment with gemcitabine and NVP-BEZ235 using a sequential application of the two compounds.

Interestingly, there is a different regulation of Survivin/BIRC5 after gemcitabine and NVP-BEZ235 administration respectively. As we have shown in this study, the anti-apoptotic protein Survivin/BIRC5 is upregulated after gemcitabine treatment and downregulated after treatment with NVP-BEZ235. It is known, that there is an association of Surivin/BIRC5 expression with gemcitabine resistance [33]. Interestingly, Survivin/BIRC5 may play a role in DNA-damage repair by interaction with members of DNA-double-strand breaks repair machinery. Rödel et al. [34] reported that Survivin/BIRC5 knockdown reduced DNA-PKcs kinase activity. Similar results were obtained by the group of Chen et al. [35] concerning the inhibition of the TORC kinases. They could demonstrate that the mTOR inhibitor rapamycin suppresses DNA double strand break repair. Consistent with this, as shown in Figure 7, sequential application of gemcitabine and NVP-BEZ235 resulted in a significant increase of double-strand breaks analysed with γH2AX assay [36].

Additionally, our data support the hypothesis that NVP-BEZ235 enhances the expression of Wnt16, which might be contradictory to the DNA damage induced by the following chemotherapy. This might be an explanation, why the sequence BEZ235◊GEM is inferior to the sequence GEM◊BEZ235 [36].

The number of cells in S-phase is increasing following first, alone or simultaneous treatment with gemcitabine, while the level of cells treated first or solely with NVP-BEZ235 is increased in G0/G1-phase, by inhibiting the transition from G1-phase to S-phase. This is remarkable as DNA-damage repair is induced in the G0/G1-phase and the effect of gemcitabine is constricted to S-phase. In accordance with the group of Manara et al. [37], we showed that chemotherapy administered before NVP-BEZ235 therapy has advantageous effects.

In summary, treatment of pancreatic cancer cells with therapeutics acting via different ways of action seems to be significantly more effective than single agent use *in vitro*, but the sequence of administration of cytostatic agents as gemcitabine and dual PI3K/mTOR inhibitors seems to be a relevant issue. Further studies need to be conducted to confirm those results *in vivo* to evaluate such regimens, which might offer a new and effective option for the treatment of pancreatic cancer in the future [38].

Acknowledgements

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