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 18 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-rasG12D/PtenloxP/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 IC50 for NVP-BEZ235. In LSL-K-rasG12D/PtenloxP/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.

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Introduction

Ovarian cancer is the most lethal of the gynecological malignancies and the 5th leading cause of cancer related deaths among women in the United States. About 21,880 new cases are expected in the United States 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 (PIP2) to phosphoinositide 3,4,5 trisphosphate (PIP3), 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 (phosphatase and tensin homolog) antagonizes the PI3K/Akt/mTOR pathway by dephosphorylating PIP3.
Translational Relevance

The oncogenic PI3K/Akt/mTOR pathway plays an important role in ovarian cancer, and therefore presents a suitable target for novel therapeutic approaches. This study evaluates the effects of dual PI3K and mTOR inhibition using NVP-BEZ235 on human ovarian carcinoma cells and on established ovarian tumor disease in an immunocompetent, transgenic mouse model. NVP-BEZ235 blocked PI3K/Akt/mTOR pathway signaling and decreased ovarian cancer cell proliferation. Furthermore, NVP-BEZ235 treatment of transgenic mice with established ovarian tumor disease resulted in significantly longer survival compared to control mice. NVP-BEZ235 also effectively inhibited growth of cisplatin-resistant ovarian cancer cells and sensitized both cisplatin-resistant and -sensitive cells to cisplatin treatment. These data provide a rationale for the therapeutic use of NVP-BEZ235 in ovarian cancer patients, and show promising evidence for efficacy in the presence of platinum-resistant disease.

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% to 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; ref. 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 2 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 antitumor 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 antitumor 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 antiangiogenic properties (14–23, 25).

In this 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, and OAW42 cells from the European Collection of Cell Cultures. HEY2, OV167, and OV207 cells were kind gifts from Dr. V. Shridhar (Mayo Clinic). 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). 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/; refs. 29, 30).

Immunoblot assay

NVP-BEZ235 and RAD001 were generously provided by NOVARTIS Pharmaceuticals. Immunoblot assays were performed using standard procedures. Briefly, cells were lysed and protein concentrations of clarified cell lysates normalized. Lysates were mixed with 4× 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]). Immuno-reactive bands were visualized by chemiluminescence (ECL-Plus; GE Healthcare Biosciences), 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 IC50 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 72 hours 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 methosulfate; 1 mg/mL) were added, and the metabolism of XTT was measured at 450 nm on an absorbance microplate reader (ELx800, Bio-Tek Instruments). Cell counting was performed on a Vi-CELL Cell Viability Analyzer (Beckman Coulter). Following treatment, adherent cells were detached with trypsin, combined with nonadherent 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 24 hours. 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). 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 Health 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)-(D-Val-DL-Leu-Dad)-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 30 hours. 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 1 hour 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 multimode 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 transmembrane filters (BD Falcon) in serum-free medium, and the lower chamber was filled with cell culture medium. Treatment was added to both chambers, and cells incubated at 37°C for 16 hours. 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 200× magnification.

**LSL-KrasG12D/+;PtenloxP/loxP ovarian cancer mouse model**  
The transgenic murine ovarian cancer model designated LSL-KrasG12D/+;PtenloxP/loxP was initially described by Dinalescu and colleagues (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 ofloxP 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 to 10 weeks after AdCre injection, animals were treated with daily oral administration of NVP-BEZ235 (40 mg/kg; n = 8) for 4 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 3 times every 12 hours. Tumor tissue was harvested 1 hour 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. Four-micrometer 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, then replaced with fresh medium with or without treatment for 24 hours. 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). 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 Health Awards CA-16042 and AI-28697, by the Jonsson Cancer Center, the UCLA AIDS Institute and the UCLA School of Medicine.

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Statistical analysis  
All statistical analyses were performed using GraphPad Prism, Version 4.00c for Macintosh, GraphPad Software, 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 IC50 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

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NVP-BEZ235 and cisplatin) treatment conditions. For all t tests, NS, not significant; *, \( P < 0.05; **, P < 0.01; ***, P < 0.001. A 2-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 3 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 (Fig. 1A). In contrast, mTOR inhibition alone using RAD001 treatment decreased pS6 and p4E-BP1, whereas Akt phosphorylation levels increased in SKOV3 and OVCAR5 cells, and remained unchanged in IGROV1 cells (Fig. 1B). The increase in Akt phosphorylation is consistent with the lack of negative feedback on p70S6K/insulin receptor substrate (IRS) signaling seen upon mTOR inhibition.

**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, HEY2, 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 (Fig. 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 \( \text{Pten} \) 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 \( \text{Pten} \) deletions (A2780, IGROV1, MOVCAR18, OAW42, and SKOV3) were highly sensitive to NVP-BEZ235 (IC\(_{50}\) range 26–70 nM). In contrast, HEY2, OV167, OV207, and OVCAR5 cells, that lack PI3K-activating mutations or \( \text{Pten} \) deletions, had an IC\(_{50}\) \( \geq \) 100 nM (IC\(_{50}\) range 100–210 nM) for NVP-BEZ235.

![Figure 1](image-url). 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 24 hours. Total cell extracts were analyzed by immunoblotting for pAkt, pS6, or p4E-BP1. Total Akt, S6, and \( \beta \)-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 72 hours. Treatment with RAD001 is shown in comparison. Results for cell proliferation are expressed as a percentage of no treatment control (% maximum) ± standard error. B, The IC50 for the effect of NVP-BEZ235 on cell growth was determined from the data in A. Cell lines with PI3K-activating mutations or Pten deletions are shown as gray columns. Insert, average IC50 for cell 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 IC50 for NVP-BEZ235. A 2-tailed Pearson’s correlation analysis showed a significant correlation between NVP-BEZ235 IC50 and relative levels of p4E-BP1 protein, but not pS6 or pAkt.
Overall, we found a statistically significant difference between the average NVP-BEZ235 IC₅₀ for cell lines with PI3K mutations or Pten deletions (IC₅₀ = 48.5 ± 8.1 nM) compared to cell lines that lack these mutations (IC₅₀ = 95.9 ± 18.4 nM; P < 0.05; Fig. 2B, insert).

We further examined whether the relative levels of pAkt, pS6, or p4E-BP1 protein, as expressed by each cell line under nonstimulated conditions, correlate with the response to NVP-BEZ235 (Fig. 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 IC₅₀ for NVP-BEZ235 was statistically significant (r² = 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: r² = 0.000006, P = 0.99; pS6: r² = 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 antitumor 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; ref. 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 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, ●).

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 3 times every 12 hours. Paraffin-embedded tumor tissue from control animals showed high levels of pAkt, pS6, and p4E-BP1 (Fig. 3B, upper). In contrast, pAkt and p4E-BP1 levels were almost undetectable in tumor tissue from NVP-BEZ235 treated mice (Fig. 3B, lower). 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 4 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.
IC50 fold more sensitive to cisplatin than SKOV3-CisR cells in proliferation assays (Fig. 4A). SKOV3 cells were about 20-fold more sensitive to cisplatin than SKOV3-CisR cells (IC50 = 1.4 ± 0.2 μg/mL vs. 28.1 ± 0.4 μg/mL, respectively), whereas OVCAR5 and OVCAR5-CisR cells showed the greatest difference in cisplatin sensitivity (IC50 = 1.8 ± 0.1 μg/mL vs. 245 ± 76 μg/mL). We confirmed differences in cisplatin sensitivity for A2780 and CP70 (IC50 = 0.32 ± 0.01 μg/mL vs. 2.17 ± 0.37 μg/mL, respectively), and OV2008 and C13* cells (IC50 = 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 4 cell lines (Fig. 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 (Fig. 4B). Interestingly, cisplatin-resistant CP70 cells were more sensitive to NVP-BEZ235 treatment compared to their cisplatin-sensitive counterpart A2780 (IC50 = 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 (IC50 = 40.2 ± 8.3 nM vs. IC50 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 (Fig. 4C, upper). 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 (Fig. 4C, lower). 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 (Fig. 4C, lower).

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 Fig. S1A). 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 Fig. S1B, upper) and cisplatin-sensitive cells (Supplementary Fig. S1B, lower) 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 pretreatment 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 Fig. S2).

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 G0/G1 phase compared to control conditions in all cisplatin-resistant cell lines (SKOV3-CisR, OVCAR5-CisR, CP70, and C13*; Fig. 5A, upper) and cisplatin-sensitive cells (SKOV3, OVCAR5, A2780, OV2008, and IGROV1; Fig. 5A, lower). The percentage of cells in the S and G2/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, because 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 (Fig. 5B, upper) and cisplatin-sensitive (Fig. 5B, lower) cells. Seven of 9 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 6 of 9 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 (Fig. 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/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 before 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

Figure 5. (Continued) B, induction of caspase 3 activity in cisplatin-resistant (upper) and cisplatin-sensitive (lower) cells treated with NVP-BEZ235. Cells were treated with cisplatin as a positive control. C, NVP-BEZ235 reduces migration of cisplatin-resistant (upper) and cisplatin-sensitive (lower) cells in a Boyden transwell dual chamber system.
model might be necessary to achieve greater antitumor 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 G0/G1 and induction of apoptosis. Furthermore, NVP-BEZ235 was able to sensitize cisplatin-sensitive and cisplatin-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 crosstalk 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 antiproliferative 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 and colleagues 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 antitumor 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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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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