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 IC50 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 IC50 values of BEZ235 for the OCCC cell lines were lower than these values for the OSAC cell lines. The IC50 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 G1 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.

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). PIK3CA, p110α (5). In response to an extracellular signal, the activated PIK3CA phosphorylates PIP2 to generate PIP3. The PIP3 recruits the PDK1 and PDK2. Activated AKT can directly activate the PI3K 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. Abrasions 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.

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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
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 Yoshiohiro 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 Yasuhiro Kiyouzuka (Nara Medical University, Kashiwara, 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% CO₂ 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 3130xl 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 (2-4x10⁴ 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, 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 Na₃VO₄ 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 (Thr37/44) 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 phosphatidyserine residues, according to the specifications of the manufacturer. Briefly, cells were suspended in 500 µl of 1X binding buffer. The cells then were stained with 5 µl Annexin V-FITC (fluorescein isothiocyanate) and 5 µl 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, 4x10⁴ 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.
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 IC50 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 IC50 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 cell 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.

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).

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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Original tumor</th>
<th>KRAS</th>
<th>PIK3CA</th>
<th>IC50 of BEZ235 (nM)</th>
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<tr>
<td>OVISE</td>
<td>Clear cell carcinoma</td>
<td>wt wt wt</td>
<td>wt</td>
<td>44</td>
</tr>
<tr>
<td>SMOV-2</td>
<td>Clear cell carcinoma</td>
<td>wt wt wt</td>
<td>3141 A&gt;A/T</td>
<td>65</td>
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<tr>
<td>KK</td>
<td>Clear cell carcinoma</td>
<td>wt wt wt</td>
<td>1634 A&gt;A/C</td>
<td>74</td>
</tr>
<tr>
<td>TU-OC-1</td>
<td>Clear cell carcinoma</td>
<td>wt wt wt</td>
<td>1624 G&gt;G/A</td>
<td>131</td>
</tr>
<tr>
<td>OVTOKO</td>
<td>Clear cell carcinoma</td>
<td>wt wt wt</td>
<td>wt</td>
<td>534</td>
</tr>
<tr>
<td>KOC-7c</td>
<td>Clear cell carcinoma</td>
<td>wt wt wt</td>
<td>wt</td>
<td>600</td>
</tr>
<tr>
<td>OVMANA</td>
<td>Clear cell carcinoma</td>
<td>wt wt wt</td>
<td>1634 A&gt;T</td>
<td>641</td>
</tr>
<tr>
<td>RMG-I</td>
<td>Clear cell carcinoma</td>
<td>wt wt wt</td>
<td>wt</td>
<td>777</td>
</tr>
<tr>
<td>KF</td>
<td>Serous adenocarcinoma</td>
<td>wt wt wt</td>
<td>wt</td>
<td>779</td>
</tr>
<tr>
<td>KOC-2s</td>
<td>Serous adenocarcinoma</td>
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<td>wt</td>
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<tr>
<td>TU-OS-3</td>
<td>Serous adenocarcinoma</td>
<td>wt wt wt</td>
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<tr>
<td>TU-OS-4</td>
<td>Serous adenocarcinoma</td>
<td>wt wt wt</td>
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<td>3,951</td>
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<tr>
<td>SHIN-3</td>
<td>Serous adenocarcinoma</td>
<td>34 G&gt;A</td>
<td>wt</td>
<td>25,400</td>
</tr>
</tbody>
</table>

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

Table II. IC50 of temsirolimus in the OCCC cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BEZ235</th>
<th>Temsirolimus</th>
</tr>
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<tbody>
<tr>
<td>OVISE</td>
<td>44</td>
<td>9,122</td>
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<tr>
<td>SMOV-2</td>
<td>64</td>
<td>8,924</td>
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<tr>
<td>KK</td>
<td>74</td>
<td>5,929</td>
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<tr>
<td>TU-OC-1</td>
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<td>OVTOKO</td>
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<td>KOC-7c</td>
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<td>9,779</td>
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<td>OVMANA</td>
<td>641</td>
<td>17,650</td>
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<tr>
<td>RMG-I</td>
<td>777</td>
<td>4,045</td>
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</table>

NVP-BEZ235 induces G1 phase arrest and apoptosis in OCCC cells. OVISE cells were arrested at the G1 phase, but did not exhibit apoptosis (denoted by an increased proportion of cells in sub-G1), after 72 h of treatment with 10 and 100 nM NVP-BEZ235 (Fig. 2A). We observed similar results of G1 arrest in the KK cells (Fig. 2A). Although the same conditions as those in the cell cycle analysis did not induce apoptosis,
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...
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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