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Growth inhibition by NVP-BEZ235, a dual PI3K/mTOR inhibitor, in hepatocellular carcinoma cell lines

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Abstract. Dysregulation of the phosphatidylinositol-3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway frequently occurs in human tumors, and is therefore considered to be a good molecular target for treatment. In hepatocellular carcinoma (HCC), overexpression of p-Akt and decrease of PTEN expression have been reported. NVP-BEZ235 is a novel dual inhibitor of PI3K and mTOR; however, its effect on HCC has not been documented. Consequently, we investigated the effects of NVP-BEZ235 on the PLC/PRF/5, HLE, JHH7 and HepG2 HCC cell lines *in vitro* and *in vivo*. NVP-BEZ235 decreased the levels of p-Akt and p-p70S6K and inhibited cell proliferation in all HCC cell lines in a dose-dependent manner. Flow cytometric analysis revealed that inhibition of cell proliferation by NVP-BEZ235 was accompanied by G1 arrest in all cell lines, and that NVP-BEZ235 induced apoptosis in PLC/PRF/5 and HLE cells. Tumor growth was suppressed without body weight loss when NVP-BEZ235 was orally administered to JHH-7 tumor-bearing mice for 11 days. These results suggest that NVP-BEZ235 is a potential new candidate for targeted HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third-leading cause of cancer-related deaths worldwide (1). HCC accounts for 85-90% of all primary liver cancers. The number of new cases of liver cancer per year has increased in many countries over the last several decades (2). Despite improvements in chemotherapy, liver resection, transcatheter arterial chemoembolization (TACE) and transplantation, many HCC patients still experience recurrence or progression after treatment. In addition, HCC is generally recognized as being chemoresistant. Therefore, the development of a novel effective therapy for HCC is urgently needed.

The phosphatidylinositol-3-kinase (PI3K)/mammalian target of the rapamycin (mTOR) pathway plays an important role in diverse cellular functions such as proliferation, tumor growth, survival and metabolism (3,4). This pathway is normally regulated by upstream receptor tyrosine kinases. Following the activation of a receptor tyrosine kinase (RTK), PI3K is activated and generates the second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (3). PIP_3 attracts PDK1 to the plasma membrane and phosphorylates Akt at Tyr^{308} (5). Full Akt activation requires phosphorylation at Ser^{473}, which is affected by the rapamycin-insensitive mTOR complex (mTORC2 containing rictor) (6). The activated Akt is able to execute a vast number of cellular operations such as cell survival and proliferation through the inhibition of cell cycle inhibitors, pro-apoptotic signals and so on (7). Another mTOR complex, rapamycin-sensitive mTOR complex (mTORC1 containing raptor) stimulates the protein synthesis machinery through the activation of its effector, p70S6K or leads to inactivation of the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), an inhibitor of translation initiation (8). In the PI3K pathway, the phosphatase and tensin homologue (PTEN) acts as a major inhibitor mediating the dephosphorylation of PIP_3 (9). Dysregulation of PI3K activity has been reported in human cancers (10-12). The causes include down-regulation or mutation of PTEN and somatic mutation or amplification of *PIK3CA*, encoding p110α of the class IA PI3K (13-15). In HCC, somatic mutation of *PIK3CA*, enhancement of Akt and phosphorylated ribosomal protein S6 and decrease of PTEN expression have been reported (16-18). These observations suggest that dysregulation of the PI3K/mTOR pathway may contribute to the survival and growth of HCC and it is therefore considered to be a good molecular target for treatment.

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NVP-BEZ235 is a novel, orally bioavailable inhibitor of pan-class I PI3K/mTOR kinase. It presents inhibition of PI3K activity by competing at its ATP-binding domain. On the mTOR, NVP-BEZ235 inhibits catalytic activity of mTOR, and the inhibition of mTORC1 and mTORC2 has also been observed (19). NVP-BEZ235 has demonstrated anti-tumor effects on various tumor types (19-22). The clinical efficacy of NVP-BEZ235 is currently being evaluated in a phase I/II clinical trial setting in patients with advanced solid malignancies supplemented by patients with advanced breast cancer. However, its effects on HCC have not been documented.

In this study, we investigated the effects of NVP-BEZ235 on HCC cell lines *in vitro* and *in vivo*. NVP-BEZ235 inhibited PI3K/mTOR modulators and cell growth of HCC cell lines dose dependently *in vitro*. Moreover, treatment with NVP-BEZ235 showed suppression of tumor growth without body weight loss in the tumor-bearing mouse model.

Materials and methods

Cell lines. The human HCC cell line HepG2 was obtained from the Riken Cell Bank, and the PLC/PRF/5, HLE and JHH7 (23) cell lines were obtained from the Health Science Research Resources Bank. All cell lines were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% penicillin-streptomycin at 37°C in a 5% CO_2 atmosphere.

Compound preparation for in vitro and in vivo studies. NVP-BEZ235 was kindly provided by Novartis Pharma. Rapamycin and LY294002 were purchased from Sigma. For *in vitro* study, these compounds were dissolved in dimethyl sulfoxide (DMSO) and cells were treated at the indicated final concentrations. The final DMSO concentration was always kept at 0.1%. For *in vivo* studies, NVP-BEZ235 was dissolved in one volume of NMP (1-methyl-2-pyrrolidone; Sigma-Aldrich) by sonication (50 mg/ml), and then nine volumes of polyethylene glycol 300 (Sigma-Aldrich) were added (final concentration 5 mg/ml).

Western blot analysis. For Western blot analyses, the cells ($2\text{-}4 \times 10^5$) were seeded in 6-well plates. The next day, the medium was discarded and replaced with 2 ml fresh medium and the cells were treated with the compounds at the indicated concentrations for 0-48 h. Floating and adhesion cells were washed twice with ice-cold PBS and then were lysed in the appropriate amount of lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 1% Nonidet® P-40 and protease inhibitor tablet (Roche Applied Sciences)]. The protein concentration was determined using a BCA™ Protein Assay Kit (Pierce). The cell lysates were separated on 7.5-12% SDS-PAGE gels and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% non-fat dry milk in TBS-T [10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.05% Tween-20] for 1 h and incubated with the following primary antibodies: anti-p-Akt, anti-Akt, anti-p-p70S6K, anti-p70S6K, anti-p-4E-BP1, anti-PTEN, anti-p110α (Cell Signaling Technology) and anti-β-actin (Sigma), followed by reaction with HRP-conjugated secondary antibodies (Jackson ImmunoResearch). The bands were visualized by ECL (GE Healthcare).

Cell proliferation assay. The cells were seeded in 96-well plates at 2×10^3 cells/100 μl per well. On the following day, the cells were treated with various concentrations of NVP-BEZ235, LY294002 or rapamycin for 24 h. After the incubation period, the cells were cultured in the presence of WST-8 reagent (Dojindo) for 2 h, followed by measurement of absorbance at 450 nm using a microplate reader (Bio-Rad).

Cell cycle analysis. The cells ($1\text{-}2 \times 10^6$) were seeded in 10-cm culture dishes. After 18-24 h, the medium was discarded and replaced with 10 ml fresh medium and the cells were treated with NVP-BEZ235 at the indicated concentration for 24 h. Floating cells and adhesion cells were collected by trypsinization and washed twice with ice-cold PBS. The cells were fixed in 70% ethanol at -20°C overnight. After fixation, the cells were washed with PBS and incubated with 50 μg/ml RNaseA (Sigma) for 30 min at 37°C. Following incubation, the cells were stained with 50 μg/ml propidium iodide (PI) (Sigma) and evaluated using flow cytometric analysis.

Apoptosis analysis. The cells (1×10^5) were seeded in 6-well culture plates. After 18-24 h, the medium was discarded and replaced with 2 ml fresh medium and the cells were treated with NVP-BEZ235 at the indicated concentration for 48 h. Then, the culture media and cells were collected and centrifuged. After washing, the cells were resuspended in 500 μl of 1X binding buffer, followed by the addition of 5 μl of Annexin V-FITC (BioVision) and 5 μl of PI (BioVision). The cells were incubated at room temperature for 5 min in the dark and assessed using flow cytometric analysis.

Tumor xenografts in nude mice. Six- to eight-week-old female BALB/c nude mice (nu^+/nu^+, n=7) were purchased from Charles River Laboratories Japan. Mice were maintained under the institutional guidelines set by the Animal Research Committee of the National Cancer Center Hospital East. Mice were housed in air-filtered laminar flow cabinets with a 12-h light cycle and access to food and water *ad libitum*. JHH7 cells (5×10^6) were subcutaneously injected into the right flank of each mouse. When the tumor volume reached 100 to 200 mm^3, the mice were administered 45 mg/kg NVP-BEZ235 in NMP/PEG300 (1:9, v/v) or NMP/PEG300 (1:9, v/v) once daily by oral gavage for 11 days. The body weight and the tumor volume were measured from the day of the first treatment. The tumor volume was estimated using the standard formula: (length x $width^2$)/2. Tumors were harvested within 1 h after the last treatment and frozen in liquid nitrogen. The frozen tissues were homogenized using a Multi-Beads Shocker® (Yasui Kikai) and lysed in lysis buffer. The lysates were used for Western blot analysis.

Results

PI3K/mTOR signaling is activated in HCC cell lines. The expression levels of PI3K/mTOR pathway-related molecules, p-Akt, p-p70S6K, PTEN and p110α, a catalytic site of PI3K, in HCC cell lines were determined by Western blotting (Fig. 1).

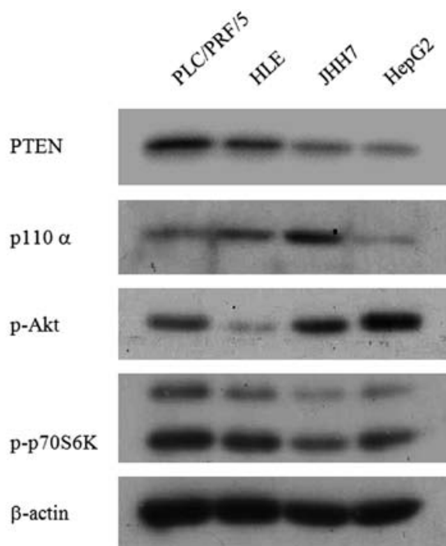

Figure 1. The PI3K/mTOR pathway is activated in HCC cell lines. Western blotting shows the expression level of PTEN, p110α, p-Akt and p-p70S6K in PLC/PRF/5, HLE, JHH7 and HepG2. Cells were cultured in fresh DMEM with 10% FBS for 12 h. β-actin was used as an internal control.

The expression level of PTEN was lower in JHH7 and HepG2, and that of p110α was the highest in JHH7 among the cell lines. As expected, p-Akt and p-p70S6K were detected in all cell lines. These results suggest that the PI3K/mTOR pathway was activated in these cell lines.

NVP-BEZ235 inhibits the PI3K/mTOR signaling pathway. Next, we evaluated the effect of NVP-BEZ235 on the expression levels of PI3K/mTOR pathway modulators in HCC cell lines by Western blot analysis. The cells were pretreated with several different concentrations of NVP-BEZ235 for 1 h and the modulator expression levels were assessed. The PI3K inhibitor LY294002 and the mTORC1 inhibitor rapamycin were used as controls. In all cell lines, the phosphorylation level of Akt and p70S6K was decreased by treatment with NVP-BEZ235 concentations ≥30 nM (Fig. 2A). Consistent with the result using NVP-BEZ235, LY294002 inhibited the phosphorylation of both modulators at 10 μM. In contrast, rapamycin inhibited the phosphorylation of p70S6K but not that of Akt at 100 nM.

The inhibitory activity of NVP-BEZ235 on PI3K/mTOR may occur in a competitive and reversible manner. Therefore, we investigated the relevance of the concentration and the duration of NVP-BEZ235 treatment. Time-course experiments were conducted to assess the effects of NVP-BEZ235 at 100 nM (Fig. 2B) and 1 μM (Fig. 2C). In PLC/PRF/5 and HLE cells, treatment with 100 nM of NVP-BEZ235 resulted in sustained inhibition of p-p70S6K exceeding 48 h, while p-Akt was inhibited up to 6 h and its recovery occured at ~12 h (Fig. 2B). The phosphorylation of Akt was almost completely inhibited for >48 h in PLC/PRF/5 and HLE cells incubated with 1 μM of NVP-BEZ235 (Fig. 2C). On the contrary, in JHH7 and HepG2 cells, 100 nM of NVP-BEZ235 inhibited p-Akt and p-p70S6K levels for around 6 h, which is shorter than the duration of the inhibition in PLC/PRF/5 and HLE cells (Fig. 2B). However, phosphorylation of Akt and p70S6K was inhibited for >48 h in all cells lines treated with 1 μM of NVP-BEZ235, except for phosphorylation of Akt in the JHH7 cell line which was observed up to ~12-18 h (Fig. 2C). In addition, p-4E-BP dose-dependently decreased in all cell lines (Fig. 2D) by NVP-BEZ235 exposure for 18 h.

NVP-BEZ235 inhibited the PI3K/mTOR pathway in a dose-dependent manner. In addition, it is suggested that PLC/PRF/5 and HLE cells were more sensitive to NVP-BEZ235 than JHH7 and HepG2 cells.

NVP-BEZ235 inhibits cell proliferation through induction of G1 arrest and apoptosis. Since the phosphorylation of Akt and p70S6K was inhibited by NVP-BEZ235, cellular viability and proliferation were evaluated by the WST-8 assay. Cells were incubated with various concentrations of NVP-BEZ235 for 48 h. Cell growth was inhibited by NVP-BEZ235 in a dose-dependent manner. Growth-inhibition 50% concentrations (GI_{50}) of PLC/PRF/5, HLE, JHH7 and HepG2 cells were 15.2 nM, 40.6 nM, 169.2 nM and >1 μM, respectively (Fig. 3A). On the other hand, LY294002 and rapamycin showed less antiproliferative activity than NVP-BEZ235, and the GI_{50}s of LY294002 and rapamycin were >10 and >1 μM, respectively (Fig. 3A).

To determine the mechanism of the antiproliferative activity of NVP-BEZ235, cell cycle analysis and apoptosis analysis were performed using flow cytometric analysis. For cell cycle analysis, the cells were incubated with NVP-BEZ235 for 24 h. The data for PLC/PRF/5 and HLE cells showed that the cells accumulated in the G1 phase of the cell cycle after treatment with NVP-BEZ235 concentrations ≥30 nM (Fig. 3B). In contrast, G1 arrest in JHH7 and HepG2 cells was observed at concentrations >300 nM (Fig. 3B). Consistent with the inhibitory activity on the phosphorylation on Akt and p70S6K, JHH7 and HepG2 cells were less sensitive than PLC/PRF/5 and HLE cells.

Furthermore, we investigated the expression level of p27, which typically causes cell arrest in the G1 phase of the cell cycle. HCC cell lines were exposed to NVP-BEZ235 for 18 h and prepared for Western blotting. The results showed that p27 levels increased in PLC/PRF/5, JHH7 and HepG2 cells, but decreased in HLE cells treated with 1 μM NVP-BEZ235 (Fig. 3C).

In addition, we assessed the apoptosis induction activity of NVP-BEZ235 using flow cytometric analysis. Cell lines were incubated with various concentrations of NVP-BEZ235 for 48 h and stained by Annexin V and PI. Annexin V^+/PI^- early apoptotic cells increased in a dose-dependent fashion in PLC/PRF/5 and HLE. On the contrary, in JHH7 and HepG2 cells, a prominent increase in apoptotic cells was not observed at the concentrations examined (Fig. 3D).

These results suggest that NVP-BEZ235 inhibited cell proliferation through the induction of G1 arrest and apoptosis in a dose-dependent manner, especially in PLC/PRF/5 and HLE cells.

NVP-BEZ235 suppresses tumor growth in vivo. The antitumor activity of NVP-BEZ235 *in vivo* was investigated in a xenograft model derived from JHH7 cells, because JHH7 is the only tumorigenic tumor among the four cell lines. NVP-BEZ235 daily oral administration at 45 mg/kg for 11

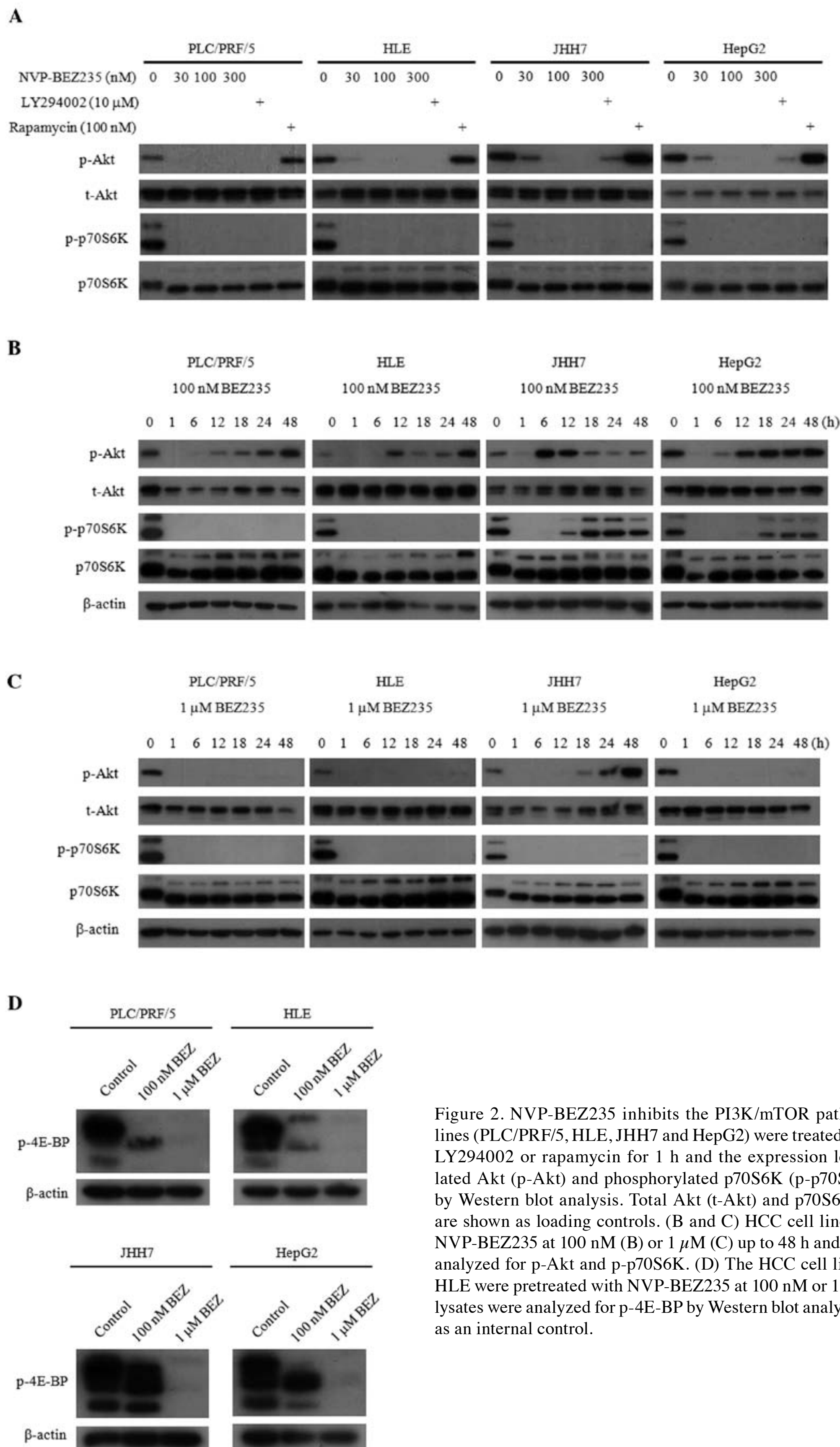

Figure 2. NVP-BEZ235 inhibits the PI3K/mTOR pathway. (A) HCC cell lines (PLC/PRF/5, HLE, JHH7 and HepG2) were treated with NVP-BEZ235, LY294002 or rapamycin for 1 h and the expression levels of phosphorylated Akt (p-Akt) and phosphorylated p70S6K (p-p70S6K) were analyzed by Western blot analysis. Total Akt (t-Akt) and p70S6K (t-p70S6K) levels are shown as loading controls. (B and C) HCC cell lines were treated with NVP-BEZ235 at 100 nM (B) or 1 μM (C) up to 48 h and the cell lysates were analyzed for p-Akt and p-p70S6K. (D) The HCC cell lines PLC/PRF/5 and HLE were pretreated with NVP-BEZ235 at 100 nM or 1 μM for 18 h and cell lysates were analyzed for p-4E-BP by Western blot analysis. β-actin was used as an internal control.

days was initiated when a xenograft achieved a volume of 100-200 mm^3. Tumor growth was significantly suppressed by NVP-BEZ235, compared with the vehicle-treated controls (P=0.032) (Fig. 4A). There were no statistical differences between the treated and control groups; loss of body weight occurred in both the control and treatment groups (Fig. 4B),

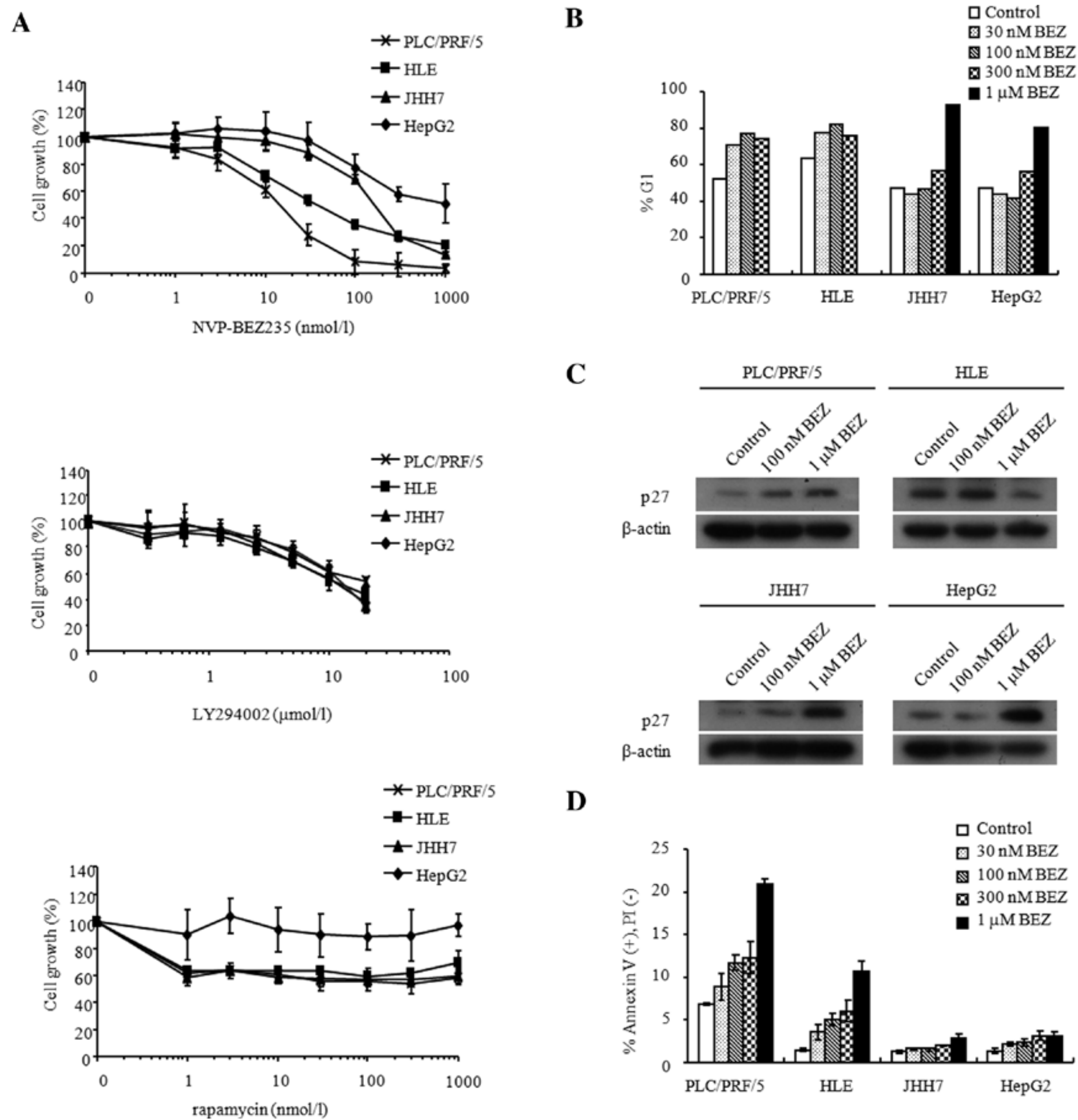

Figure 3. NVP-BEZ235 blocks cell proliferation and induces G1 cell cycle arrest and apoptosis in HCC cell lines. (A) HCC cells ($2x10^3$/well) were incubated with various concentrations of NVP-BEZ235, LY294002 or rapamycin and cell viability was determined after 48 h by the WST-8 assay. The data are presented as the percent of viable cells vs. non-treated cells (n=6). (B) The cells were treated with various concentrations of NVP-BEZ235 for 24 h, and cell cycle analysis was performed by PI staining. The data show the percentage of the G1 phase population in each cell line. (C) The cells were treated with 100 nM or 1 μM of NVP-BEZ235 for 18 h, and lysates were analyzed by Western blot analysis for p27. β-actin was used as an internal control. (D) Cells ($1x10^5$/well) were treated with various concentrations of NVP-BEZ235. After 48 h, the cells were harvested and stained with Annexin V and PI. Annexin V^+/PI^- cells were detected using flow cytometric analysis. The percentage of Annexin V^+/PI^- (early apoptotic) cells is shown.

which may have been due to the tumor. At the end of the experiment, we measured the level of p-Akt in tumors excised from the mice at 1 h post-drug administration by Western blot analysis. The phosphorylation of Akt was decreased in the treatment group (Fig. 4C). These results indicate that NVP-BEZ235 suppresses HCC tumor growth through the down-regulation of PI3K *in vivo*.

Discussion

Dysregulation of growth factors, receptors and their downstream signaling pathway components have been reported in HCC (1). We focused on the PI3K/mTOR pathway and hypothesized that it might be a candidate therapeutic target in HCC. NVP-BEZ235 reduces the kinase activity of both PI3K and mTOR (19). Previous reports show the effect of NVP-BEZ235 on several types of tumors (19-22). In this study, we examined the efficacy of NVP-BEZ235 against HCC cell lines.

Indeed, the PI3K/mTOR pathway is activated in HCC cell lines. NVP-BEZ235 completely inhibited the phosphorylation of Akt and p70S6K in all cell lines treated with NVP-BEZ235 for 1 h at 100 nM. However, time-course experiments revealed a difference in sensitivity to NVP-BEZ235 among the cell lines. JHH7 and HepG2 cells recovered phosphorylation of Akt and p70S6K earlier than PLC/PRF/5 and HLE cells. JHH7 and HepG2 cells expressed a low level of PTEN that acts as an inhibitor of the PI3K pathway. In addition, the expression level of p110α, a catalytic site of PI3K, was up-regulated to a greater

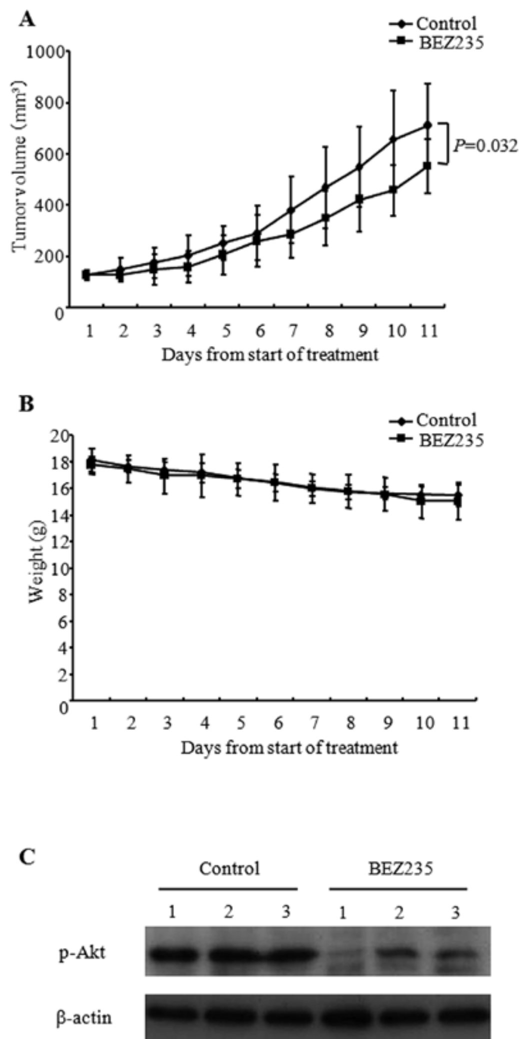

Figure 4. Effect of NVP-BEZ235 on tumor growth *in vivo*. (A and B) JHH7 tumor-bearing mice were treated with NVP-BEZ235 (45 mg/kg) or with the vehicle control once per day for 11 days (n=7). Tumor volume (A) and body weight (B) were recorded during the course of the experiment. (C) One hour after the last treatment, the JHH7 xenograft was removed and the expression level of p-Akt was analyzed by Western blot analysis.

degree in JHH7 than in the others. These factors may have contributed to the earlier phosphorylation recovery of Akt and p70S6K in JHH7 and HepG2 cells.

NVP-BEZ235 inhibited cell proliferation in a dose-dependent manner. In the cell proliferation assay, consistent with the time-course experiments, PLC/PRF/5 and HLE cells showed higher sensitivity to NVP-BEZ235; the GI_{50}s were <50 nM. On the contrary, JHH7 and HepG2 showed lower sensitivity; the GI_{50}s were >150 nM. Next, we compared the antiproliferative activity of NVP-BEZ235 with the PI3K inhibitor LY294002. It was interesting that dual inhibition of PI3K and mTOR by NVP-BEZ235 was more effective than treatment with the PI3K inhibitor or the mTORC1 inhibitor alone.

In addition, the results of cell cycle analysis and apoptosis assay showed different tendencies between the high-sensitivity group, PLC/PRF/5 and HLE, and the low-sensitivity group, JHH7 and HepG2 cells. G1 arrest and apoptosis were induced by a lower concentration of NVP-BEZ235 (<30 nM) in PLC/PRF/5 and HLE cells. On the contrary, JHH7 and HepG2 cells required a high concentration of NVP-BEZ235 (>300 nM) for G1 arrest; moreover, apoptosis was not significantly induced. These results suggest that the antiproliferation was due to G1 arrest and apoptosis in the high-sensitivity group, but only G1 arrest in the low-sensitivity group. In addition, NVP-BEZ235 inhibited the phosphorylation of p70S6K and 4EBP through the inhibition of mTOR. The phosphorylation of these molecules results in protein synthesis. This result may also have contributed to the antiproliferative activity of NVP-BEZ235. The p27 expression level was analyzed to investigate G1 arrest. The p27 expression level was higher than that of the control cells in the treated PLC/PRF/5, JHH7 and HepG2 cells, but not in HLE cells. There are other molecules regulating the cell cycle that are related to the PI3K/mTOR pathway; therefore, further study of cell-cycle molecules such as p21 is required.

Moreover, it remains unclear why apoptosis was not significantly induced in JHH7 and HepG2 cells. In JHH7 and HepG2 cells, phosphorylation of Akt was significantly inhibited, at least at 1 μM; indeed, cell cycle arrest was observed at 1 μM. NVP-BEZ235 induced apoptosis through the inhibition of PI3K/mTOR, which is located upstream from pro- or anti-apoptotic molecules. Therefore, if downstream molecules that regulate apoptosis were dysregulated, NVP-BEZ235 may not be able to fully induce apoptosis. Indeed, HCC has been reported to overexpress Bcl-x_L, an anti-apoptotic member of the Bcl-2 family (24), and to down-regulate pro-apoptotic members, Bcl-x_S and Bax, with dysfunction in the p53 pathway (25). Therefore, further study is required on the dysregulation of pro- or anti-apoptotic molecules such as Bcl-x_L, Bax or Bcl-x_S in JHH7 and HepG2.

NVP-BEZ235 significantly suppressed tumor growth *in vivo* in JHH7, which was less sensitive to NVP-BEZ235 treatment *in vitro*. Moreover, there was no significant difference in the loss of body weight between the treated and control groups. These results suggest that NVP-BEZ235 is effective and tolerable in HCC therapy.

In summary, this study demonstrated that NVP-BEZ235 inhibits the PI3K/mTOR pathway in HCC cell lines both *in vitro* and *in vivo*. NVP-BEZ235 is a potential therapeutic option for the treatment of HCC. However, HCC cell lines showed slightly lower sensitivity to NVP-BEZ235 in this study compared to other cancer cell lines in previous reports (19-22). Further investigation is needed to clarify the effectiveness of NVP-BEZ235 in HCC.

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