**Dual PI3K/mTOR inhibitors induce rapid over-activation of the MEK/ERK pathway in human pancreatic cancer cells through**

**suppression of mTORC2**

**Supplementary Data**

**Files in this Data Supplement:**

* **Supplementary Figure Legends- Contains Supplementary Figure Legends S1-S6.**
* **Supplementary Figures:**

**Supplemental Figure S1. Structures of Inhibitors NVP-BEZ235; PKI-587; GDC-0980 and PD0325901**

**Supplemental Figure S2. NPV-BEZ235 induces over-activation of ERK phosphorylation in MiaPaca-2 and PANC-1 cells**.

**Supplemental Figure S3. The MEK inhibitor PD 0325901 suppresses ERK over-activation induced by NPV-BEZ235 at nanomolar concentrations.**

**Supplemental Figure S4. Overactivation of MEK phosphorylation by combination of dual PI3K/mTOR kinase inhibitor NPV-BEZ235 and MEK inhibitor PD0325901.**

**Supplemental Figure S5. Dual PI3K/mTOR kinase inhibitors NVP-BEZ235, PKI-587 and GDC-0980 suppress PIP3 accumulation in PDAC cells in different times of stimulation**.

**Supplemental Figure S6. NPV-BEZ235 enhances ERK pathway activation through a pathway that does not require EGFR, HER2, insulin receptor or IGF-1R**

**Supplementary Figure Legends**

**Supplemental Figure S1. Structures of Inhibitors NVP-BEZ235; PKI-587; GDC-0980 and PD0325901**

**Supplemental Figure S2. NPV-BEZ235 induces over-activation of ERK phosphorylation in MiaPaCa-2 and PANC-1 cells**.

 **A and B**: Cultures of MiaPaCa-2 cells (panel A) and PANC-1 (panel B) were incubated in the absence or in the presence of NPV-BEZ235 (0,005-1 μM) for 2h. Then, the cells were stimulated for another 2h with 5 nM neurotensin and 10 ng/ml insulin and lysed with SDS–PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect the phosphorylated state of S6 at Ser 240/244, AKT at Ser473 and at Thr308 and ERK at Thr202 and Tyr204. Immunoblotting with antibodies that recognize total S6, AKT and ERK was used to verify that the cell treatments did not change the total level of these proteins.

**Supplemental Figure S3. The MEK inhibitor PD 0325901 suppresses ERK over-activation induced by NPV-BEZ235 at nanomolar concentrations.**

**A:** Cultures of MiaPaCa-2 cells were incubated in 0.1µM of NPV-BEZ235 in the absence or presence of increasing doses of PD0325901 (0.005 – 0.1 μ M) for 2h. Then, the cells were stimulated for 60 min with 5 nM neurotensin and 10 ng/ml insulin and lysed with SDS–PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect phosphorylated state of ERK at Thr202 and Tyr204 and total ERK.

**B:** Cultures of MiaPaCa-2 cells were incubated in the presence of increasing doses of NPV-BEZ235 (0.01 to 1 µM) with or without 0.05 μ M of PD0325901 for 2h. Then, the cells were stimulated for 60 min with 5 nM neurotensin and 10 ng/ml insulin and lysed with SDS–PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies that detect phosphorylated state of ERK at Thr202 and Tyr204and total ERK.

**Supplemental Figure S4. Overactivation of MEK phosphorylation by combination of dual PI3K/mTOR kinase inhibitor NPV-BEZ235 and MEK inhibitor PD0325901.**

**A** and **B**: Cultures of MiaPaCa-2 cells (**A**) and PANC-1 (**B**) cells were incubated in the presence of NPV-BEZ235 and/or PD0325901 at indicated concentrations for 2 h. The cultures were then stimulated for 2 h with 5 nM neurotensin and 10 ng/ml insulin and lysed with SDS–PAGE sample buffer. **C and D**: Cultures of MiaPaCa-2 (panel **C**) and PANC-1 (panel **D**) cells were also incubated for 2 h in the in the presence of NPV-BEZ235 and/or PD0325901 at indicated concentrations. Then, the cells were stimulated for 2 h with 2% fetal bovine serum (serum) and lysed with SDS–PAGE sample buffer. All samples were analyzed by SDS-PAGE and immunoblotting with an antibody that detects the phosphorylated state of MEK at Ser217/221 and total MEK.

**Supplemental Figure S5. Dual PI3K/mTOR kinase inhibitors NVP-BEZ235, PKI-587 and GDC-0980 suppress PIP3 accumulation in PDAC cells in different times of stimulation**.

 **A:** MiaPaCa-2 cells were transiently transfected with a plasmid encoding a fusion protein between GFP and the PH domain of AKT (AKT-PH-GFP). The cultures were incubated in the absence or presence of NVP-BEZ235 (BEZ) at 0.1 μ M for 1h prior to stimulation with 5 nM neurotensin and 10 ng/ml insulin for 0, 15, 30 and 60 min. The intracellular distribution of AKT-PH-GFP was monitored under a fluorescence microscope. The selected cells displayed in the figures were representative of 90% of the population of GFP-positive cells.

 **B:** MiaPaCa-2 cells were transiently transfected with a plasmid encoding a fusion protein between GFP and the PH domain of AKT (AKT-PH-GFP). The cultures were incubated in the absence or in the presence of PKI-587 (PKI) or GDC-0980 (GDC) both at 0.1 μ M for 1h prior to stimulation with 5 nM neurotensin and 10 ng/ml insulin for 15 min.

**Supplemental Figure S6. NPV-BEZ235 enhances ERK pathway activation through a pathway that does not require EGFR, HER2, insulin receptor or IGF-1R**

**A:** Verification that the inhibitors, at the concentrations used, prevented EGF or IGF-induced ERK activation in parallel MiaPaCa-2 cultures. Cells were incubated with inhibitor as described in Figure 5. Then, the cells were stimulated for 15 min with 50 ng/ml of EGF or IGF and lysed with SDS–PAGE sample buffer. Immunoblotting with antibodies that recognize phosphorylated ERK at Thr202 and Tyr204 was used to verify effectiveness of above drugs and total ERK was used to verify equal loading.

**B, C and D:** Cells were incubated and stimulated as described in Figure 5. Quantification of fold increase phosphorylated ERK at Thr202 and Tyr204 using Multi Gauge V3.0. after AG-1478 (AG) on panel A, Lapatinib (Lp) in panel B and OSI-906 (OSI) in panel C.