Title

Dual blockade of PI3K/AKT/mTOR (NVP-BEZ235) and Ras/Raf/MEK (AZD6244) pathways synergistically inhibit growth of primary endometrioid endometrial carcinoma cultures, whereas NVP-BEZ235 reduces tumor growth in the corresponding xenograft models

Authors

Stefanie Schrauwen[a], Jeroen Depreeuw[a,b,c], Lieve Coenegrachts[a], Els Hermans[a], Diether Lambrechts[b,c], Frédéric Amant[a].

Stefanie Schrauwen[a]

[a]KU Leuven – University of Leuven, University Hospitals Leuven, Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, B-3000 Leuven, Belgium

Email address: Stefanie.Schrauwen@med.kuleuven.be

Jeroen Depreeuw[a,b,c]

[a]KU Leuven – University of Leuven, University Hospitals Leuven, Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, B-3000 Leuven, Belgium

[b]KU Leuven, Department of Oncology, Laboratory for Translational Genetics, B-3000 Leuven, Belgium

[c]VIB, Vesalius Research Center (VRC), B-3000 Leuven, Belgium

Email address: Jeroen.Depreeuw@vib-kuleuven.be

Lieve Coenegrachts[a]

[a]KU Leuven – University of Leuven, University Hospitals Leuven, Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, B-3000 Leuven, Belgium

Email address: coenegracthslieve@hotmail.com

Els Hermans[a]

[a]KU Leuven – University of Leuven, University Hospitals Leuven, Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, B-3000 Leuven, Belgium

Email address: Els.Hermans@uzleuven.be

Diether Lambrechts[b,c]

[b]KU Leuven, Department of Oncology, Laboratory for Translational Genetics, B-3000 Leuven, Belgium

[c]VIB, Vesalius Research Center (VRC), B-3000 Leuven, Belgium

Email address: Diether.Lambrechts@vib-kuleuven.be

Frédéric Amant[a]

[a]KU Leuven – University of Leuven, University Hospitals Leuven, Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, B-3000 Leuven, Belgium

Email address: Frederic.Amant@uzleuven.be

Corresponding author

Prof. Frédéric Amant, MD, PhD

KU Leuven – University of Leuven, University Hospitals Leuven

Division of Gynecologic Oncology

Department of Obstetrics and Gynecology

UZ Herestraat 49 - bus 7003

3000 Leuven

Telephone number: +32 16 3 44635

Fax number: +32 16 3 44629

E-mail address: Frederic.Amant@uzleuven.be

Abstract

Objectives. Endometrial carcinoma (EC) is the most common gynecological cancer in the Western World. Treatment options are limited for advanced and recurrent disease. Therefore, new treatment options are necessary. Inhibition of the PI3K/AKT/mTOR and/or the Ras/Raf/MEK pathways are suggested to be clinically relevant. However, the knowledge about the effect of combination targeted therapy in EC is limited. The aim of this study was to investigate the effect of these therapies on primary endometrioid EC cell cultures *in vitro* and *in vivo*.

Methods. Primary microsatellite instable endometrioid EC cell cultures were incubated with Temsirolimus (mTORC1 inhibitor), NVP-BKM120 (pan-PI3K inhibitor), NVP-BEZ235 (pan-PI3K/mTOR inhibitor), or AZD6244 (MEK1/2 inhibitor) as single treatment. *In vitro*, the effect of NVP-BEZ235 with or without AZD6244 was determined for cell viability, cell cycle arrest, apoptosis induction, and cell signaling. *In vivo,* the effect of NVP-BEZ35 was investigated for 2 subcutaneous xenograft models of the corresponding primary cultures.

Results. NVP-BEZ235 was the most potent PI3K/AKT/mTOR pathway inhibitor. NVP-BEZ235 and AZD6244 reduced cell viability and induced cell cycle arrest and apoptosis, by reduction of p-AKT, p-S6, and p-ERK levels. Combination treatment showed a synergistic effect. *In vivo*, NVP-BEZ235 reduced tumor growth and inhibited p-S6 expression. The effects of the compounds were independent of the mutation profile of the cell cultures used.

Conclusions. A synergistic antitumor effect was shown for NVP-BEZ235 and AZD6244 in primary endometrioid EC cells *in vitro*. In addition, NVP-BEZ235 induced reduction of tumor growth *in vivo*. Therefore, targeted therapies seems a promising treatment strategy for EC.

Highlights

- NVP-BEZ235 (dual pan-PI3K/mTOR inhibitor) is the most potent PI3K/AKT/mTOR pathway inhibitor for primary endometrioid endometrial carcinoma cell cultures
- NVP-BEZ235 could reduce tumor growth in xenograft models based on primary endometrioid endometrial carcinoma cell cultures

- Combination treatment with NVP-BEZ235 (dual pan-PI3K/mTOR inhibitor) and AZD6244 (MEK inhibitor) shows a synergistic antitumor effect *in vitro*

Keywords

Endometrioid endometrial cancer; primary cell culture; targeted therapy; NVP-BEZ235; AZD6244

Introduction

Endometrial cancer is the most common gynecologic malignancy in the Western World and the fourth most common cancer in women after breast, lung, and colorectal cancers. Since 2008, the incidence of endometrial cancer has increased by 21% and the mortality rate per 100.000 cases has increased by more than 100% during the past 20 years[1, 2]. Endometrial carcinoma (EC) is the most common endometrial cancer subtype. Endometrioid EC (type I) accounts for 85% of EC cases and is associated with good prognosis. Only 15-20% of the cases show recurrences. In contrast, non-endometrioid EC (type II), like serous and clear cell carcinoma, are aggressive tumors and account for more than 50% of the recurrences[3]. Patients with advanced or recurrent disease have a poor median survival, since conventional cytotoxic and radiation therapy are not effective at this stage[4-6]. Therefore, new treatment options are necessary.

Advances in the understanding of tumor biology have established the critical role of targeted therapy as treatment options for cancer. In addition, an increasing number of preclinical studies suggest that the combination of targeted agents is a more promising strategy compared to single agent treatment, due to feedback loops and cross-talk between signaling pathways.

The phosphatidylinositol 3-kinase enzyme/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) and Ras/Raf/MEK pathways play a critical role in cell proliferation, survival, differentiation, metabolism and motility in response to extracellular cues. Within the PI3K/AKT/mTOR pathway there are feedback loops present. mTOR can increase p70S6K, which in turn phosphorylates and inhibits insulin receptor substrate 1, a protein upstream of PI3K/AKT. Therefore, mTOR inhibition can lead to activation of the PI3K/AKT pathway[7]. In addition, the Ras/Raf/MEK pathway cross-activates and cross-inhibits PI3K/mTORC1 signaling by regulating PI3K, tuberous sclerosis complex 2, and mTORC1[8]. Both

pathways are commonly deregulated in EC. Therefore, inhibition of both pathways could be a potential new treatment strategy for EC.

Many valuable inhibitors targeting one protein (single inhibitors) or two proteins at the same time (dual inhibitors) in these pathways have been recently developed. Temsirolimus (CCI-779) and Everolimus, both mTORC1 inhibitors, showed clinical activity in recurrent and metastatic EC patients[9, 10]. However, no correlation between the mutation profile of the primary tumor and response was determined[11]. NVP-BKM120 is a pan-PI3K inhibitor, which showed antitumor activity in a variety of tumor cell lines and xenograft models of cancers with and without aberrant PI3K pathway activation, including primary human EC xenograft models[12, 13]. In addition, phase I clinical studies showed that NVP-BKM120 is well tolerated in patients[14, 15]. NVP-BEZ235 is a pan-PI3K/mTOR inhibitor, which induces anticancer effects in several human cancer cell lines and xenograft models, such as models based on commercial endometrioid EC cell lines[16, 17]. In addition, this inhibitor was the first PI3K/mTOR dual inhibitor to enter clinical trials[18]. AZD6244 (ARRY-142886) is a potent and selective inhibitor of MEK1/2 kinases[19]. It is currently in phase II clinical development as a single agent, including recurrent and persistent EC, or as a combination treatment[20-22]. Preclinical and clinical studies showed that inhibition of both pathways induces a synergistic effect in several solid malignancies[23-25]. However, there is a lack of knowledge about the response to dual blockade of the PI3K/AKT/mTOR and Ras/Raf/MEK pathways in EC.

For the moment, no appropriate preclinical model system exists for EC. Therefore, we established primary EC cell cultures, as described by Schrauwen et al.[26]. These cell cultures were established directly from patient tumors and closely resemble the heterogeneity and genomic features of the primary tumor. They better reflect treatment-response of patients compared to commercial cell lines[27, 28]. In addition, these cultures can be used for *in vivo* screening of new treatments, by using them to establish subcutaneous (s.c.) xenograft models. However, limitations of these models are that they are not metastatic and the tumors develop in an immunocompromised environment.

There is a lack of knowledge about the effect of blockade of the PI3K/AKT/mTOR and the Ras/Raf/MEK pathways in EC. Therefore the aim of this study is to determine if targeted therapies related to these pathways could be a new treatment option for EC. To the best of our knowledge, we are the first one who used primary endometrioid EC cell cultures to determine the *in vitro* response to targeted therapies as single agent treatments and in combination. Three PI3K and/or mTOR inhibitors

(Temsirolimus, NVP-BKM120, and NVP-BEZ235), and a MEK1/2 inhibitor (AZD6244) were used, which already showed promising results in clinical trials. In addition, the *in vivo* response was analyzed for two s.c. xenograft mouse models treated with NVP-BEZ235.

Materials and methods

Establishment of primary endometrial carcinoma cell cultures

Establishment of primary endometrial carcinoma cell cultures is described by Schrauwen et al.[26]. Briefly, biopsies were collected from chemotherapy-naïve patients undergoing surgery for EC at the Division of Gynecologic Oncology, University Hospitals Leuven. Tissues were fresh-frozen, formalin-fixed, or used for cell culture. For cell culture, tissues were minced and digested with collagenase type IV (1 mg/ml; Roche, Mannheim, Germany) in RPMI 1640 medium with Pencillin/Streptomycin, Fungizone and DNAse I (0.1 mg/ml; Roche, Mannheim, Germany) for 3h at 37°C. Red blood cells were lysed using Ammonium Chloride (Stem Cell Technologies, Vancouver, Canada). Single cells were cultured in culture medium, comprising RPMI 1640 medium, 20% Fetal Bovine Serum (FBS), 2 mM L-Glutamine, 100 U/ml Pencillin/Streptomycin, 1 µg/ml Fungizone, and 10 µg/ml gentamycin (all from Life Technologies, Paisley, UK). Mesenchymal cells were removed using mouse anti-human CD90 antibody (Clone AS02; Dianova, Hamburg, Germany) and Mouse Pan IgG Dynabeads (Life Technologies, Oslo, Norway). All cells were cultured in a humidified atmosphere containing 5% CO_2. Cells were routinely monitored for mycoplasma. The study was approved by the local ethical committees in accordance with the principles of the Declaration of Helsinki and all patients gave their written informed consent.

DNA extraction and somatic mutation profiling

As described by Schrauwen et al.[26], the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used to extract DNA from EC cell cultures, according to the manufacturer's instructions. Hotspot mutation profiling was performed of the established EC cell cultures. Selected mutations in *KRAS*, *PIK3CA*, *MSH6, TP53*, *NRAS*, *BRAF*, *PTEN* and *CTNNB1* were determined, based on the COSMIC database.

In addition, microsatellite instable (MSI) status of primary EC cell cultures was determined. Briefly, Sequenom Massarray profiling was used to analyze 59 mononucleotide homopolymers for single nucleotide insertions or deletions. Cell cultures were determined as MSI if positive for 8 or more insertions or deletions, and microsatellite stable (MSS) if positive for less than 8 markers.

Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method. Cells were seeded in culture medium containing 10% FBS at a concentration of 5.000 cells/well (n=4) in 96-well plates. After 8h, the medium was replaced by 1% FBS culture medium. Twenty-four hours after seeding, cells were treated with NVP-BEZ235 (PI3K/mTOR inhibitor), Temsirolimus (mTOR inhibitor), NVP-BKM120 (PI3K inhibitor), or AZD6244 (MEK1/2 inhibitor) (all compounds: 0-100 µM, n=4) (all from Selleck Chemicals, Munich, Germany). Cell viability was determined at 24h, 48h, and 72h, by adding of 40 µl of MTT solution (2.5 mg/ml; Sigma-Aldrich, St. Louis, USA) to each well and further incubation for 2h. The medium was removed, and the blue crystals were dissolved in 120 µl 83% dimethyl sulfoxide/17% Sorenson's glycine buffer. The absorbance was measured with a microplate reader at 540 nm wavelength. Cell growth was reported as the percentage of absorbance relative to untreated controls. Each experiment was performed 3 times in triplicate.

Based on these results, the IC_{50} values of the compounds were calculated for each cell culture at 72h using CompuSyn. software (Biosoft, Cambridge, UK).

For the analysis of multiple drug treatments, combination index (CI) was calculated according to the Chou-Talalay method, using a fixed dose ratio[29]. Cells were treated with 0.25x, 0.5x, 1x, 2x, and 4x their respective IC_{50}'s of the compounds for 72h. Cell viability was measured using the MTT assay and CI was calculated using CompuSyn. Software (Biosoft, Cambridge, UK). In this analysis, synergy was defined as CI values lower than 1.0, antagonism as CI values higher than 1.0 and additivity as CI values equal to 1.0.

.

Cell cycle and apoptosis assay

For cell cycle and apoptosis assays, cells were seeded in 6-well plates ($1.5x10^5$ cells/well, n=1) and treated for 24h and 48h. Cells were treated with NVP-BEZ235, AZD6244, or the combination of both compounds. The range of concentrations used was based on calculated IC_{50} values.

To analyze the cell cycle, cells were fixed with ice-cold 70% ethanol and stained with propidium iode (PI)/RNase A staining buffer, containing 10 µg PI/ml PBS (Sigma-Aldrich, St. Louis, USA) and 20 µg RNase A/ml PBS (Sigma-Aldrich, St. Louis, USA).

Apoptosis was determined by double staining with FITC-conjugated Annexin V and PI according to the manufacturer's instructions (BD Pharmingen™, Erembodegem, Belgium).

Cell cycle distribution and the percentage of apoptotic cells were analyzed using a flow cytometer (Becton Dickinson, San Jose, USA) and FlowJo 7.6.5 software (Tree Star Inc., Ashland, USA). Experiments were repeated twice.

In vivo evaluation of the efficacy of targeted treatment

Cell-derived xenograft models were established by subcutaneous (s.c.) injection of $5x10^6$ cells in 200 µl PBS per mouse. Mice were treated with either vehicle (10% NMP/90% PEG) (both from Sigma-Aldrich, St. Louis, USA), NVP-BEZ325 (40 mg/kg; daily, p.o.) or carboplatin (50 mg/kg, 1x/week, i.p.) (Hospira, Antwerp, Belgium) (n=7 per group) for 21 days. Control animals received the equivalent volume of vehicle. Mice were weighed every week and evaluated for adverse effects. Tumor size was measured 2x/week with a caliper and the tumor volume was calculated using the following formula: $V = L \times W^2 \times (\pi/6)$ (V, volume; L, length; W, width). Mice were scarified 1h after last dose. Harvested tumors were weighed, and fragments were fresh-frozen, or formal-fixed and paraffin-embedded for further analyses.

Western blotting

Total protein lysates were obtained from single cell suspensions and tumor tissues post-treatment. Cells were seeded in 10% FBS culture medium in 100 mm^2 plates (500.000 cells/plate, n=2). After 8h, medium was replaced by 1% FBS culture medium. Twenty-four hours after seeding, cells were treated with either NVP-BEZ235 or AZD6244 or both. The range of concentrations used was based on the IC_{50} values. Total protein lysates were prepared from single cell suspensions at 6h and 24h after treatment-initiation and from tumor tissues using the mammalian cell lysis MCL1 kit (Sigma-Aldrich, St.

Louis, USA) according to the manufacturer's instructions. The Pierce BCA protein assay kit (Thermo Scientific, Waltham, USA) was used to determine protein concentrations. Equal amounts of protein were run on any kD Mini Protein TGX gel (Bio-Rad, Hercules, USA) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, USA). Next, the membranes were probed with primary and secondary antibodies. The following primary antibodies were used: AKT, p-AKT$^{(Ser473)}$, S6, p-S6$^{(Ser235/Ser236)}$, ERK1/2, p-ERK1/2$^{(Thr202/Tyr204)}$ (all at 1/1000), and β-actin (1/2500)(all from Cell Signaling Technologies, Danvers, USA). Anti-rabbit or -mouse horseradish peroxidase-conjugated antibodies (Jackson ImmunoResearch, West Grove, USA) were used as secondary antibody. The enhanced chemiluminescence system (Thermo Scientific, Waltham, USA) and FUJI mini-LAS 4000-plus imaging system (GE Healthcare, Diegem, Belgium) were used to visualize protein expression.

Statistics

Results are shown as mean ± standard error of mean (SEM). Two-way ANOVA was performed followed by Bonferroni posttest to determine significant differences between treatment groups with regard to *in vitro* cell viability, cell cycle status, and apoptosis induction. One-way ANOVA was performed for repeated measurements followed by a Tukey's multiple comparison test to analyze differences in therapy response over time between treatment groups *in vivo*. In addition, one-way ANOVA followed by Tukey's multiple comparison test was performed to assess differences in tumor weight between treatment groups. Analyses were performed using GraphPad Prism 5 software (GraphPad Inc., La Jolla, USA). *P*-values of $p<0.05$ were considered to be statistically significant.

Results

Single treatment of NVP-BEZ235 and AZD6244 reduced cell viability

We used three primary EC cell cultures as described by Schrauwen et al.[26]. The histologic and genetic characteristics are described in table 1.

Since these cell cultures showed mutations in the PI3K/AKT/mTOR and Ras/Raf/MEK pathways, we selected compounds which have an inhibitory effect on proteins involved in these pathways and are already in clinical trials. For inhibition of the PI3K/AKT/mTOR pathway, we selected NVP-BEZ235 (pan-PI3K/mTOR inhibitor), Temsirolimus (mTORC1 inhibitor), and NVP-BKM120 (PI3K inhibitor). For

inhibition of the Ras/Raf/MEK pathway we used AZD6244 (MEK1/2 inhibitor). First, we determined the most potent PI3K/AKT/mTOR pathway inhibitor by treating the three cell cultures with the different PI3K/AKT/mTOR pathway inhibitors for 72h (all compounds 0-100 μM). NVP-BEZ235 was the only inhibitor that decreased cell viability in a dose- and time-dependent manner for all 3 cell cultures (Figure 1). AZD6244 induced a significant reduction of cell viability for all 3 cell cultures at the highest concentration at 72h (Figure 1). The cell cultures were less sensitive for this compound compared to NVP-BEZ235 (Table 1). No correlation between mutation profile and response to targeted therapy was demonstrated.

Combination treatment of NVP-BEZ235 and AZD6244 showed a synergistic inhibitory effect on cell viability

We investigated potential synergistic effects of inhibiting both the PI3K/AKT/mTOR and the Ras/Raf/MEK pathways, by treating with NVP-BEZ235 and AZD6244. The anti-proliferative effect of this combination was measured in all 3 cell cultures by calculating the CI. The 3 cell cultures were treated with both NVP-BEZ235 and AZD6244 at 0.25x, 0.5x, 1x, 2x and 4x their respective IC_{50}'s for 72h. For all cell cultures, a synergistic effect was determined by combination of 1x of their respective IC_{50}'s of each compound, NPV-BEZ235 and AZD6244 (Supplementary file 1).

NVP-BEZ235 and AZD6244 induced cell cycle arrest

We investigated the effect of NVP-BEZ325 and AZD6244 as single treatments and in combination on cell cycle arrest for 48h. Since combination treatment with 1x IC_{50} values of NVP-BEZ235 and AZD6244 showed a synergistic effect on the cell viability of all cell cultures, the cell cultures were treated with the respective IC_{50} values of each compound (Table 1). As shown in figure 2A, at 24h no cell cycle arrest was induced for all cell cultures. However, only for 2 out of 3 cell cultures, PC-EM001 and PC-EM002, a significant effect on the cell population was determined at 48h. For PC-EM001, NVP-BEZ235 reduced the number of cells of the S phase compared to control (p<0.05). PC-EM002 showed an increased percentage of the population in the G1 phase of cells treated with NVP-BEZ235 or AZD6244 compared to control (p<0.05 vs p<0.01). In addition, AZD6244 reduced significantly the number of cells in the S phase compared to control (p<0.05).Combination treatment showed no enhanced effect compared to single treatment or control (Figure 2B).

NVP-BEZ235 and AZD6244 induced apoptosis

We analyzed apoptosis induction by NVP-BEZ235 and AZD6244 as single treatments and in combination. Early apoptosis was induced in only 2 cell cultures, PC-EM002 and PC-EM004, by single and combination treatment after 24h (Figure 3A). Also here, cells were treated with the respective 1x IC_{50} values of NVP-BEZ235 and AZD6244 as single treatment or combined (Table 1).

PC-EM004 showed an increase in apoptosis, only for the combination treatment, compared to control (p<0.01) and compared to single treatment of AZD6244 (p<0.05). After 48h of treatment, AZD6244 also induced apoptosis (p<0.05). In contrast, PC-EM002 showed a significant increase in early apoptosis for both NVP-BEZ235 and AZD6244, as single treatments compared to control (p<0.05 vs p<0.01) after 24h. Moreover, combination treatment showed a significant increase in apoptosis compared to control (p<0.001), NVP-BEZ235 (p<0.001), and AZD6244 (p<0.001) as single treatments. Late apoptosis was induced by combination treatment. After 48h, the same effects were observed. Interestingly, for PC-EM002 a higher percentage of cells was present in the apoptosis phase, compared to 24h. In contrast, NVP-BEZ235 induced no apoptosis in PC-EM004 at 48h. We therefore conclude that both NVP-BEZ235 and AZD6244 induce apoptosis. Moreover, for PC-EM002 and PC-EM004 a synergistic effect was found for combination treatment (Figure 3B).

NVP-BEZ235 and AZD6244 reduced phosphorylation of proteins related to the PI3K/AKT/mTOR and Ras/Raf/MEK pathway

To evaluate the short and long term effects of NVP-BEZ235 and AZD6244 on PI3K/AKT/mTOR signaling and Ras/Raf/MEK signaling, the phosphorylation status of AKT, S6, and ERK were analyzed. As shown in figure 4, the total protein expression of AKT, S6, and ERK remained unchanged after treatment with NVP-BEZ235 and AZD6244 for all cell cultures. The primary cell cultures were treated with the respective IC_{50} values of related compound (Table 1). PC-EM001 cells were treated with a low concentration of NVP-BEZ235 ($1.8E^{-4}$µM). This compound showed no difference in phosphorylation of AKT and S6. In contrast, cells treated with AZD6244 alone or in combination with NVP-BEZ235 had decreased levels of p-S6 and p-ERK1/2. In PC-EM002 and PC-EM004 cultures, cells were treated with 0.20µM and 2.09µM NVP-BEZ235, respectively. NVP-BEZ235 decreased p-AKT and p-S6 levels, whereas AZD6244 induced dephosphorylation of p-S6 and p-ERK1/2. Combination treatment with

NVP-BEZ235 and AZD6244 inhibited the phoshorylation of p-AKT, p-S6 and p-ERK1/2 for both cell cultures. Therefore, the effect of NVP-BEZ235 and AZD6244 on the PI3K/AKT/mTOR and Ras/Raf/MEK pathways seems to be related to the concentration used rather than the mutation profile of the cell cultures.

Effect of NVP-BEZ235 on tumor growth in nude mice xenografts

Lastly, we evaluated the effect of NVP-BEZ235 on tumor growth *in vivo*. This compound was used since it was the most potent compound *in vitro*. We established 2 primary s.c. xenograft mouse models generated from PC-EM002 and PC-EM004 cell cultures, since PC-EM002 cells harbored a PI3KCA mutation and the PC-EM004 cells the wild-type genes. We treated the mice with NVP-BEZ235, carboplatin (a chemotherapeutic), or vehicle. For both models, NVP-BEZ235 and carboplatin significantly reduced tumor growth compared to the placebo-treated group. Interestingly, PC-EM004 mice treated with NVP-BEZ235 showed a greater reduction of tumor growth compared to PC-EM002 mice. No difference was determined between the growth-inhibitory effect of NVP-BEZ235 and carboplatin (Figure 5A). This was confirmed by the wet tumor weight at sacrifice (Figure 5B).

Protein expression in the tumor tissue was investigated by Western Blot. For both models, no effect on phosphorylation of AKT was determined. In contrast, dephosphorylation of S6 was shown for NVP-BEZ235 treated mice of both xenograft models and for PC-EM004 mice treated with carboplatin. No difference in protein expression of AKT, S6, and β-actin was determined all mice (Figure 5C).

Discussion

To our knowledge, this is the first study using primary *in vitro* and *in vivo* EC models to test the following targeted therapies: Temsirolimus, NVP-BKM120, NVP-BEZ235 and AZD6244. Our results suggest a synergistic antitumor effect of NVP-BEZ235 and AZD6244 in primary endometrioid EC cell cultures, with regard to cell viability and apoptosis, possibly. Interestingly, our results also suggest that the sensitivity of primary endometrioid EC cell cultures towards either PI3K/mTOR or MEK inhibitors is independent of mutated genes involved in related pathways. In addition, NVP-BEZ235 was the most potent inhibitor of the PI3K/AKT/mTOR pathway. *In vivo*, NVP-BEZ235 reduced tumor growth in mice harboring tumors with different mutation profiles.

Treatment options for advanced and recurrent EC are limited. Therefore, new therapies are necessary. Targeted therapy shows promising response in clinical trials. Therefore, targeted therapies against the PI3K/AKT/mTOR and Ras/Raf/MEK pathways are an interested new treatment strategy for EC, especially by blockade of both pathways to prevent compensatory feedback loops.

In this study we used primary preclinical models. Primary cell cultures better represent the histological and genetic heterogeneity of the primary tumor more compared to commercial cell lines[27, 28]. Therefore, our results better reflect the response of patients on these treatments compared to models based on immortalized cell lines.

We showed that dual inhibition of the PI3K/AKT/mTOR pathway, by blocking PI3K and mTOR with NVP-BEZ235 is more effective compared to inhibition of only PI3K or mTORC1. These results are in accordance with the results of Shoji et al. and Oishi et al., who used commercial EC cell lines and ovarian clear cell carcinoma cell lines, respectively[17, 30]. In hepatocellular carcinoma, however, Kirstein et al. indicated that NVP-BKM120 has a higher antitumor activity compared to RAD001 (mTORC1 inhibitor) and NVP-BEZ235[31].

MSI tumors show high frequent differentiating mutation profiles and therefore it is difficult to determine driver and passenger mutations. Our results are interesting since all primary cell cultures were sensitive to NVP-BEZ235 and AZD6244 and these compounds induced a synergistic effect with regard to cell viability and apoptosis, despite the MSI status. Low concentrations of NVP-BEZ235 ($1.8E^{-4}$µM) did not affect phosphorylation of AKT and S6. In contrast, high concentrations (0.20µM and 2.09µM) reduced p-AKT and p-S6 levels. Therefore, induced effects seem related to the concentration ranges used rather than the IC_{50} values. Moreover, also *in vivo* no correlation was determined between the mutation profile of tumors and response to NVP-BEZ235. This is in accordance with previous results for other type of cancers, in which the presence of KRAS gene mutation was not predictive of sensitivity to MEK or PI3K inhibitors[32-34]. In addition, the response of EC xenografts to NVP-BKM120 was independent of the presence of a PIK3CA gene mutation[12]. However, we have to take into account that we used a limited number of primary endometrioid EC cell cultures. To investigate the *in vitro* and *in vivo* responses on combination of targeted therapies, further studies should include a larger panel of type I and type II EC, including MSS tumors. In addition, the use of MSS tumors could be useful to determine biomarkers able to predict the response to targeted therapies.

In vitro, NVP-BEZ235 was a more potent compound on PC-EM002 cells than on PC-EM004 cells. In contrast, *in vivo* results showed a higher tumor growth reduction for mice bearing PC-EM004 tumors compared to PC-EM002 mice. Other studies demonstrated also this phenomenon that *in vitro* sensitivity to mTORC1 inhibitors does not correlate with *in vivo* sensitivity[35, 36]. Fuereder et al. showed that *in vivo* efficacy of NVP-BEZ235 is not correlated with PI3K/mTOR target regulation, hotspot mutations in *PIK3CA*, *Ras* or *BRAF* in gastric tumor xenograf models, based on commercial cell lines[35].

In vivo, NVP-BEZ235 showed the same effect on tumor growth inhibition as carboplatin. It is shown that NVP-BEZ235 synergizes with chemotherapy *in vitro* and *in vivo* for several cancers[37, 38]. In addition, combination of dual PI3K/mTOR inhibitors with radiotherapy can improve radiosensitivity in radioresistant prostate cancer cells[39]. Therefore, combination treatment of (neo)adjuvant therapy and targeted therapy for advanced and recurrent EC could be an interesting strategy, especially for patients harboring a chemo- or radioresistant tumor. Further comprehensive studies should be performed to test the safety and efficacy *in vitro* and *in vivo* of these treatments before clinical application.

Although the present study has several limitations such as the small number of cell cultures tested *in vitro* and *in vivo*, it demonstrated a synergistic interaction between NVP-BEZ235 and AZD6244 in primary endometrioid EC cells *in vitro*. In addition, NVP-BEZ235 showed the same anti-proliferative effects as carboplatin *in vivo*. Therefore, it will be interesting to determine the synergistic effect of targeted therapies and currently used therapies. Remarkably, the antitumor effects of single and combination treatment of targeted therapies *in vitro* and *in vivo* were independent of mutations related to the PI3K/AKT/mTOR and Ras/Raf/MEK pathway. Moreover, they showed anticancer effects in MSI defined tumors. Altogether, targeted therapies against PI3K and/or mTOR seem a promising treatment strategy for EC.

Conflict of Interest Statement

No conflicts of interest

Acknowledgments

This study was supported by a grant of the Belgian Foundation against Cancer (Grant No. KPC_29_005). In addition, we would like to thank Anticancer Fund for scientific and financial support.

References

[1] Siegel R., Ma J., Zou Z., Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* ;**64**:9-29;2014

[2] Sorosky J.I. Endometrial cancer. *Obstet Gynecol* ;**120**:383-97;2012

[3] Amant F., Moerman P., Neven P., Timmerman D., Van L.E., Vergote I. Endometrial cancer. *Lancet* ;**366**:491-505;2005

[4] Emons G., Heyl W. Hormonal treatment of endometrial cancer. *J Cancer Res Clin Oncol* ;**126**:619-23;2000

[5] Humber C.E., Tierney J.F., Symonds R.P., Collingwood M., Kirwan J., Williams C. et al. Chemotherapy for advanced, recurrent or metastatic endometrial cancer: a systematic review of Cochrane collaboration. *Ann Oncol* ;**18**:409-20;2007

[6] Salvesen H.B., Haldorsen I.S., Trovik J. Markers for individualised therapy in endometrial carcinoma. *Lancet Oncol* ;**13**:e353-e361;2012

[7] Cloughesy T.F., Yoshimoto K., Nghiemphu P., Brown K., Dang J., Zhu S. et al. Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma. *PLoS Med* ;**5**:e8;2008

[8] Mendoza M.C., Er E.E., Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci* ;**36**:320-8;2011

[9] Oza A.M., Elit L., Tsao M.S., Kamel-Reid S., Biagi J., Provencher D.M. et al. Phase II study of temsirolimus in women with recurrent or metastatic endometrial cancer: a trial of the NCIC Clinical Trials Group. *J Clin Oncol* ;**29**:3278-85;2011

[10] Ray-Coquard I., Favier L., Weber B., Roemer-Becuwe C., Bougnoux P., Fabbro M. et al. Everolimus as second- or third-line treatment of advanced endometrial cancer: ENDORAD, a phase II trial of GINECO. *Br J Cancer* ;**108**:1771-7;2013

[11] Mackay H.J., Eisenhauer E.A., Kamel-Reid S., Tsao M., Clarke B., Karakasis K. et al. Molecular determinants of outcome with mammalian target of rapamycin inhibition in endometrial cancer. *Cancer* ;**120**:603-10;2014

[12] Bradford L.S., Rauh-Hain A., Clark R.M., Groeneweg J.W., Zhang L., Borger D. et al. Assessing the efficacy of targeting the phosphatidylinositol 3-kinase/AKT/mTOR signaling pathway in endometrial cancer. *Gynecol Oncol* ;**133**:346-52;2014

[13] Maira S.M., Pecchi S., Huang A., Burger M., Knapp M., Sterker D. et al. Identification and characterization of NVP-BKM120, an orally available pan class I PI3-Kinase inhibitor. *Mol Cancer Ther* ;2011

[14] Bendell J.C., Rodon J., Burris H.A., de J.M., Verweij J., Birle D. et al. Phase I, dose-escalation study of BKM120, an oral pan-Class I PI3K inhibitor, in patients with advanced solid tumors. *J Clin Oncol* ;**30**:282-90;2012

[15] Rodon J., Brana I., Siu L.L., De Jonge M.J., Homji N., Mills D. et al. Phase I dose-escalation and -expansion study of buparlisib (BKM120), an oral pan-Class I PI3K inhibitor, in patients with advanced solid tumors. *Invest New Drugs* ;**32**:670-81;2014

[16] Maira S.M., Stauffer F., Brueggen J., Furet P., Schnell C., Fritsch C. et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Mol Cancer Ther* ;**7**:1851-63;2008

[17] Shoji K., Oda K., Kashiyama T., Ikeda Y., Nakagawa S., Sone K. et al. Genotype-dependent efficacy of a dual PI3K/mTOR inhibitor, NVP-BEZ235, and an mTOR inhibitor, RAD001, in endometrial carcinomas. *PLoS One* ;**7**:e37431;2012

[18] Maira S.M., Stauffer F., Schnell C., Garcia-Echeverria C. PI3K inhibitors for cancer treatment: where do we stand? *Biochem Soc Trans* ;**37**:265-72;2009

[19] Yeh T.C., Marsh V., Bernat B.A., Ballard J., Colwell H., Evans R.J. et al. Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase kinase 1/2 inhibitor. *Clin Cancer Res* ;**13**:1576-83;2007

[20] Carvajal R.D., Sosman J.A., Quevedo J.F., Milhem M.M., Joshua A.M., Kudchadkar R.R. et al. Effect of selumetinib vs chemotherapy on progression-free survival in uveal melanoma: a randomized clinical trial. *JAMA* ;**311**:2397-405;2014

[21] Hochster H.S., Uboha N., Messersmith W., Gold P.J., ONeil B.H., Cohen D. et al. Phase II study of selumetinib (AZD6244, ARRY-142886) plus irinotecan as second-line therapy in patients with K-RAS mutated colorectal cancer. *Cancer Chemother Pharmacol* ;2014

[22] Paolo M., Assunta S., Antonio R., Claudia S.P., Anna B.M., Clorinda S. et al. Selumetinib in advanced non small cell lung cancer (NSCLC) harbouring KRAS mutation: endless clinical challenge to KRAS-mutant NSCLC. *Rev Recent Clin Trials* ;**8**:93-100;2013

[23] Bedard P.L., Tabernero J., Janku F., Wainberg Z.A., Paz-Ares L., Vansteenkiste J. et al. Ph lb dose escalation study of oral pan-PI3K inhibitor buparlisib (BKM120) with oral MEK1/2 inhibitor trametinib (GSK1120212) in patients with advanced solid tumours. *Clin Cancer Res* ;2014

[24] Khalili J.S., Yu X., Wang J., Hayes B.C., Davies M.A., Lizee G. et al. Combination small molecule MEK and PI3K inhibition enhances uveal melanoma cell death in a mutant. *Clin Cancer Res* ;**18**:4345-55;2012

[25] Shimizu T., Tolcher A.W., Papadopoulos K.P., Beeram M., Rasco D.W., Smith L.S. et al. The clinical effect of the dual-targeting strategy involving PI3K/AKT/mTOR and RAS/MEK/ERK pathways in patients with advanced cancer. *Clin Cancer Res* ;**18**:2316-25;2012

[26] Schrauwen S., Coenegrachts L., Depreeuw J., Luyten C., Verbist G., Debruyne D. et al. Microsatellite instable and microsatellite stable primary endometrial carcinoma cells and their

492 subcutaneous and orthotopic xenografts recapitulate the characteristics of the corresponding
493 primary tumor. *Int J Gynecol Cancer* ;**25**:363-71;2015

494 [27] Maletzki C., Stier S., Gruenert U., Gock M., Ostwald C., Prall F. et al. Establishment,
495 characterization and chemosensitivity of three mismatch repair deficient cell lines from
496 sporadic and inherited colorectal carcinomas. *PLoS One* ;**7**:e52485;2012

497 [28] Sos M.L., Michel K., Zander T., Weiss J., Frommolt P., Peifer M. et al. Predicting drug
498 susceptibility of non-small cell lung cancers based on genetic lesions. *J Clin Invest* ;**119**:1727-
499 40;2009

500 [29] Chou T.C. Theoretical basis, experimental design, and computerized simulation of synergism
501 and antagonism in drug combination studies. *Pharmacol Rev* ;**58**:621-81;2006

502 [30] Oishi T., Itamochi H., Kudoh A., Nonaka M., Kato M., Nishimura M. et al. The PI3K/mTOR
503 dual inhibitor NVP-BEZ235 reduces the growth of ovarian clear cell carcinoma. *Oncol Rep*
504 ;**32**:553-8;2014

505 [31] Kirstein M.M., Boukouris A.E., Pothiraju D., Buitrago-Molina L.E., Marhenke S., Schutt J. et al.
506 Activity of the mTOR inhibitor RAD001, the dual mTOR and PI3-kinase inhibitor BEZ235 and
507 the PI3-kinase inhibitor BKM120 in hepatocellular carcinoma. *Liver Int* ;**33**:780-93;2013

508 [32] Davies B.R., Logie A., McKay J.S., Martin P., Steele S., Jenkins R. et al. AZD6244 (ARRY-
509 142886), a potent inhibitor of mitogen-activated protein kinase/extracellular signal-regulated
510 kinase kinase 1/2 kinases: mechanism of action in vivo, pharmacokinetic/pharmacodynamic
511 relationship, and potential for combination in preclinical models. *Mol Cancer Ther* ;**6**:2209-
512 19;2007

513 [33] Tentler J.J., Nallapareddy S., Tan A.C., Spreafico A., Pitts T.M., Morelli M.P. et al.
514 Identification of predictive markers of response to the MEK1/2 inhibitor selumetinib (AZD6244)
515 in K-ras-mutated colorectal cancer. *Mol Cancer Ther* ;**9**:3351-62;2010

[34] Zhong H., Sanchez C., Spitrzer D., Plambeck-Suess S., Gibbs J., Hawkins W.G. et al. Synergistic effects of concurrent blockade of PI3K and MEK pathways in pancreatic cancer preclinical models. *PLoS One* ;**8**:e77243;2013

[35] Fuereder T., Wanek T., Pflegerl P., Jaeger-Lansky A., Hoeflmayer D., Strommer S. et al. Gastric cancer growth control by BEZ235 in vivo does not correlate with PI3K/mTOR target inhibition but with [18F]FLT uptake. *Clin Cancer Res* ;**17**:5322-32;2011

[36] Geoerger B., Kerr K., Tang C.B., Fung K.M., Powell B., Sutton L.N. et al. Antitumor activity of the rapamycin analog CCI-779 in human primitive neuroectodermal tumor/medulloblastoma models as single agent and in combination chemotherapy. *Cancer Res* ;**61**:1527-32;2001

[37] Moon d.G., Lee S.E., Oh M.M., Lee S.C., Jeong S.J., Hong S.K. et al. NVP-BEZ235, a dual PI3K/mTOR inhibitor synergistically potentiates the antitumor effects of cisplatin in bladder cancer cells. *Int J Oncol* ;**45**:1027-35;2014

[38] Yang F., Qian X.J., Qin W., Deng R., Wu X.Q., Qin J. et al. Dual phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 has a therapeutic potential and sensitizes cisplatin in nasopharyngeal carcinoma. *PLoS One* ;**8**:e59879;2013

[39] Chang L., Graham P.H., Hao J., Ni J., Bucci J., Cozzi P.J. et al. PI3K/Akt/mTOR pathway inhibitors enhance radiosensitivity in radioresistant prostate cancer cells through inducing apoptosis, reducing autophagy, suppressing NHEJ and HR repair pathways. *Cell Death Dis* ;**5**:e1437;2014

Figures and legends

Table 1. IC_{50} values of three primary endometrioid EC cell cultures for PI3K and/or mTOR inhibitors and a MEK inhibitor (AZD6244) at 72h.

Figure 1. The effect of NVP-BEZ235, Temsirolimus, NVP-BKM120 and AZD6244 on cell viability in primary endometrial carcinoma cell cultures. Dose response curves of NVP-BEZ235, Temsirolimus, NVP-BKM120, and AZD6244 for all primary cell cultures are shown. All cell cultures

were treated with increasing doses of the 4 compounds (0-100 µM) (n=4 wells per concentration) for 72h. Changes in cell proliferation were examined by MTT assay. Each data point represents the mean ± SEM of triplicate experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to control, two-way ANOVA with Bonferroni post hoc test.

Figure 2. The induction of cell cycle arrest by NVP-BEZ235 and AZD6244 on in primary endometrial carcinoma cell cultures. Three primary EC cell cultures were treated with NVP-BEZ235, AZD6244 as single or combination treatment. Cell cultures were treated with the respective IC_{50} values of NVP-BEZ235 and AZD6244. Cell cycle distribution was determined at 24h, and 48h. A) At 24h no cell cycle arrest was induced for all cell cultures. B) After 48h, NVP-BEZ235 and AZD6244 increased the population of cells in the S phase for PC-EM001 and PC-EM002. In addition, an increased percentage of cell in G1 phase of PC-EM002 was determined for NVP-BEZ235 and AZD6244 treatment. Data represent the mean ± SEM of duplicate experiments after 48h of treatment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to each treatment group, two-way ANOVA with Bonferroni post hoc test.

Figure 3. The effect of NVP-BEZ235 and AZD6244 on early and late apoptosis in primary endometrial carcinoma cell cultures. Three primary EC cell cultures were treated with NVP-BEZ235 combined with AZD6244 or as single treatments. Cell cultures were treated with the respective IC_{50} values of NVP-BEZ235 and AZD6244. Induction of apoptosis was determined at 24h, and 48h. A) For two cell cultures, PC-EM002 and PC-EM004, early apoptosis was induced at 24h. PC-EM002 also showed late apoptosis in response to combination treatment at 24h. B) After 48h, an increased apoptosis was induced in PC-EM002 cells. Data represent the mean ± SEM of duplicate experiments after 24h and 48h of treatment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to each treatment group, two-way ANOVA with Bonferroni post hoc test.

Figure 4. The influence of NVP-BEZ235 and AZD6244 on activation of cell signaling pathways. Cells were incubated with NVP-BEZ235 and/or AZD6244 for 6h and 24h. Cell cultures were treated with the respective IC_{50} values of NVP-BEZ235 and AZD6244. Phosphorylation of AKT, S6, and ERK1/2 was analyzed by Western Blotting. In the PC-EM001 cell culture, only AZD6244 showed an

574 effect on phosphorylation of investigated proteins, by dephosphorylation of p-S6$^{(Ser235/236)}$ and p-
575 ERK1/2$^{(Thr202/Tyr204)}$. In PC-EM002 and PC-EM004 cultures, NVP-BEZ235 reduced p-AKT$^{(Ser473)}$ and p-
576 S6$^{(Ser235/236)}$ levels and AZD6244 decreased p-S6$^{(Ser235/236)}$ and p-ERK$^{(Thr202/Tyr204)}$ levels. Combination
577 treatment showed dephosphorylation of all 3 proteins for both cell cultures. Effects were determined
578 for at least 24h.
579
580 **Figure 5. The effect of NVP-BEZ235 on tumor growth *in vivo*.** PC-EM002 and PC-EM004 bearing
581 mice were treated with vehicle (10% NMP/90% PEG), NVP-BEZ235 (40 mg/kg; daily, p.o,), or
582 carboplatin (50 mg/kg, 1x/week, i.p.) (n=7). A) NVP-BEZ235 reduced tumor growth for PC-EM002 and
583 even stabilized tumor growth for PC-EM004. The effect of targeted therapy was comparable to
584 chemotherapy. One-way ANOVA for repeated measurements followed by Tukey's multiple
585 comparison test was performed. B) Wet tumor weights were significantly different between mice
586 treated with a compound and mice treated with placebo. One-way ANOVA followed by Tukey's
587 multiple comparison test was performed. C) For both xenograft models a reduction of p-S6 was
588 demonstrated for tumors of mice treated with NVP-BEZ235.
589 Results are presented as mean ± SEM. ** p<0.01, *** p<0.001 compared to placebo group.
590
591 **S1. Overview combination index of primary endometrioid endometrial carcinoma cell cultures**
592 **treated with a combination of NPV-BEZ235 and AZD6244 for 72h.** Combination index: Synergistic
593 effect is indicated in blue and antagonistic effect is indicated in orange.

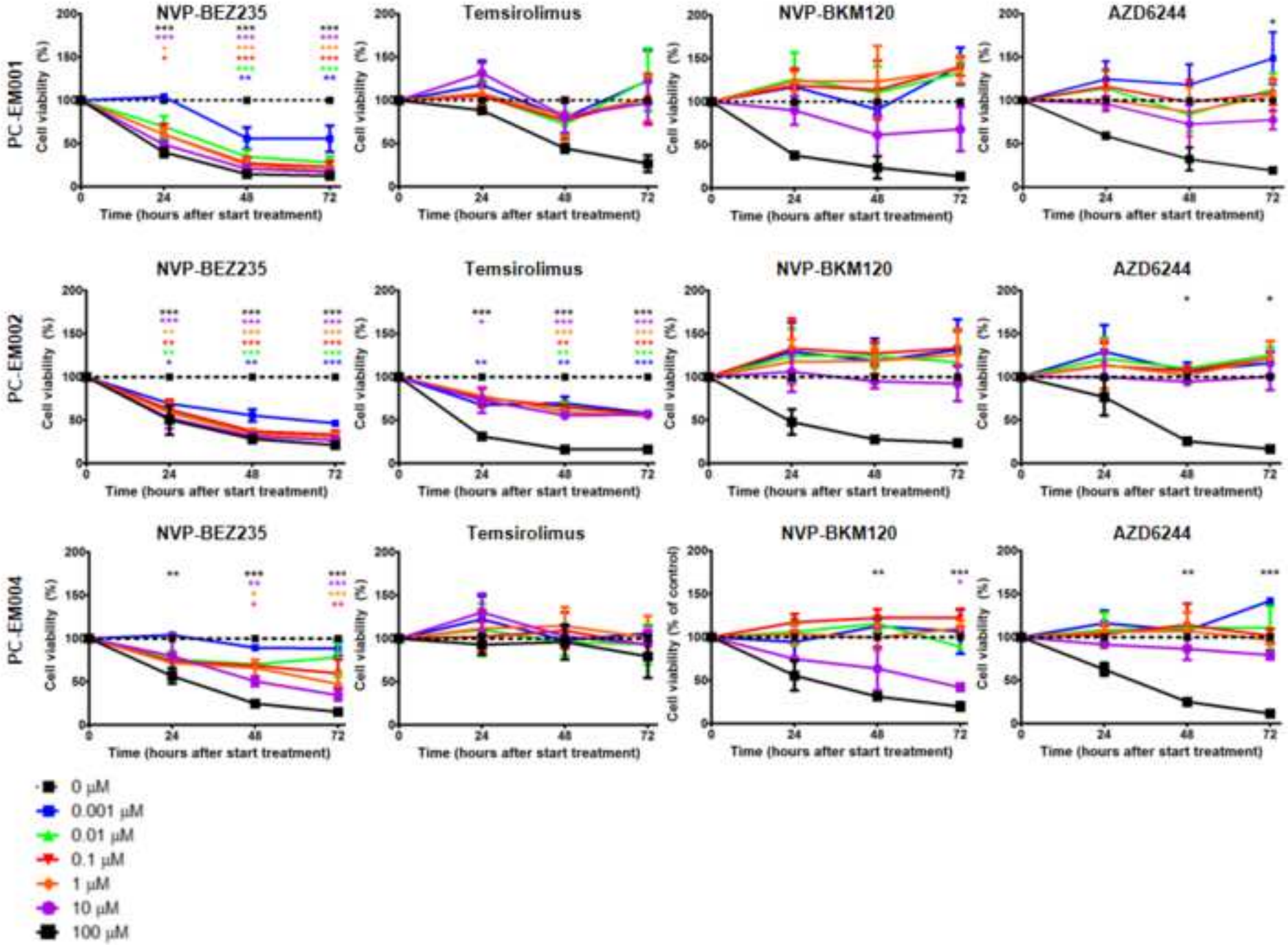
4. Table 1

Click here to download 4. Table: BEZ-AZD_Table 1_20150304_SS.docx

ID cell culture	Histopathology	Grade	FIGO Stage (2009)	Mutation profile	NVP-BEZ235 (µM)	AZD6244 (µM)
PC-EM001	Endometrioid	2	II	KRAS_G35 ACT (GT)	1.80E-4	27.00
PC-EM002	Dedifferentiated endometrioid	3	II	PIK3CA_277T (TC) PTEN_697 (CT)	0.20	79.70
PC-EM004	Mixed endometrioid/serous	3	IA	/	2.09	41.55

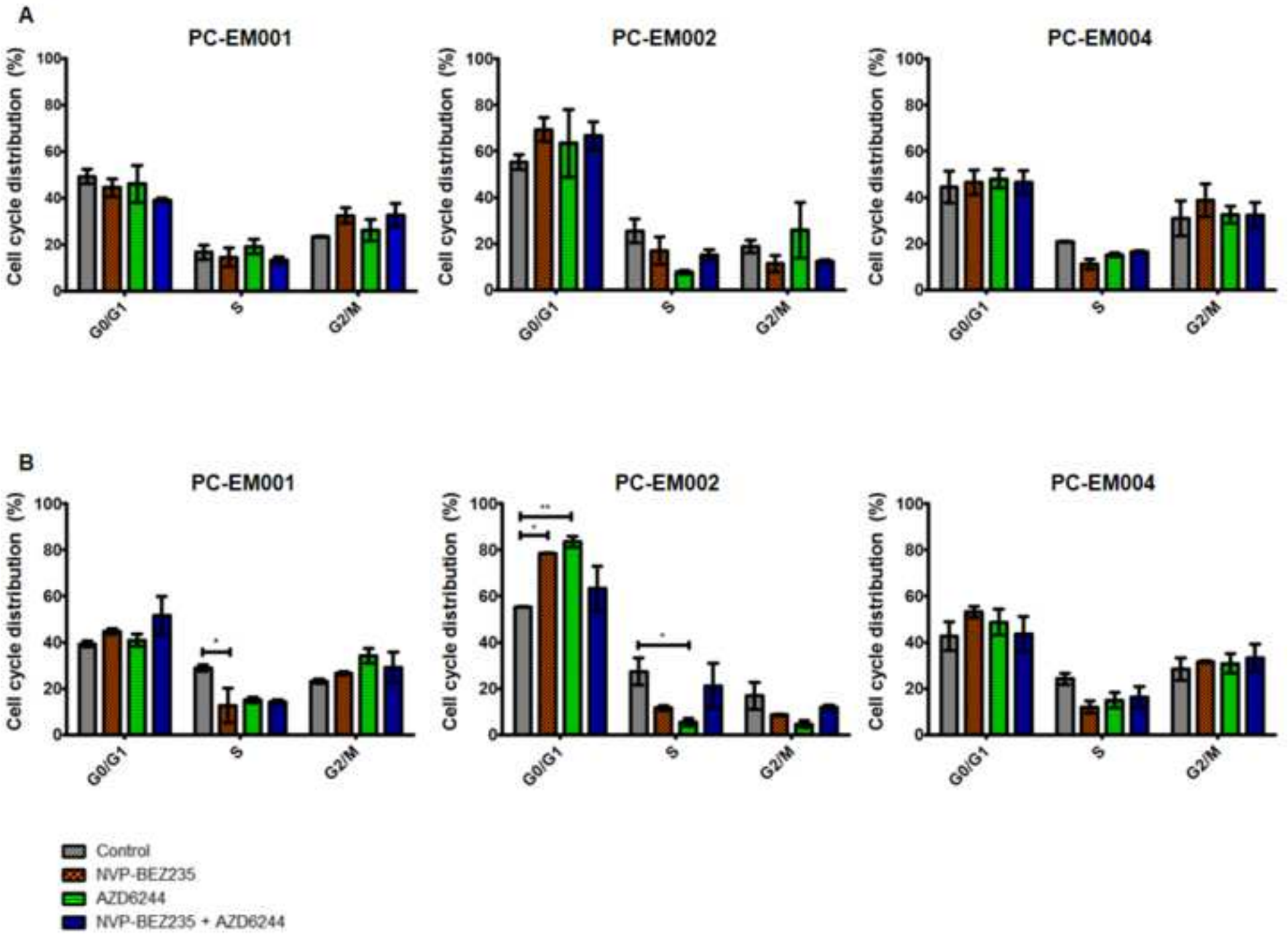
5. Figure 1

Click here to download high resolution image



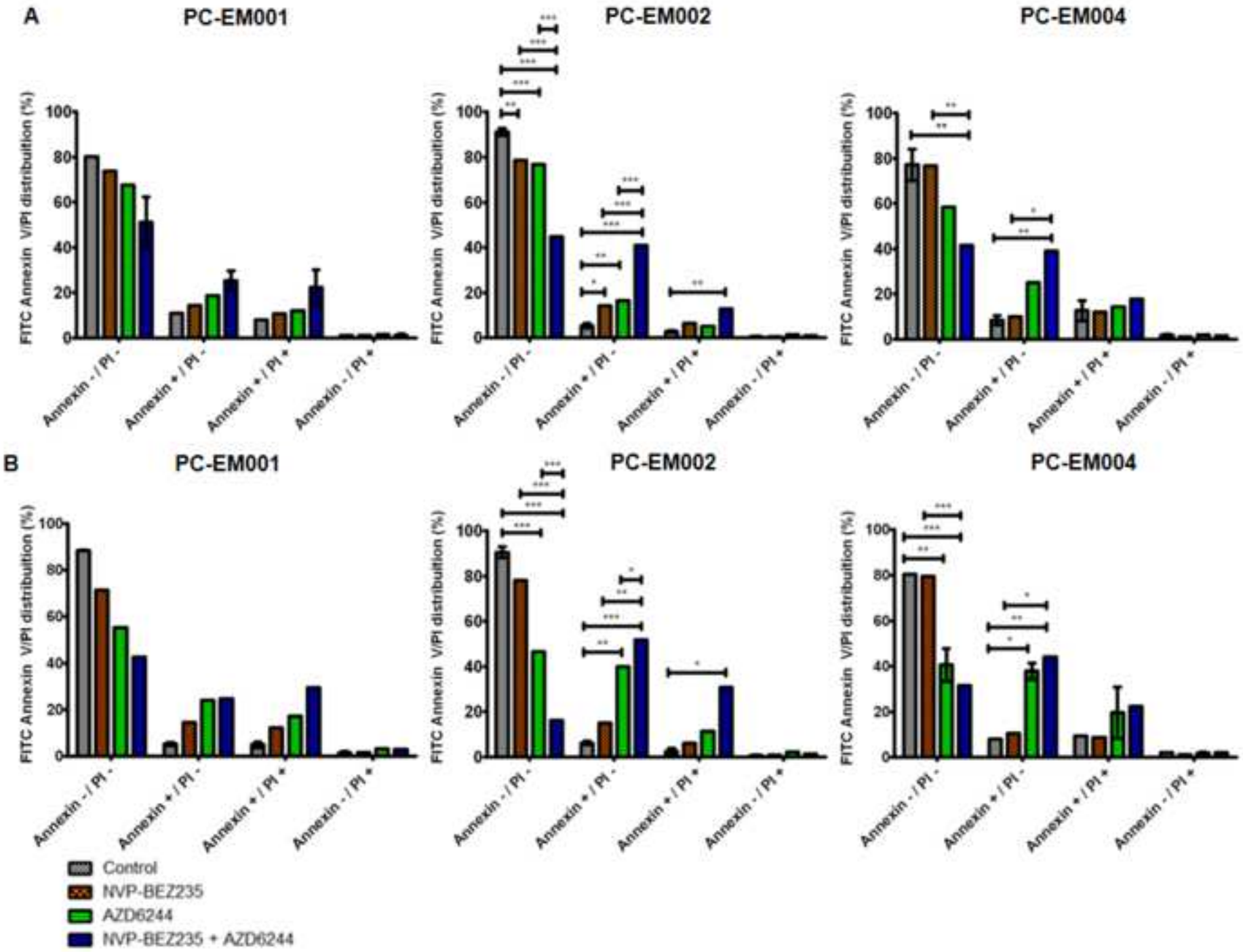
5. Figure 2

Click here to download high resolution image



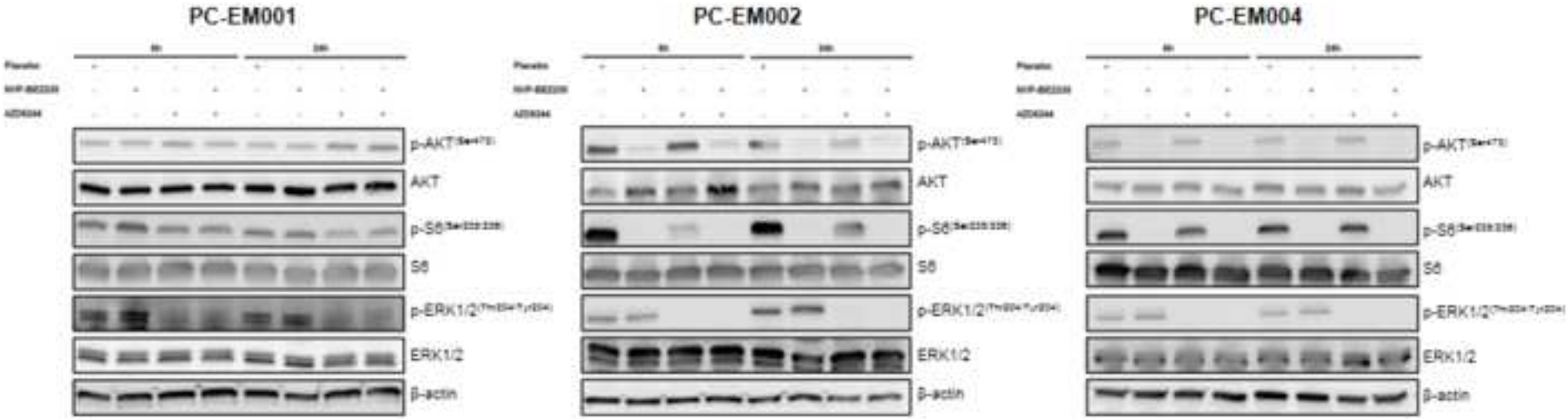
5. Figure 3

Click here to download high resolution image



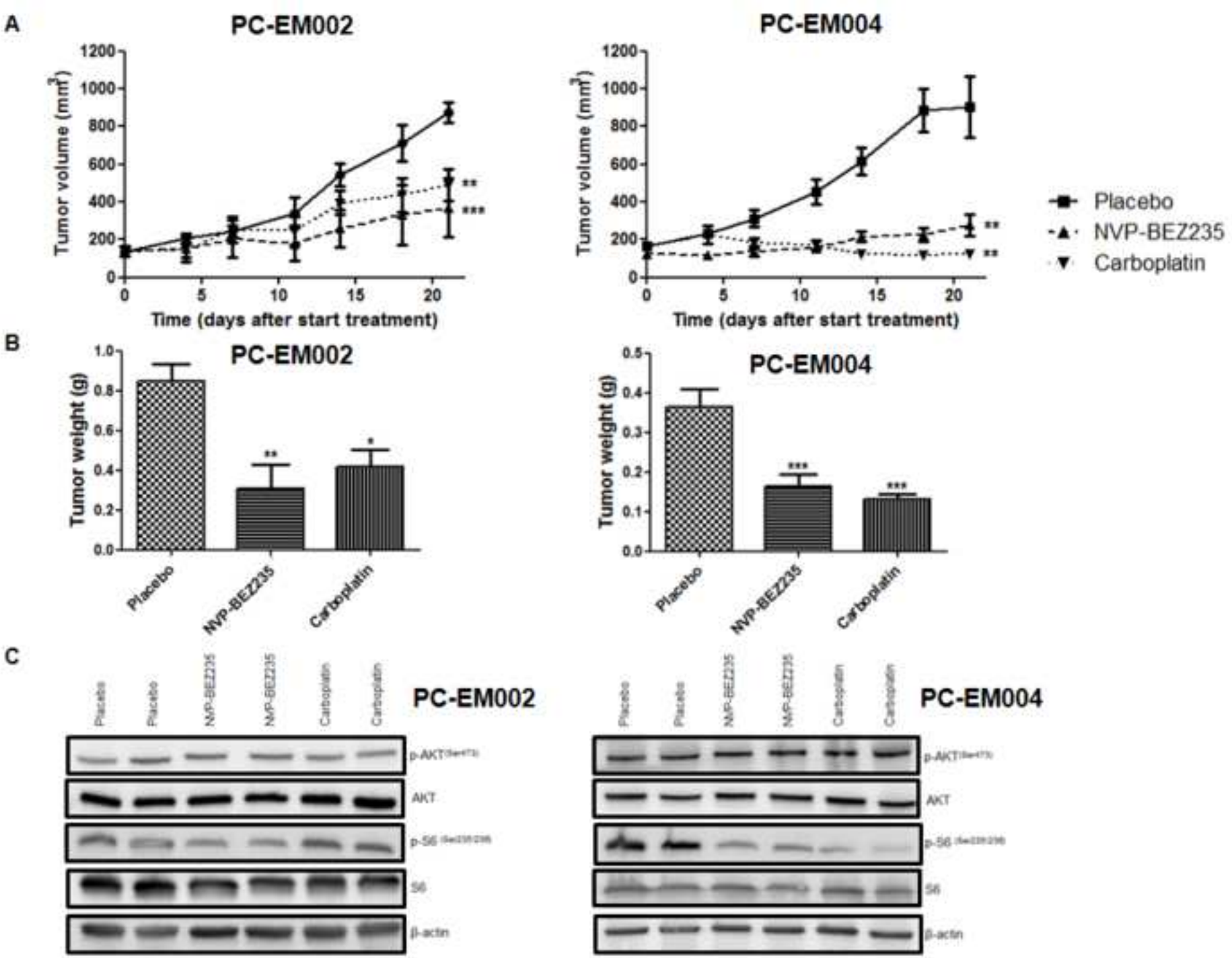
5. Figure 4

Click here to download high resolution image



5. Figure 5

Click here to download high resolution image



6. Supplementary Material

Click here to download 6. Supplementary Material: BEZ-AZD_Supplementary file 1_20150304_SS.pdf

Supplementary file 1

PC-EM001

NVP-BEZ235 (µM)

$7.20E^{-4}$	1.33	1.26	1.22	1.11	1.00	
$3.60E^{-4}$	0.77	0.77	0.69	0.64	0.52	
$1.80E^{-4}$	1.21	0.56	0.45	0.43	0.32	
$0.90E^{-4}$	2.18	1.09	0.41	0.47	0.23	
$0.45E^{-4}$	5.70	2.17	1.32	0.80	0.22	
	6.80	**13.50**	**27.00**	**54.00**	**108.00**	**AZD6244 (µM)**

PC-EM002

NVP-BEZ235 (µM)

0.80	0.10	0.19	0.36	0.67	1.34	
0.40	0.11	0.19	0.35	0.66	1.35	
0.20	0.10	0.19	0.35	0.67	1.37	
0.10	0.11	0.19	0.36	0.67	1.36	
0.05	0.11	0.19	0.36	0.68	1.36	
	19.93	**39.85**	**79.70**	**159.40**	**318.80**	**AZD6244 (µM)**

PC-EM004

NVP-BEZ235 (µM)

8.40	0.06	0.10	0.17	0.26	0.46	
4.20	0.07	0.11	0.17	0.25	0.47	
2.10	0.08	0.12	0.19	0.29	0.47	
1.05	0.07	0.13	0.22	0.29	0.48	
0.53	0.07	0.12	0.22	0.31	0.47	
	10.39	**20.78**	**41.55**	**83.10**	**166.20**	**AZD6244 (µM)**