The PI3K/mTOR dual inhibitor NVP-BEZ235 reduces the growth of ovarian clear cell carcinoma

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Abstract. Patients with clear cell carcinoma of the ovary (OCCC) have poor survival due to resistance to standard chemotherapy. OCCC has frequent activating mutations of the *PIK3CA* gene. The present study was conducted to clarify the efficacy of the inhibition of the PI3K-AKT-mTOR pathway in OCCC. We used 8 OCCC cell lines and 5 ovarian serous adenocarcinoma (OSAC) cell lines. The mutation status of the *PIK3CA* and *KRAS* genes was examined by direct sequencing. The IC_{50} values of NVP-BEZ235 (BEZ235) and temsirolimus were determined by WST-8 assay. Protein expression levels of PI3K-AKT-mTOR pathway molecules were examined by western blotting. Cell cycle distribution was analyzed by flow cytometry. Annexin V staining was used for detecting apoptosis. We also investigated the effects of BEZ235 on OCCC tumor growth in a nude mouse xenograft model. Four of the 8 OCCC cell lines showed a *PIK3CA* mutation while none of the 5 OSAC cell lines showed a mutation. The IC_{50} values of BEZ235 for the OCCC cell lines were lower than these values for the OSAC cell lines. The IC_{50} value of temsirolimus was higher than BEZ235 in the OCCC cell lines. The *PIK3CA* mutation was more frequently noted in OCCC than OSAC cells, but the sensitivity of these cell lines to BEZ235 or temsirolimus was not related to the mutation status. pHER3 and pAkt proteins were expressed more frequently in OCCC compared with OSAC. However, protein expression levels were distributed widely, and were not related to the sensitivity. Treatment with BEZ235 suppressed expression of pAkt, although treatment with temsirolimus did not. OCCC cells exhibited G_1 phase arrest after treatment with BEZ235 and apoptosis with a higher concentration of the agent. BEZ235 significantly inhibited tumor growth in mice bearing OVISE and TU-OC-1 cell tumors. The present study indicated that the PI3K-AKT-mTOR pathway is a potential target for OCCC, and that BEZ235 warrants investigation as a therapeutic agent.

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Abbreviations: PI3K, phosphatidylinositol 3-kinase; PDK1, phosphatidylinositol-dependent kinase 1; mTOR, mammalian target of rapamycin; OCCC, ovarian clear cell carcinoma; OSAC, ovarian serous adenocarcinoma

Key words: clear cell carcinoma, PI3K, mTOR, ovarian carcinoma

Introduction

Clear cell carcinoma of the ovary (OCCC) is recognized in the World Health Organization classification of ovarian tumors as a distinct histological entity. Its clinical behavior is distinctly different from other epithelial ovarian cancers (1). OCCC accounts for 3.7-12.1% of epithelial ovarian cancers (2,3). We found that response rates for platinum-based chemotherapy were 11.1% for OCCC and 72.5% for serous adenocarcinoma (SAC), suggesting that OCCC resists conventional platinum-based chemotherapy (4). A novel therapeutic strategy is needed to improve the prognosis of patients with OCCC.

PIK3CA is located at the 3q26.3 locus and encodes the catalytic subunit of the phosphatidylinositol 3-kinase (PI3K), p110α (5). In response to an extracellular signal, the activated p110α phosphorylates PIP2 to generate PIP3. The PIP3 recruits AKT to the plasma membrane, where it is phosphorylated and activated by phosphatidylinositol-dependent kinase 1 (PDK1) and PDK2. Activated AKT can directly activate the mammalian target of rapamycin (mTOR) by phosphorylation at Ser2448. mTOR is a serine/threonine kinase that acts as an effector in the PI3K/Akt pathway. Aberrations of the PI3K pathway are frequently present in many different types of cancer. A number of studies have shown amplification or mutations of the *PIK3CA* gene in ovarian cancers (6-8). AKT and mTOR are also hyperactivated in ovarian cancer (9,10). Additionally, a high frequency of activating mutations of *PIK3CA* has been observed in OCCC (11).

NVP-BEZ235 is an imidazoquinoline derivative that potently and reversibly inhibits class 1 PI3K and mTOR catalytic activity by competing at its ATP-binding site (12). It has been demonstrated to reduce tumor growth in several xenograft models and is currently in clinical trials (12-14). The present study was conducted to clarify the efficacy of NVP-BEZ235 treatment on OCCC.

Materials and methods

Cell lines and cell cultures. Eight human OCCC cell lines (OVISE, SMOV-2, KK, TU-OC-1, OVTOKO, KOC-7c, RMG-I and OVMANA) and five OSAC cell lines (KF, KOC-2s, TU-OS-3, TU-OS-4 and SHIN-3) were used. Cells were obtained as follows: OVISE and OVTOKO from Dr Hiroshi Minaguchi (Yokohama City University, Yokohama, Japan); SMOV-2 from Dr Tomohiro Iida (St. Marianna University, Kawasaki, Japan); KK and KF from Dr Yoshihiro Kikuchi (National Defense Medical College, Tokorozawa, Japan); KOC-7c and KOC-2s from Dr Toru Sugiyama (Kurume University, Kurume, Japan); RMG-I from Dr Shiro Nozawa (Keio University, Tokyo, Japan); and SHIN-3 from Dr Yasuhiko Kiyozuka (Nara Medical University, Kashihara, Japan). TU-OC-1, TU-OS-3, and TU-OS-4 cells were established by our department (15,16). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37˚C.

Mutation screening. Screening for mutations was performed as previously described (17). Genomic DNA was purified from all cell lines using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA). PCR primers used to amplify the sequence of interest (exons 9 and 20 of the *PIK3CA* gene, exons 2 and 3 of the *KRAS* gene) were the same as reported in the literature (18,19). DNA was amplified in reactions of 30 sec at 94˚C; 30 sec at 55˚C; followed by 90 sec at 72˚C for 30 cycles. Then, PCR products were subjected to sequencing using BigDye Terminator v3.1 Cycle Sequencing kit and an Applied Biosystems 3130x1 Genetic Analyzer (Applied Biosystems Foster City, CA, USA).

Reagents. NVP-BEZ235 and temsirolimus were purchased from LC Laboratories (Woburn, MA, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at -20˚C for the *in vitro* experiments. The drugs were diluted in fresh medium immediately before each experiment. In all the experiments, the final DMSO concentration was <0.1%.

Dose-response studies. The cytotoxicities of NVP-BEZ235 and temsirolimus were assessed by the WST-8 assay using Cell Counting Kit-8 (Dojindo Laboratories, Tabaru, Japan) as previously described (17). Cells (2-4x10^3 cells/80 μl) were seeded into each well of a 96-well tissue culture plate, cultured overnight, and then treated with 20 μl of NVP-BEZ235 or temsirolimus solution at a final concentration of 0.001, 0.01, 0.1, 1 or 10 μM for 72 h. After that, 20 μl of Cell Counting Kit-8 solution was added to each well, and the plates were incubated for another 1-2 h. Absorbance was measured at 450 nm with a microplate reader (iMark Microplate Absorbance Reader). Cell viability was calculated as the percentage of cells killed by the treatment. All experiments were conducted in triplicate. Median inhibitory concentrations were determined from these calculations.

Western blot analysis. Cells were washed twice with ice-cold PBS. Cell pellets were then lysed in a buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4 and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics)] as previously described (17). Protein concentrations were measured against a standardized control using a protein assay kit (Bio-Rad Laboratories). A total of 50 mg protein was separated by electrophoresis on a 5-20% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). The antibodies were as follows: rabbit anti-erbB3 antibody (C17) (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-β-actin (AC-40) antibody (1:1,000; Sigma-Aldrich, St. Louis, MO, USA); and anti-phospho-erbB3 (Tyr1289) (21D3) antibody (1:1,000), rabbit anti-AKT antibody (1:1,000), rabbit anti-phospho-AKT (Ser473) antibody (1:500), rabbit anti-mTOR antibody (1:500), rabbit anti-phospho-mTOR (Ser2448) antibody (1:500), rabbit anti-p70S6K antibody (1:500), rabbit anti-phospho-p70S6K (Thr389) antibody (1:500), rabbit anti-4E-BP1 antibody (1:1,000) and rabbit anti-phospho-4E-BP1 (Thr37/49) antibody (1:1,000) (all from Cell Signaling Technology (Danvers, MA, USA). Signals were detected with secondary anti-mouse or anti-rabbit immunoglobulin G antibody coupled with horseradish peroxidase, using an Ez-Capture II chemiluminescent imaging system (ATTO, Tokyo, Japan).

Cell cycle distribution analysis. Cell cycle distribution was analyzed by flow cytometry. Briefly, cells were plated in a 6-well plate, cultured overnight, and then treated with NVP-BEZ235 or left untreated for 48 or 72 h (final concentration of 10 or 100 nM). Floating and adherent cells were fixed overnight in ice-cold 70% ethanol. The cells were then resuspended in PBS containing propidium iodide (PI, 25 μg/ml) supplemented with 0.1% RNase A and incubated at 37˚C for 30 min. DNA content was measured with a FACSCalibur flow cytometer with CellQuest software (Becton-Dickinson, Franklin Lakes, NJ, USA). Cell fit analysis determined the percentage of the cell count in a specific phase of the cell cycle.

Annexin V staining. The Annexin V-FITC Apoptosis Detection kit (BioVision, Mountain View, CA, USA) was used to assess apoptosis as the externalization of phosphatidylserine residues, according to the specifications of the manufacturer. Briefly, cells were suspended in 500 ml of 1X binding buffer. The cells then were stained with 5 ml Annexin V-FITC (fluorescein isothiocyanate) and 5 ml PI (50 mg/ml) for 5 min in the dark at room temperature. Finally, the cells were analyzed with a flow cytometer (FACSCalibur; Becton-Dickinson).

Ovarian cancer xenograft model. OVISE or TU-OC-1 cells in log-phase growth were trypsinized, washed twice with PBS and centrifuged at 250 x g. For subcutaneous tumor development, 4x10^6 viable cells (in 0.1 ml of PBS) were injected subcutaneously under aseptic conditions into female athymic mice. Seven days after the injection, we confirmed the development of measurable tumors, and then treatment was initiated with NVP-BEZ235 at doses of 25 or 50 mg/kg/day, and continued for 3 weeks. Mice treated with vehicle (10% 1-methyl-2-pyrrolidone-90% polyethylene glycol 300) were used as the control group. All agents were administered by oral gavage. Ten mice were used in each experimental group. The tumor volume was measured with a caliper twice weekly. The body weight of mice was also measured twice weekly.

Table I. Characteristics of the OCCC and OSAC cell lines.

Cell line	Original tumor	*KRAS*		*PIK3CA*		IC_{50} of BEZ235 (nM)
		Exon 2	Exon 3	Exon 9	Exon 20	
OVISE	Clear cell carcinoma	wt	wt	wt	wt	44
SMOV-2	Clear cell carcinoma	wt	wt		3141 A>A/T	65
KK	Clear cell carcinoma	wt	wt	1634 A>A/C	wt	74
TU-OC-1	Clear cell carcinoma	wt	wt	1624 G>G/A	wt	131
OVTOKO	Clear cell carcinoma	wt	wt	wt	wt	534
KOC-7c	Clear cell carcinoma	wt	wt	wt	wt	600
OVMANA	Clear cell carcinoma	wt	wt	1634 A>T	wt	641
RMG-I	Clear cell carcinoma	wt	wt	wt	wt	777
KF	Serous adenocarcinoma	wt	wt	wt	wt	779
KOC-2s	Serous adenocarcinoma	wt	wt	wt	wt	989
TU-OS-3	Serous adenocarcinoma	wt	wt	wt	wt	1,004
TU-OS-4	Serous adenocarcinoma	wt	wt	wt	wt	3,951
SHIN-3	Serous adenocarcinoma	34 G>A	wt	wt	wt	25,400

OCCC, ovarian clear cell carcinoma; OSAC, ovarian serous adenocarcinoma; wt, wild-type.

Statistical analysis. Statistical analyses were performed with Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as means ± 1 standard error. Means for all data were compared by one-way analysis of variance with *post hoc* testing or by unpaired t-test. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

Identification of PIK3CA and KRAS mutations in OCCC and OSAC cell lines. We first screened the mutation status of *PIK3CA* (exons 9 and 20) and *KRAS* (exons 2 and 3) in the 8 OCCC and 5 OSAC cell lines. Four out of the 8 OCCC cell lines showed a *PIK3CA* mutation while none of the 5 OSAC cell lines showed the mutation (Table I). One of the 5 OSAC cell lines showed a *KRAS* mutation (34G>A) while none of the 8 OCCC cell lines showed this mutation.

Sensitivity to NVP-BEZ235 or temsirolimus. The IC_{50} values of NVP-BEZ235 in the OCCC cell lines were lower than these values in the OSAC cell lines (Table I). In the OCCC cell lines, the IC_{50} of temsirolimus was higher than that of BEZ235 (Table II). Although the *PIK3CA* mutation was more frequently noted in OCCC than OSAC, the sensitivity of these cell lines to NVP-BEZ235 or temsirolimus was not related to the mutation status.

Expression levels of PI3K-Akt-mTOR pathway molecules in the OCCC and OSAC cell lines. Comparison of the OCCC and OSAC cell lines showed that pHER3 and pAkt expression was more frequent in OCCC than OSAC (Fig. 1A). That is, 7 of the 8 OCCC cell lines expressed pHER3 whereas 2 of the 5 OSAC cells lines exhibited expression. Similarly, 6 of the 8 OCCC cell lines expressed pAkt while 2 of the 5 OSAC cell lines did. The protein expression levels were distributed widely, and did not relate to the sensitivity to NVP-BEZ235 or temsirolimus.

Table II. IC_{50} of temsirolimus in the OCCC cell lines.

Cell line	IC_{50} (nM)	
	BEZ235	Temsirolimus
OVISE	44	9,122
SMOV-2	64	8,924
KK	74	5,929
TU-OC-1	131	7,224
OVTOKO	534	12,776
KOC-7c	600	9,779
OVMANA	641	17,650
RMG-I	777	4,045

When OVISE cells were treated with NVP-BEZ235, expression levels of p-p70S6K and p4E-BP1 were suppressed in a dose-dependent manner (Fig. 1B). Treatment with temsirolimus incompletely suppressed p-p70S6K and p4E-BP1 expression in the OVISE cells. Moreover, treatment with NVP-BEZ235 suppressed pAKT expression, while treatment with temsirolimus did not. Similar results were observed in the KK cells (Fig. 1C).

NVP-BEZ235 induces G_1 phase arrest and apoptosis in OCCC cells. OVISE cells were arrested at the G_1 phase, but did not exhibit apoptosis (denoted by an increased proportion of cells in sub-G_1), after 72 h of treatment with 10 and 100 nM NVP-BEZ235 (Fig. 2A). We observed similar results of G_1 arrest in the KK cells (Fig. 2A). Although the same conditions as those in the cell cycle analysis did not induce apoptosis,

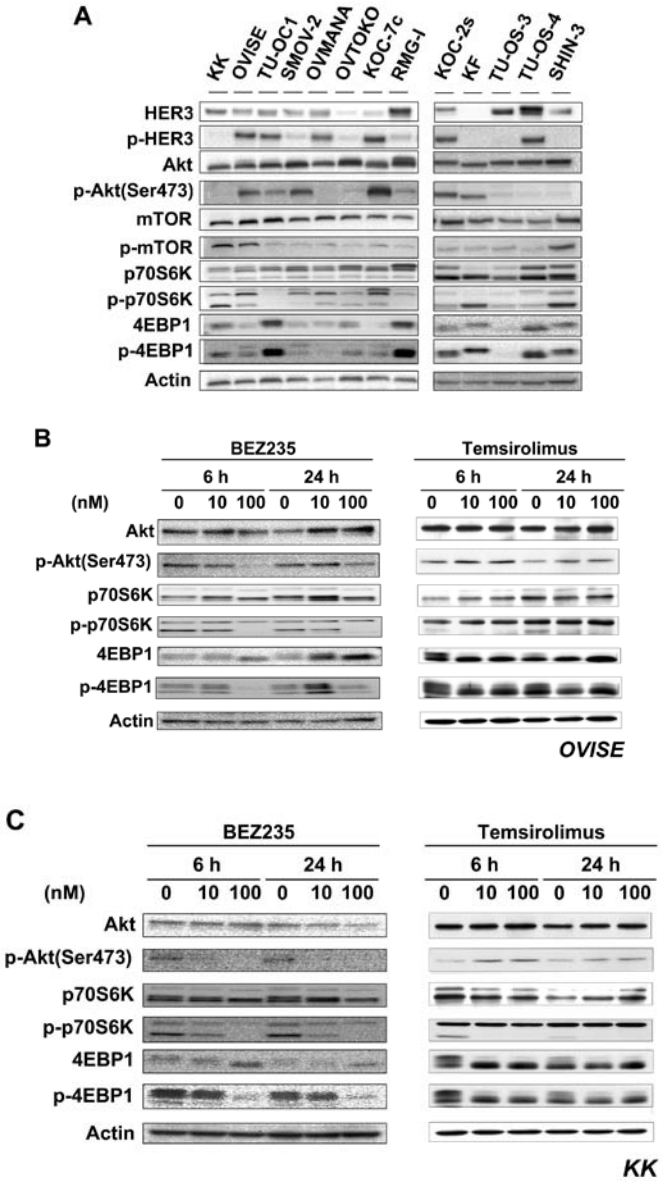

Figure 1. (A) Baseline expression of PI3K-Akt-mTOR pathway molecules in the OCCC and OSAC cell lines. Eight OCCC cell lines (KK, OVISE, TU-OC-1, SMOV-2, OVMANA, OVTOKO, KOC-7c and RMG-I) and 5 OSAC cell lines (KOC-2s, KF, TU-OS-3, TU-OS-4 and SHIN-3) were cultured in DMEM/F12 medium with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37˚C. Western blot analysis was performed to detect the expression levels of HER3, p-HER3, Akt, p-Akt, mTOR, p-mTOR, p70S6K, p-p70S6K, 4E-BP1 and p-4E-BP1. β-actin was used as a loading control. Each experiment was repeated 3 times independently. (B and C) NVP-BEZ235 suppressed pAkt expression in OCCC cells. Two OCCC cell lines (OVISE and KK) were plated in 6-well plates. The protein samples were collected after treatment with 10 and 100 nM NVP-BEZ235 or temsirolimus for 6 or 24 h. Western blot analysis was performed to detect Akt, p-Akt, p70S6K, p-p70S6K, 4E-BP1 and p-4E-BP1 expression. β-actin was used as a loading control.

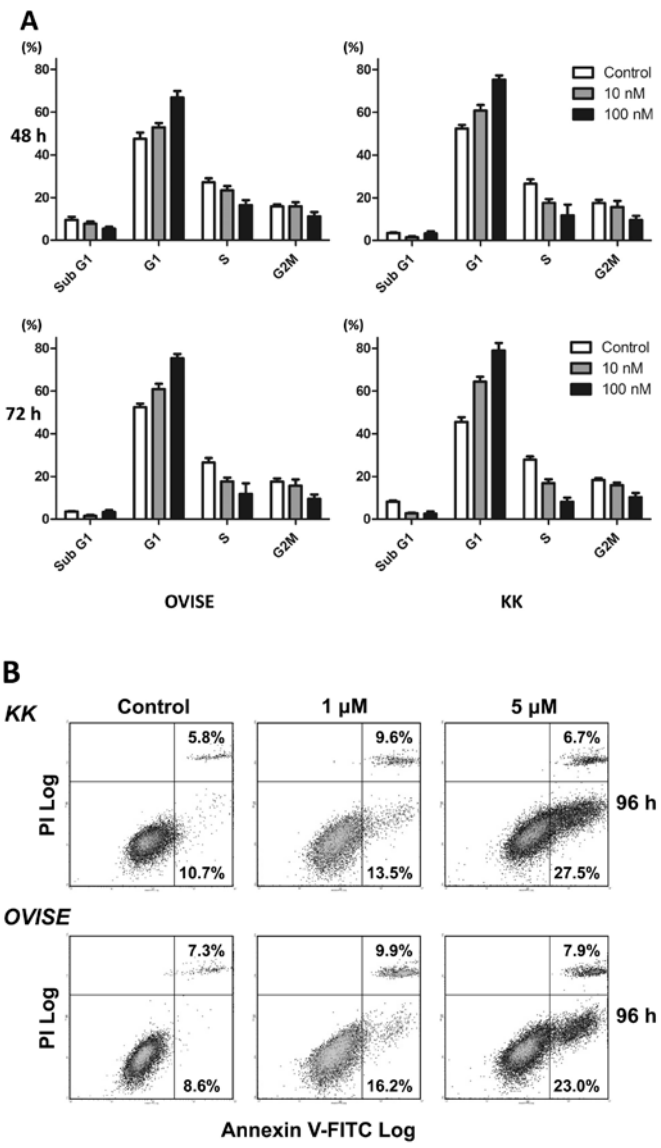

Figure 2. (A) NVP-BEZ235 induced G_1 phase arrest in the OCCC cells. OVISE or KK cells were treated with 10 or 100 nM NVP-BEZ235 for 48 or 72 h. FACScan analysis was performed to detect the cell cycle distribution. (B) NVP-BEZ235 induced apoptosis in the OCCC cells. OVISE and KK cells were treated with 1 or 5 μM NVP-BEZ235 for 96 h. Apoptosis was determined by the Annexin V-FITC Apoptosis Detection kit. Early apoptotic cells were scored as Annexin V-FITC-positive and propidium iodide (PI)-negative to exclude necrotic cells.

treatment of OVISE cells with 1 or 5 μM of NVP-BEZ235 for 96 h increased the number of Annexin V-positive and PI-negative cells (Fig. 2B). Similar results were observed in the KK cells (Fig. 2B).

NVP-BEZ235 suppresses tumor growth in an OCCC xenograft model. To assess short-term systemic toxicity of the agent, we recorded body weight changes of mice in addition to visual observation. After treatment, no mice had detectable changes in body weight, implying that there was no severe toxicity (Fig. 3A). At doses of 25 or 50 mg/kg/day, NVP-BEZ235 significantly inhibited subcutaneous tumor growth in mice bearing OVISE cells ($P<0.05$ for 25 mg/kg/day, $P<0.01$ for 50 mg/kg/day) (Fig. 3B). TU-OC-1 tumor volume in the 50 mg/kg/day group was significantly lower than that of the vehicle control although that in the 25 mg/kg/day group was not ($P<0.01$ for 50 mg/kg/day) (Fig. 3C).

Discussion

Many authors have reported poorer prognoses for patients with advanced stage OCCC (4,20,21). Low survival rates in OCCC

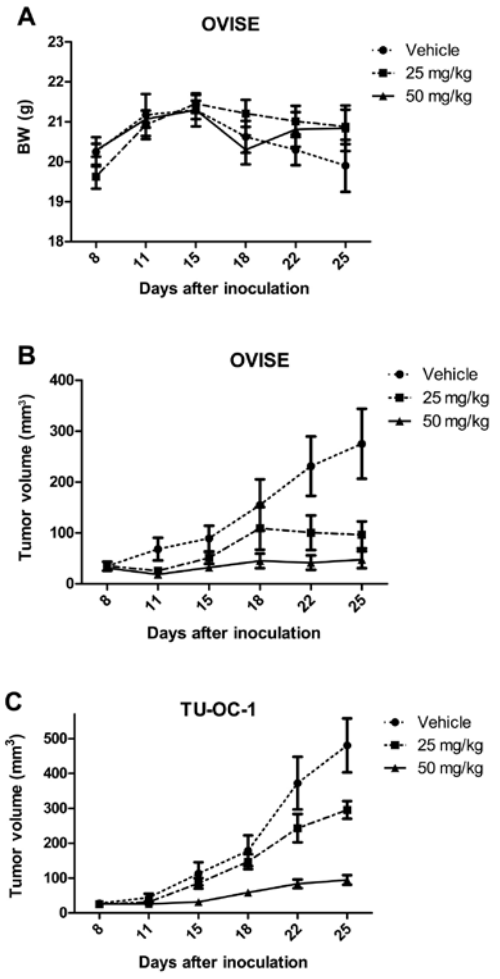

Figure 3. NVP-BEZ235 inhibits tumorigenicity in an OCCC xenograft model without producing toxic effects. Female athymic nude mice were injected subcutaneously with $4x10^6$ OVISE or TU-OC-1 cells. The experimental groups were treated with vehicle control (10% 1-methyl-2-pyrrolidone-90% polyethylene glycol 300), or 25 or 50 mg/kg/day NVP-BEZ235 for 3 weeks beginning 7 days after cell injection. (A) Mean body weight in each treatment group. (B and C) The tumor volume in OVISE or TU-OC-1 xenografts was measured with a caliper twice weekly. Error bars represent the standard error.

may, in part, reflect its lack of sensitivity to platinum-based chemotherapy. There are no antineoplastic agents definitely active and effective to treat OCCC. Therefore, novel therapeutic strategies, including targeted therapy, are needed to improve the prognosis of patients with OCCC.

It is known that mutations of *PIK3CA* are common molecular genetic alterations identified in OCCC (11). Expression of phospho-mTOR was found to be more prominent in OCCC than in OSAC (22). mTOR exists in two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. The downstream targets of mTORC1 are p70 ribosomal S6 kinase 1 (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), both of which are crucial to the regulation of protein synthesis.

In practice, inhibitors of mTORC1, such as temsirolimus and everolimus, have been used for renal cell carcinoma. A phase II study (GOG268) combining temsirolimus with carboplatin and paclitaxel following temsirolimus consolidation as first-line therapy is underway in patients with OCCC (23). However, the efficacy of mTORC1 blockade may be attenuated due to the loss of an mTORC1-dependent negative feedback loop on PI3K signaling and the mTORC2-mediated activation of Akt (24). p70S6K inhibits insulin receptor substrate 1 (IRS-1) by phosphorylating it, by inducing it to degrade and by altering its localization (25,26). The inhibition of IRS by p70S6K attenuates PI3K-AKT activation. Rapamycin (and its analogs temsirolimus and everolimus) stops this negative feedback loop from the p70S6K to the PI3K signaling pathway, resulting in activation of proliferative and prosurvival effectors such as AKT.

NVP-BEZ235 is a dual pan-class I PI3K and an mTOR kinase inhibitor that has the possible advantage of inhibiting PI3K, mTORC1 and mTORC2. Therefore, it should turn off this pathway completely and overcome feedback inhibition that is normally observed with mTORC1 inhibitors (e.g. rapamycin analogs). It is known that NVP-BEZ235 displays significant antitumor activities in glioblastoma, lung, breast, renal cell and uterine endometrial carcinomas (12,14,13,27).

In the present study, IC_{50} of temsirolimus was markedly higher than NVP-BEZ235 in all OCCC cell lines. In contrast, NVP-BEZ235 effectively suppressed proliferation of OCCC cells. Additionally, treatment with temsirolimus increased expression of pAKT while p-p70S6K and p4E-BP1 were suppressed. Treatment with NVP-BEZ235 suppressed pAkt, p-p70S6K and p4E-BP1. Accordingly, NVP-BEZ235 may be the more effective agent.

We found that NVP-BEZ235 suppressed tumor growth in an OCCC xenograft model. A few authors have reported on the antitumor activity of this compound in ovarian carcinoma. Montero *et al* (28) showed that NVP-BEZ235 effectively suppressed proliferation of 4 ovarian carcinoma cell lines which were not derived from OCCC. Santiskulvong *et al* (29), investigated the *in vivo* effects of NVP-BEZ235 on an immunocompetent transgenic murine ovarian endometrioid adenocarcinoma model. They also examined *in vitro* activity of the compound in 17 human ovarian carcinoma cell lines including 2 OCCC cell lines (ES-2 and OV207). Unfortunately, these studies did not focus on OCCC. Recently, Rahman *et al* (30) investigated the frequency of *PIK3CA* mutations in patients with OCCC and the relationship between the mutations and clinicopathological or biological variables. They also examined the *in vitro* sensitivity of 9 OCCC cell lines to LY294002, temsirolimus and NVP-BEZ235. No relationship was observed between the mutation status and sensitivity to these inhibitors. We also examined the mutation status of *PIK3CA* and *KRAS* genes and baseline protein expression levels of the PI3K/Akt/mTOR pathway molecules. Although the *PIK3CA* mutation was more common in OCCC than in OSAC in our series, there were no relationships between the mutation status or protein expression levels and sensitivity to NVP-BEZ235. These findings supported those of a previous report (30).

Our results revealed that NVP-BEZ235 effectively suppressed not only p-p70S6K and p4E-BP1, but also pAKT expression in OCCC cell lines and suppressed tumor growth in an OCCC xenograft model. This is the first report to demonstrate the efficacy of NVP-BEZ235 in OCCC.

We conclude that the PI3K-AKT-mTOR pathway is a potential therapeutic target for OCCC and that NVP-BEZ235 warrants investigation as a therapeutic agent.

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