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Dual Targeting of Phosphoinositide 3-Kinase and Mammalian Target of Rapamycin Using NVP-BEZ235 as a Novel Therapeutic Approach in Human Ovarian Carcinoma

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Abstract

Purpose—This study evaluates the effect of dual PI3K and mTOR inhibition using NVP-BEZ235 in preclinical models of ovarian cancer as a potential novel therapeutic strategy.

Experimental Design—Inhibition of PI3K/Akt/mTOR signaling by NVP-BEZ235 was demonstrated by immunoblotting. The effect on cell proliferation was assessed in eighteen ovarian cancer cell lines, including four pairs of syngeneic cisplatin-sensitive and cisplatin-resistant cell lines. The *in vivo* effects of NVP-BEZ235 on established tumor growth were evaluated using an immunocompetent, transgenic murine ovarian cancer model (LSL-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$).

Results—NVP-BEZ235 decreased cell proliferation in all ovarian cancer cell lines assayed and sensitized cisplatin-resistant cells to the cytotoxic effects of cisplatin. Cell lines with *PI3K* activating mutations or *Pten* deletions were significantly more sensitive to the effect of NVP-BEZ235 than cell lines without these mutations ($p < 0.05$). A statistically significant correlation was found between relative levels of p4E-BP1 and the IC_{50} for NVP-BEZ235. In LSL-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice with established intraperitoneal tumor disease, oral administration of NVP-BEZ235 decreased pAkt, p4E-BP1 and Ki67 in tumor tissue, and resulted in significantly longer survival compared to control animals ($p < 0.05$). NVP-BEZ235 also induced cell cycle arrest, caspase 3 activity, and reduced cell migration.

Conclusions—Targeting PI3K and mTOR simultaneously using NVP-BEZ235 effectively inhibits ovarian cancer cell growth even in the presence of platinum resistance and prolongs survival of mice with intra-abdominal ovarian tumor disease. We propose that dual PI3K and mTOR inhibition using NVP-BEZ235 may be an effective novel therapeutic approach in patients with ovarian cancer.

Keywords

ovarian cancer; NVP-BEZ235; PI3K; mTOR; platinum resistance

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INTRODUCTION

Ovarian cancer is the most lethal of the gynecological malignancies and the 5th leading cause of cancer related deaths amongst women in the US. About 21,880 new cases are expected in the US in 2010, and 13,850 patients are estimated to succumb to the disease (1). Approximately two thirds of all patients are diagnosed with advanced disease with a 5-year survival rate of about 30% (2–4). The presence of primary platinum-resistant disease in 15% of all patients, and the development of platinum-resistance in recurrent ovarian cancer presents a major therapeutic challenge.

Recently, the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway has been the focus of novel targeted therapeutic agents. This pathway plays an important role in many biological processes, including cell proliferation, growth, and survival (5–7). Activated PI3K phosphorylates phosphoinositide 3,4 bisphosphate (PIP_2) to phosphoinositide 3,4,5 trisphosphate (PIP_3), which recruits the serine/threonine kinase Akt to the membrane where it is phosphorylated and activated. Akt activates several downstream targets including mTOR, which regulates translation initiation by phosphorylating p70S6 kinase and 4E-binding protein 1 (4E-BP1). p70S6 kinase phosphorylates the ribosomal protein S6 with subsequent enhancement of translation of mRNAs bearing a 5′-terminal oligopyrimidine tract. Phosphorylation of 4E-BP1 causes its dissociation from the eukaryotic initiation factor-4E (eIF-4E), resulting in enhanced cap-dependent protein synthesis. The tumor suppressor protein PTEN antagonizes the PI3K/Akt/mTOR pathway by dephosphorylating PIP_3.

PI3K/Akt/mTOR pathway activation in ovarian cancer has been demonstrated in several studies (8–12). Overexpression of phosphorylated Akt in human ovarian tissue by immunohistochemistry is found in 50% of papillary serous ovarian cancers, and in up to 70–80% of endometrioid and clear cell adenocarcinomas of the ovary (12). About 40% of primary ovarian tumors show amplification of the gene encoding the kinase active p110α subunit of PI3K (*PIK3CA*) (11). Expression of phosphorylated 4E-BP1 in ovarian tumor specimens has been associated with a poor prognosis (13).

NVP-BEZ235 is a novel therapeutic agent that targets two molecules in the PI3K/Akt/mTOR pathway, PI3K and mTOR (14). It is an ATP-competitive pan-class I PI3K inhibitor that is effective against p110α with hotspot mutations, and likewise inhibits both mammalian target of rapamycin complex 1 (mTORC1) and mTORC2 (14–16). Previous studies have demonstrated the efficacy of NVP-BEZ235 as an anti-tumor agent *in vitro* and *in vivo* in glioblastoma, multiple myeloma, melanoma, lymphomas, sarcomas, breast and lung cancer models (14–26). In a human sarcoma model, the drug was effective in inhibiting liver metastasis (24,25). NVP-BEZ235 was shown to decrease the population of cells enriched in prostate cancer progenitors and their sphere-forming capacity (27). NVP-BEZ235 treatment has demonstrated anti-tumor efficacy in combination with chemotherapeutic agents and ionizing radiation (18,20–22,25,28). The effects of this dual PI3K/mTOR inhibitor have been attributed to the induction of cell cycle arrest, apoptosis, and to its anti-angiogenic properties (14–23,25).

In the present study, we demonstrate that dual inhibition of PI3K and mTOR using NVP-BEZ235 inhibits cell growth in a panel of 18 cisplatin-sensitive and cisplatin-resistant human ovarian carcinoma cell lines. The presence of *PI3K* activating mutations or *Pten* deletions and elevated levels of p4E-BP1 protein were significantly correlated with increased sensitivity to NVP-BEZ235. In a transgenic, immunocompetent mouse model of ovarian cancer, NVP-BEZ235 inhibited PI3K/Akt/mTOR pathway signaling in tumor tissue

and prolonged the survival of mice with established tumor disease. NVP-BEZ235 also induced cell cycle arrest, caspase 3 activity, and reduced cell migration.

MATERIALS AND METHODS

Cell Lines

The cell lines A2780 and IGROV1 were obtained from the National Cancer Institute (Frederick, MD). SKOV3, ES-2 and TOV-112D cells were obtained from the American Type Culture Collection (Rockville, MD), and OAW42 cells from the European Collection of Cell Cultures (Salisbury, UK). HEYC2, OV167 and OV207 cells were kind gifts from Dr. V. Shridhar (Mayo Clinic; Rochester, MN). The cell line PE01 was a kind gift from Dr. S.P. Langdon (Edinburgh Cancer Research Center, University of Edinburgh; Edinburgh, UK). MCAS cells were from the Japanese Health Science Research Resources Bank (Osaka, Japan). The cell lines C13*, CP70 and OV2008 were kind gifts from Dr. B. Karlan (Cedars Sinai; Los Angeles, CA). OVCAR5 cells were a kind gift from Dr. T. Lane (University of California, Los Angeles; Los Angeles, CA). The mutational status of each cell line was queried in the literature and in the Catalogue of Somatic Mutations in Cancer (COSMIC, http://www.sanger.ac.uk/genetics/CGP/cosmic/) (29,30). The individuality of each cell line was checked by mitochondrial DNA sequencing. SKOV3-CisR and OVCAR5-CisR cells were established by exposing the parental SKOV3 and OVCAR5 cell lines, respectively, to increasing concentrations of cisplatin over 12 months. The MOVCAR18 cell line was established from ascites-derived cells harvested from an ovarian tumor bearing, transgenic mouse (LSL-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$).

Immunoblot Assay

NVP-BEZ235 and RAD001 were generously provided by NOVARTIS Pharmaceuticals, (Basel, Switzerland). Immunoblot assays were performed using standard procedures. Briefly, cells were lysed and protein concentrations of clarified cell lysates normalized. Lysates were mixed with 4X Laemmli buffer and analyzed by SDS-PAGE followed by immunoblotting. Antibodies used were from Cell Signaling Technology (phospho-4E-BP1 (T37/46), phospho-Akt (S473), Akt, phospho-S6 (S240/244), S6) or Santa Cruz Biotechnology (β-actin; Santa Cruz, CA). Immuno-reactive bands were visualized by chemifluorescence (ECL-Plus; GE Healthcare Biosciences; Piscataway, NJ), captured with a Typhoon® 9400 scanner (GE Healthcare Biosciences), and quantified using ImageQuant® software (GE Healthcare Biosciences). Blots were stripped and reprobed for β-actin to ensure equal protein loading. For the correlation between NVP-BEZ235 IC_{50} and pAkt, pS6 or p4E-BP1 the basal level of pAkt, pS6 or p4E-BP1 was expressed relative to the corresponding level of β-actin.

Cell Proliferation Assays

Cells seeded in 96- (XTT) or 24- (Vi-CELL) well plates were incubated overnight, followed by drug treatment for 72h at 37°C. Cell viability was assessed according to standard procedures using the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) cell viability assay. XTT (1 mg/ml) and PMS (phenazine methosulphate; 1 mg/ml) were added, and the metabolism of XTT was measured at 450 nm on an absorbance microplate reader (ELx800, Bio-Tek Instruments; Winooski, VT). Cell counting was performed on a Vi-CELL Cell Viability Analyzer (Beckman Coulter; Brea, CA). Following treatment, adherent cells were detached with trypsin, combined with non-adherent cells, and counted.

Flow Cytometric Analysis

Cell cycle progression was studied using flow cytometry. Cells plated in 12-well plates were incubated overnight at 37°C, then replaced with fresh medium with or without treatment for 24h. Chromosomal DNA was stained with propidium iodide (100 μg/ml), and DNA quantity analyzed on a Becton Dickenson FACScan flow cytometer using the CellQuest software package (BD Biosciences; San Jose, CA). Flow cytometry was performed in the UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility that is supported by the National Institutes of Heath Awards CA-16042 and AI-28697, by the Jonsson Cancer Center, the UCLA AIDS Institute and the UCLA School of Medicine.

Caspase 3 Activity Assay

Induction of apoptosis was determined by caspase 3 cleavage of the fluorogenic substrate Ac-DEVD-AMC (Ac(N-acetyl)-DEVD-(7-amino-4-methylcoumarin)) according to the manufacturer's protocol (BD Biosciences). Cells were plated in 12-well plates, followed by drug treatment for 24 or 30h. Protein concentrations of clarified cell lysates were normalized and combined with Ac-DEVD-AMC substrate and caspase assay buffer (20 mM HEPES, 10% glycerol and 2 mM DTT) for 1h at 37°C. Fluorescence was read in 96-well plates with an excitation wavelength of 360 nm and emission wavelength of 460 nm on a BioTek Synergy 2 multi-mode microplate reader using the Gen5™ data analysis software (Bio-Tek Instruments).

Cell Migration Assay

Cell migration assays were performed using the Boyden transwell dual chamber system. Cells were plated into the upper chamber of cell culture inserts with 8.0 μm transparent PET membranes (BD Falcon; Franklin Lakes, NJ) in serum-free medium, and the lower chamber was filled with culture medium. Treatment was added to both chambers, and cells incubated at 37°C for 16h. Cells remaining in the upper chamber were removed, and the membranes fixed and stained with crystal violet. The number of migrating cells on the underside of the membrane was manually counted as the sum of 3 randomly selected fields at a 200X magnification.

LSL-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ Ovarian Cancer Mouse Model

The transgenic murine ovarian cancer model designated LSL-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ was initially described by Dinalescu et al (31). Two genomic modifications allow conditional deletion of a functional sequence within exon 5 of the *Pten* gene, and expression of mutated, constitutively active *K-ras* (G12D). Both modifications are contingent upon Cre protein-mediated recombination of loxP sites. To achieve Cre expression in the murine ovarian epithelium, a replication incompetent, recombinant adenovirus (AdCre) was injected into the ovarian bursa at 2.5×10^7 PFUs (plaque forming units) in a total volume of 5 μl. AdCre was generated in the laboratory of Dr. A. Berk (UCLA Department of Microbiology, Immunology, and Molecular Genetics, Los Angeles, CA). Upon diagnosis of tumor disease 8 – 10 weeks after AdCre injection, animals were treated with daily oral administration of NVP-BEZ235 (40 mg/kg) (n = 8) for four weeks and followed for survival. The control group consisted of untreated animals and animals treated with placebo (n = 13). For target validation experiments, NVP-BEZ235 (40 mg/kg) was administered orally three times every twelve hours. Tumor tissue was harvested 1h after administration of the last dose of drug, paraffin embedded, and subjected to immunohistochemistry.

Immunohistochemistry

Mouse tissues were fixed in 10% formaldehyde for routine histopathology. 4 μm sections were de-paraffinized and hydrated through a gradient of ethanol. Following antigen retrieval, slides were blocked with 5% goat serum, incubated overnight at 4°C with the respective primary antibodies (phospho-4E-BP1 (T37/46), phospho-Akt (S473) (736E11), and phospho-S6 (S240/244) from Cell Signaling; Ki-67 from Vector Laboratories (Burlingame, CA)), washed, and incubated with biotinylated goat anti-rabbit secondary for 1h at room temperature. Antibody binding was demonstrated using the avidin-biotin complex labeling method (Vectastain ABC kit; Vector Laboratories). The sections were then incubated with diaminobenzidine (DAB Peroxidase Substrate kit; Vector Laboratories) and H_2O_2 at room temperature for 5 – 8 minutes, and counterstained with hematoxylin.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism, Version 4.00c for Macintosh, GraphPad Sofware San Diego CA, USA, www.graphpad.com. A nonlinear regression curve fit (one phase exponential decay) was used to analyze NVP-BEZ235 and cisplatin dose response experiments. Two-tailed unpaired t-tests were used to calculate the significance of differences between the mean IC_{50} for NVP-BEZ235 in cell lines with or without *PI3K* activating mutations or *Pten* deletions, caspase 3 activity, cell migration, and single (NVP-BEZ235 or cisplatin) versus combination (NVP-BEZ235 and cisplatin) treatment conditions. For all t-tests, NS = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. A two-tailed Pearson's correlation was used to correlate the IC_{50} for NVP-BEZ235 with the basal expression level of pAkt, pS6 and p4E-BP1. Kaplan Meier curves were constructed for survival analysis in mouse experiments. The difference in survival between the treatment groups was calculated using a log-rank/Mantel Cox test.

RESULTS

NVP-BEZ235 inhibits PI3K/Akt/mTOR pathway signaling in human ovarian cancer cell lines

We examined the effect of dual PI3K/mTOR inhibition using NVP-BEZ235 on PI3K/Akt/mTOR pathway signaling in three human ovarian cancer cell lines. Treatment of IGROV1, SKOV3 or OVCAR5 cells with NVP-BEZ235 decreased phosphorylation of Akt (pAkt), S6 (pS6) and 4E-BP1 (p4E-BP1) in a dose-dependent manner in all cell lines (Figure 1A). In contrast, mTOR inhibition alone using RAD001 treatment decreased pS6 and p4E-BP1, while Akt phosphorylation levels increased in SKOV3 and OVCAR5 cells, and remained unchanged in IGROV1 cells (Figure 1B). This latter observation is consistent with the mTOR inhibition mediated lack of the negative feedback loop on p70S6K/insulin receptor substrate (IRS) signaling that leads to increased Akt phosphorylation.

NVP-BEZ235 inhibits proliferation of ovarian cancer cell lines

To study the effects of NVP-BEZ235 on cell proliferation, we used an ovarian cancer cell line panel of human (A2780, ES-2, HEYC2, IGROV1, MCAS, OAW42, OV167, OV207, OV2008, OVCAR5, PE01, SKOV3 and TOV-112D) and murine (MOVCAR18) cell lines. NVP-BEZ235 decreased cell proliferation in all cell lines in a dose-dependent manner (Figure 2A). In contrast, the decrease in cell proliferation induced by RAD001 was markedly less compared to NVP-BEZ235 at equimolar concentrations.

We next studied the relationship between the presence of activating mutations in *PI3K* or *Pten* deletions in each cell line and their sensitivity to NVP-BEZ235. The IC_{50} (concentration of drug that results in a decrease of cell proliferation by 50%) for the effect of NVP-BEZ235 on cell proliferation was calculated for each cell line. Cell lines with an IC_{50} < 100 nM were considered to be highly sensitive to drug treatment, compared to cell lines

with an $IC_{50} \geq 100$ nM, which were considered less sensitive. Figure 2B shows that all cell lines with *PI3K* activating mutations or *Pten* deletions (A2780, IGROV1, MOVCAR18, OAW42 and SKOV3) were highly sensitive to NVP-BEZ235 (IC_{50} range 26 – 70 nM). In contrast, HEYC2, OV167, OV207 and OVCAR5 cells, that lack *PI3K* activating mutations or *Pten* deletions, had an $IC_{50} \geq 100$ nM (IC_{50} range 100 – 210 nM) for NVP-BEZ235. Overall, we found a statistically significant difference between the average NVP-BEZ235 IC_{50} for cell lines with *PI3K* mutations or *Pten* deletions ($IC_{50} = 48.5 \pm 8.1$ nM) compared to cell lines that lack these mutations ($IC_{50} = 95.9 \pm 18.4$ nM; $p < 0.05$) (Figure 2B, insert).

We further examined whether the relative levels of pAkt, pS6 or p4E-BP1 protein, as expressed by each cell line under non-stimulated conditions, correlate with the response to NVP-BEZ235 (Figure 2C). We found that cell lines with high levels of p4E-BP1 protein showed a greater sensitivity to NVP-BEZ235 than cell lines with low p4E-BP1 levels. The correlation between the relative p4E-BP1 protein levels and the IC_{50} for NVP-BEZ235 was statistically significant ($r^2 = 0.46$, $p < 0.01$). In contrast, we could not demonstrate a correlation between relative levels of pAkt or pS6 and sensitivity of cells to NVP-BEZ235 (pAkt: $r^2 = 0.000006$, p=0.99; pS6: $r^2 = 0.10$, p=0.28). These data suggest that p4E-BP1 protein levels might serve as a molecular predictor of response to NVP-BEZ235.

NVP-BEZ235 increases survival in an ovarian cancer mouse model

We evaluated the anti-tumor effects of NVP-BEZ235 in the transgenic and immunocompetent LSL-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mouse model. The development of murine ovarian tumor disease in this model is contingent upon the conditional deletion of *Pten* and the concomitant expression of a constitutively active form of the *K-ras* oncogene (*K-ras* G12D) (31). Deletion of *Pten* and activation of *K-ras* is achieved via the expression of Cre protein in the ovarian epithelium by a Cre expressing recombinant adenovirus (AdCre). Figure 3A shows an example of ovarian tumor disease that developed 10 weeks after AdCre injection of the right ovary in a control animal. In this experiment, the contralateral ovary was injected with control adenovirus expressing green fluorescent protein (AdGFP) and failed to develop tumor disease.

To determine whether NVP-BEZ235 could inhibit PI3K/Akt/mTOR signaling *in vivo*, LSL-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice with established ovarian tumor disease were treated orally with NVP-BEZ235 three times every 12h. Paraffin embedded tumor tissue from control animals showed high levels of pAkt, pS6 and p4E-BP1 (Figure 3B, upper panel). In contrast, pAkt and p4E-BP1 levels were almost undetectable in tumor tissue from NVP-BEZ235 treated mice (Figure 3B, lower panel). The expression of pS6 was detectable after NVP-BEZ235 treatment, albeit at a lower level. Staining of tumor tissue for the proliferation marker Ki67 decreased markedly upon NVP-BEZ235 treatment.

We next examined the effects of NVP-BEZ235 on survival of mice with established, intra-abdominal tumor disease using the LSL-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ model. Treatment with daily, oral NVP-BEZ235 at 40 mg/kg was initiated after the development of palpable ovarian tumors and ascites, and continued for four weeks. The early effects of the inhibitor on intraperitoneal tumor growth were evident within the first 2 weeks after initiation of treatment. All animals showed a marked decrease in abdominal distention due to reduced ascites formation. More importantly, the median survival of mice treated with NVP-BEZ235 was significantly longer compared to survival of control animals based on Kaplan Meier survival curve analysis (105 days vs. 58 days, respectively; $p < 0.01$) (Figure 3C). The hazard ratio for death in the control group was 4.02 (95% CI = 1.47 – 10.98). These results demonstrate that NVP-BEZ235 was able to control intra-peritoneal tumor disease in mice, resulting in significantly improved survival.

NVP-BEZ235 inhibits proliferation of cisplatin-resistant human ovarian cancer cells

To examine the efficacy of NVP-BEZ235 in cisplatin-resistant cells, we developed cisplatin-resistant, isogenic clones of SKOV3 and OVCAR5 cells (designated SKOV3-CisR and OVCAR5-CisR, respectively). In addition, we used two pairs of cisplatin-sensitive (A2780 and OV2008) and their cisplatin-resistant clones (CP70 and C13*, respectively) previously established by other investigators (32).

The level of cisplatin resistance was assayed using cell proliferation assays (Figure 4A). SKOV3 cells were about 20 fold more sensitive to cisplatin than SKOV3-CisR cells (IC_{50} = 1.4 ± 0.2 μg/ml vs. 28.1 ± 0.4 μg/ml respectively), while OVCAR5 and OVCAR5-CisR cells showed the greatest difference in cisplatin sensitivity (IC_{50} = 1.8 ± 0.1 μg/ml vs 245 ± 76 μg/ml). We confirmed differences in cisplatin sensitivity for A2780 and CP70 (IC_{50} = 0.32 ± 0.01 μg/ml vs. 2.17 ± 0.37 μg/ml, respectively), and OV2008 and C13* cells (IC_{50} = 0.059 ± 0.002 μg/ml vs. 0.49 ± 0.06 μg/ml, respectively).

Treatment of cisplatin-resistant SKOV3-CisR, OVCAR5-CisR, CP70 and C13* cells with NVP-BEZ235 resulted in inhibition of cell proliferation in all four cell lines (Figure 4B). When compared to their respective cisplatin-sensitive isogenic cell lines, SKOV3-CisR cells and C13* cells showed a very similar response to NVP-BEZ235 treatment (Figure 4B). Interestingly, cisplatin-resistant CP70 cells were more sensitive to NVP-BEZ235 treatment compared to their cisplatin-sensitive counterpart A2780 (IC_{50} = 4.6 ± 0.4 nM vs. 37.2 ± 0.5 nM, respectively). In contrast, we found that cisplatin-sensitive OVCAR5 cells responded better to NVP-BEZ235 compared to OVCAR5-CisR cells (IC_{50} = 40.2 ± 8.3 nM vs. IC_{50} 518 ± 173 nM).

NVP-BEZ235 sensitizes ovarian cancer cells to cisplatin

We next determined whether NVP-BEZ235 could increase the cytotoxic effects of cisplatin on cisplatin-resistant human ovarian cancer cells. SKOV3-CisR, OVCAR5-CisR, CP70 and C13* cells were treated with cisplatin alone or in combination with NVP-BEZ235. In all cell lines, inhibition of cell proliferation by cisplatin was significantly greater in the presence of NVP-BEZ235 (Figure 4C, upper panel). For example, in SKOV3-CisR cells, treatment with 1 μg/ml cisplatin resulted in a minor decrease of cell proliferation to 89.4 ± 1.9% compared to control cells. However, combination treatment of 1 μg/ml cisplatin and 25 nM NVP-BEZ235 reduced cell proliferation to 60.6 ± 3.8% ($p < 0.001$ compared to cisplatin alone). A sensitizing effect was also observed in OVCAR5-CisR cells that showed significantly reduced cell proliferation with the combination of 50 nM NVP-BEZ235 and cisplatin (68.0 ± 6.9%) compared to cisplatin treatment alone (93.4 ± 6.0%). Combination treatment of the cisplatin-resistant cell lines CP70 and C13* showed similar sensitizing effects..

We examined whether treatment of cisplatin-sensitive cells with the combination of cisplatin and NVP-BEZ235 also resulted in cisplatin sensitization (Figure 4C lower panel). In SKOV3 cells, the addition of NVP-BEZ235 to cisplatin decreased cell proliferation to a significantly greater extent compared to cisplatin treatment alone (85.8 ± 2.7% (cisplatin) vs. 57.7 ± 1.6% (cisplatin + NVP-BEZ235); $p < 0.001$ (1 μg/ml)). Similar results were found in OVCAR5 cells that were more sensitive to cisplatin when treated in combination with NVP-BEZ235 (76.4 ± 2.7% vs. 39.0 ± 3.2 %; $p < 0.001$ (1 μg/ml cisplatin)). The data for combination treatment of the cisplatin-sensitive cell lines A2780 and OV2008 showed similar sensitizing effects (Figure 4C, lower panel).

We next investigated whether the cisplatin sensitizing effect of NVP-BEZ235 requires continuous exposure to cisplatin, or can also be demonstrated with shorter cisplatin treatment. We therefore first determined the sensitivity of cisplatin-resistant and cisplatin-sensitive cells to 2 hours of cisplatin treatment (Supplementary Figure 1A). We then

performed all combination experiments using 2 hours exposure to cisplatin and continuous treatment with NVP-BEZ235. The results confirmed that NVP-BEZ235 is able to sensitize cisplatin-resistant (Supplementary Figure 1B, upper panel). and cisplatin-sensitive cells (Supplementary Figure 1B, lower panel) to the cytotoxic effects of cisplatin treatment even with short-term exposure.

We further studied whether we could optimize the effect of combination treatment on cell proliferation by sequencing cisplatin and NVP-BEZ235 treatment. We performed combination treatment under various conditions including pre-treatment of cells with NVP-BEZ235 followed by cisplatin compared to cisplatin followed by NVP-BEZ235. Experiments were performed in SKOV3-CisR and SKOV3 cells. The results show that concomitant NVP-BEZ235 and cisplatin treatment had equal or greater effects on cell proliferation compared to any of the sequential treatments (Supplementary Figure 2).

NVP-BEZ235 effect on cell cycle progression, induction of caspase 3 activity and migration

We next examined the effects of NVP-BEZ235 on cell cycle progression. NVP-BEZ235 caused an increase in the percentage of cells in the G_0/G_1 phase compared to control conditions in all cisplatin-resistant cell lines (SKOV3-CisR, OVCAR5-CisR, CP70 and C13*; Figure 5A, upper panel) and cisplatin-sensitive cells (SKOV3, OVCAR5, A2780, OV2008 and IGROV1; Figure 5A, lower panel). The percentage of cells in the S and G_2/M phases showed a proportional decrease.

To determine whether NVP-BEZ235 induces the activity of caspase 3 as an important mediator of apoptosis, cells were treated with increasing concentrations of NVP-BEZ235 (50, 100 and 250 nM). Cisplatin was used as a positive control and for comparison, since platinum-based agents are the most effective and frequently used chemotherapeutic agents for the treatment of ovarian cancer patients. NVP-BEZ235 induced an increase in caspase 3 activity in the cisplatin-resistant (Figure 5B, upper panel) and cisplatin-sensitive (Figure 5B, lower panel) cells. Seven out of the nine cell lines tested (OVCAR5-CisR, CP70, C13*, SKOV3, A2780, OV2008 and IGROV1) showed induction of caspase 3 activity at concentrations as low as 50 nM. Only OVCAR5 and SKOV3-CisR cells required concentrations of NVP-BEZ235 higher than 50 nM. Interestingly, the level of caspase 3 activity induced by NVP-BEZ235 was comparable to or higher than the level observed under cisplatin treatment in some cell lines.

We finally determined the effect of NVP-BEZ235 on cell migration in cisplatin-resistant and cisplatin-sensitive cells using the Boyden transwell dual chamber system. NVP-BEZ235 treatment caused a decrease in the average number of migrated cells compared to untreated cells in all cell lines tested. Migration was inhibited in six out of nine cell lines (OVCAR5, C13*, SKOV3, A2780, OV2008 and IGROV1) using 50 nM NVP-BEZ235. In OVCAR5-CisR, SKOV3-CisR and CP70 cells, inhibition of migration required concentrations of NVP-BEZ235 higher than 50 nM (Figure 5C).

DISCUSSION

This study is the first to provide evidence for the efficacy of the novel dual PI3K/mTOR inhibitor NVP-BEZ235 in preclinical models of ovarian cancer. We demonstrate that NVP-BEZ235 effectively blocked PI3K/Akt/mTOR pathway signaling, decreased cell proliferation, and sensitized cells to cisplatin treatment. In immunocompetent mice with established ovarian tumor disease, oral administration of NVP-BEZ235 decreased pAkt, p4E-BP1 and Ki67 in tumor tissue, and resulted in significantly longer survival compared to

control animals. Importantly, NVP-BEZ235 was effective in both platinum-sensitive and platinum-resistant cell models.

Targeting the PI3K/Akt/mTOR pathway in cancer disease has been a focus of drug development recently, but the optimal therapeutic strategy has yet to be identified. Prior studies including studies from our laboratory in preclinical models of ovarian cancer have mainly investigated the effects of either PI3K or mTOR inhibition alone (8,33–40). For example, the mTOR inhibitor RAD001 showed a delay in the onset and progression of tumor growth in a transgenic ovarian cancer mouse model, and clinical trials with RAD001 are ongoing (38,41). However, mTOR inhibition alone can increase Akt phosphorylation levels as demonstrated in our study and described by others prior (42–44). This effect is attributed to the mTOR inhibition-mediated lack of the negative feedback loop on p70S6K/insulin receptor substrate (IRS) signaling and subsequent increase in Akt phosphorylation. In contrast to mTOR inhibition alone, we showed that dual PI3K/mTOR inhibition using NVP-BEZ235 reduced, rather than increased, pAkt levels and caused a greater decrease in the proliferation of ovarian cancer cell lines. These data are consistent with prior observations that showed a similar, more pronounced effect of NVP-BEZ235 compared to RAD001 on inhibition of cell growth in other tumor models (16,45).

The validation of our cell culture data in a transgenic animal model of ovarian cancer provides evidence for the promising *in vivo* effects of NVP-BEZ235. In contrast to most other studies that use human ovarian cancer xenografts in nude or SCID mice models, we chose to test the efficacy of NVP-BEZ235 in an immunocompetent model of murine, epithelial ovarian cancer. In addition, we initiated treatment of mice only upon diagnosis of intra-abdominal tumor disease rather than prior to development of gross tumor disease, further strengthening our *in vivo* observations. Our data show that oral treatment with NVP-BEZ235 was able to abrogate Akt and 4E-BP1 phosphorylation in ovarian tumor tissue. In addition, a statistically significant prolongation of survival of mice with established tumor disease was observed. Further studies will be necessary to optimize treatment regimens and possibly induce a more complete tumor regression. Concomitant inhibition of the Ras/ERK pathway in this model might be necessary to achieve greater anti-tumor effects.

Ovarian cancer is a chemotherapy-sensitive disease, and about 85% of patients respond to first line treatment with platinum-based chemotherapeutic agents. However, the presence of primary platinum-resistant disease, and the development of platinum resistance in recurrent ovarian cancer presents a major therapeutic challenge. Unfortunately, at the current time, there is no truly effective treatment strategy for platinum-resistant disease. We found that NVP-BEZ235 effectively decreased the proliferation of not only cisplatin-sensitive ovarian cancer cell lines, but cisplatin-resistant cells as well. The decrease in proliferation is likely due to an arrest of cells in G_0/G_1 and induction of apoptosis. Furthermore, NVP-BEZ235 was able to sensitize cisplatin-sensitive and -resistant cells to the effects of cisplatin on cell proliferation, suggesting that dual PI3K/mTOR inhibition might be able to overcome mechanisms of resistance to platinum agents. This effect could be partially due to the inhibition of cisplatin-induced Akt activation (46–48).

A major challenge in the clinical use of PI3K/Akt/mTOR pathway inhibitors is the identification of patients who will likely respond to the treatment. We found that ovarian cancer cell lines with activating *PI3K* or *Pten* deletion mutations were particularly sensitive to NVP-BEZ235. However, the absence of PI3K/Akt pathway activating mutations did not preclude a response to NVP-BEZ235. Our findings are supported by previous studies that showed NVP-BEZ235 to be effective in cell lines and tumor models with a variety of oncogenic pathway mutations, including *K-ras* and *B-raf* (16,28). It is possible that crosstalks between the PI3K/Akt and other oncogenic pathways causes activation of mTOR

and hence confers sensitivity to the mTORC1/2 inhibitory effect of NVP-BEZ235. Our data showing that the level of 4E-BP1 protein phosphorylation correlates with the anti-proliferative effect of NVP-BEZ235 treatment might support this hypothesis. The 4E-BP1 proteins play a crucial role in regulating cell proliferation as recently demonstrated by Dowling et al. using 4E-BP1 double knockout mouse embryo fibroblasts (49).

In summary, our data show that dual PI3K/Akt/mTOR inhibition using NVP-BEZ235 in ovarian cancer models has growth-inhibitory and anti-tumor effects, and is able to sensitize cells to platinum agents. We propose NVP-BEZ235 treatment as a promising therapeutic strategy in ovarian cancer patients that might be effective even in the presence of platinum resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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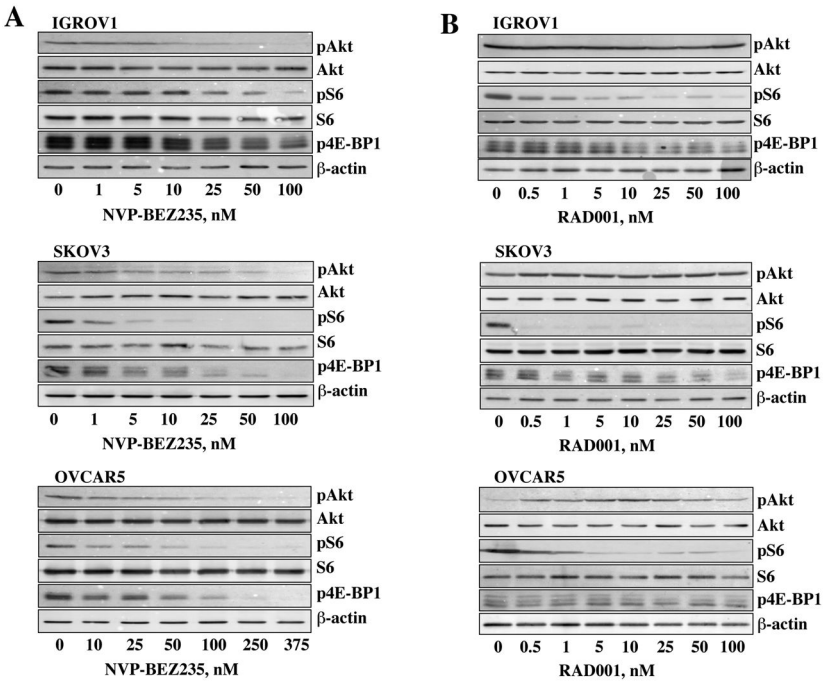

Figure 1. NVP-BEZ235 blocks phosphorylation of Akt, S6 and 4E-BP1

A and B. IGROV1, SKOV3 and OVCAR5 cells were serum-starved overnight, then treated with NVP-BEZ235 **(A)** or RAD001 **(B)** in culture medium for 24h. Total cell extracts were analyzed by immunoblotting for pAkt, pS6 or p4E-BP1. Total Akt, S6 and β-actin levels are shown as loading controls.

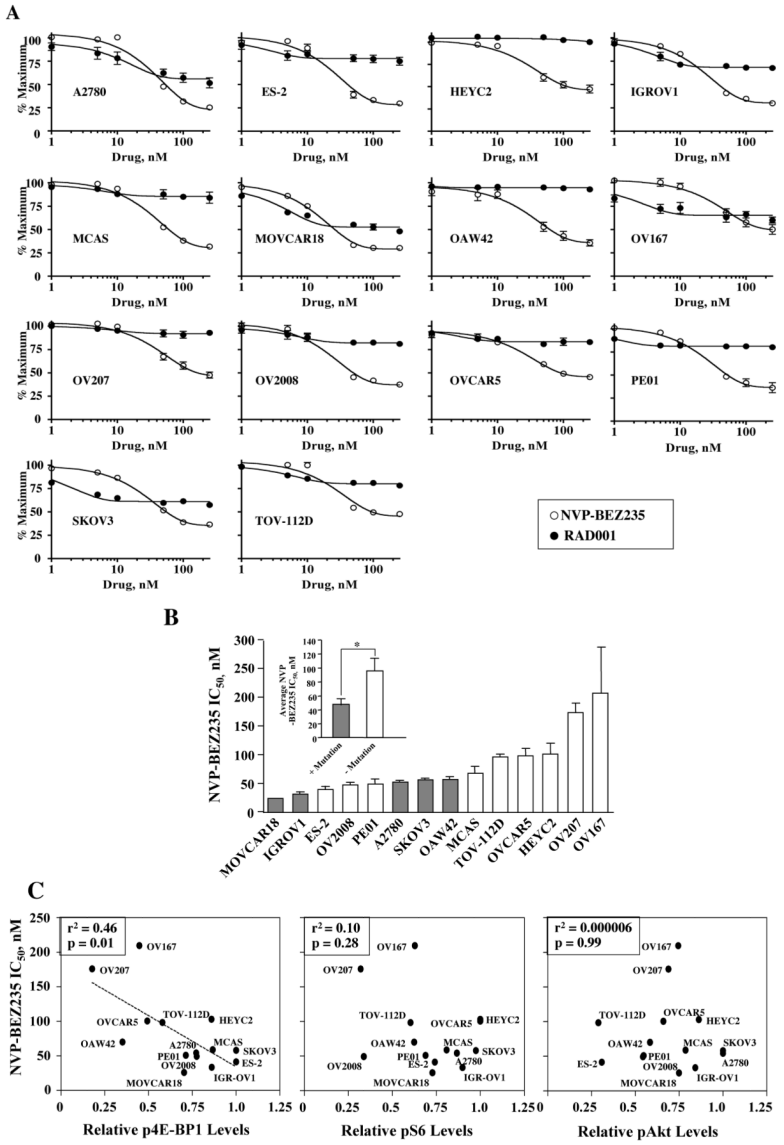

Figure 2. NVP-BEZ235 decreases ovarian cancer cell proliferation. Cells mutated in the PI3K/Akt pathway or with high p4E-BP1 levels demonstrate increased sensitivity

A. Ovarian cancer cells were treated with NVP-BEZ235 for 72h. Treatment with RAD001 is shown in comparison. Results for cell proliferation are expressed as a percentage of no treatment control (% maximum) ± the standard error.

B. The IC_{50} for the effect of NVP-BEZ235 on cell growth was determined from the data in Figure 2A. Cell lines with *PI3K* activating mutations or *Pten* deletions are shown as gray columns. *Insert*, Average IC_{50} for cells lines with (gray columns) and without (white columns) *PI3K* mutations or *Pten* deletions.

C. Correlation of basal p4E-BP1, pS6 or pAkt protein levels with the IC_{50} for NVP-BEZ235. A two-tailed Pearson's correlation analysis showed a significant correlation between NVP-BEZ235 IC_{50} and relative levels of p4E-BP1 protein, but not pS6 or pAkt.

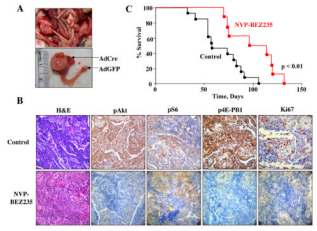

Figure 3. NVP-BEZ235 blocks pAkt and p4E-BP1, reduces Ki67 expression, and prolongs survival in LSL-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice bearing ovarian tumors

A. Ovarian tumor formation in the right ovarian bursa of LSL-*K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice injected with Cre expressing recombinant adenovirus (AdCre). The left ovary was injected with a control, GFP expressing adenovirus (AdGFP) and lacked formation of tumor.

B. Mice with established ovarian tumors were treated with 40 mg/kg NVP-BEZ235 3 times every 12 hours. Tumors were harvested and immunohistochemistry was performed for the indicated proteins. Images were taken at 200 × magnification.

C. Mice with established tumor disease were treated daily for 4 weeks with 40 mg/kg NVP-BEZ235 (red line, ■), and survival compared to control animals (black line, ●).

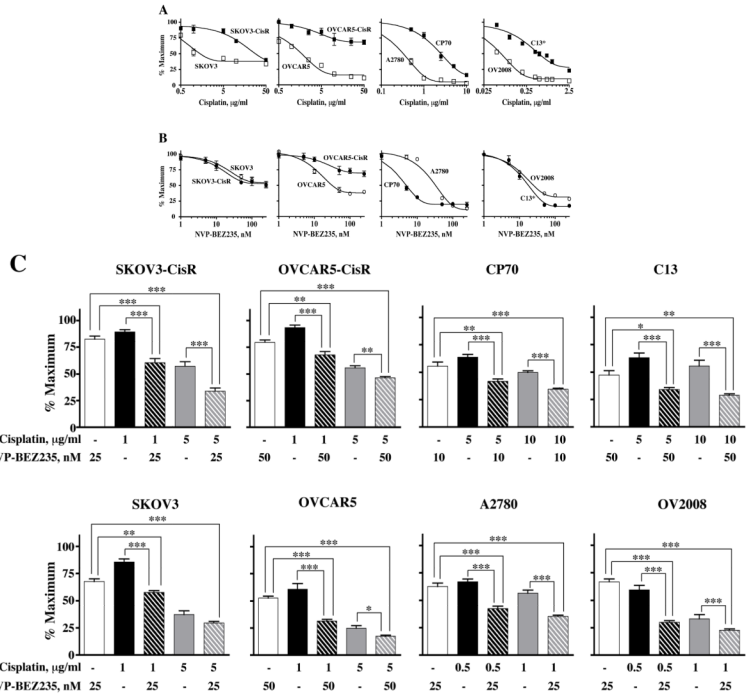

Figure 4. NVP-BEZ235 decreases cell proliferation in cisplatin-resistant ovarian cancer cells and sensitizes ovarian cancer cells to cisplatin

A. Cisplatin sensitivity of SKOV3, OVCAR5, A2780 and OV2008 cells and their corresponding isogenic cisplatin-resistant cells, SKOV3-CisR, OVCAR5-CisR, CP70 and C13*, respectively.

B. Effect of NVP-BEZ235 on proliferation of cisplatin-resistant ovarian cancer cells.

C. NVP-BEZ235 sensitizes cells to cisplatin in cisplatin-resistant (upper panel) and cisplatin-sensitive (lower panel) cells treated as indicated with cisplatin with or without NVP-BEZ235.

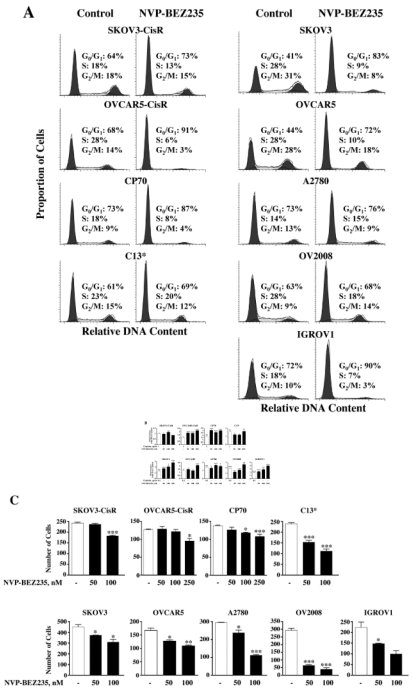

Figure 5. NVP-BEZ235 blocks cell cycle progression, induces caspase 3 activity and decreases cell migration

A. Cisplatin-resistant (SKOV3-CisR, OVCAR5-CisR, CP70, C13*) (upper panel) and cisplatin-sensitive (SKOV3, OVCAR5, A2780, OV2008, IGROV1) (lower panel) cells accumulate in G_0/G_1 phase after 24h treatment with 50 nM NVP-BEZ235.

B. Induction of caspase 3 activity in cisplatin-resistant (upper panel) and cisplatin-sensitive (lower panel) cells treated with NVP-BEZ235. Cells were treated with cisplatin as a positive control.

C. NVP-BEZ235 reduces migration of cisplatin-resistant (upper panel) and cisplatin-sensitive (lower panel) cells in a Boyden transwell dual chamber system.