

Effect of the dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 against human Merkel cell carcinoma MKL-1 cells

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Abstract. Merkel cell carcinoma (MCC) is an aggressive skin cancer with an increasing incidence. Aberrant activation of the phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is common in human cancers and has been revealed to play an important function in cell proliferation, metabolism and tumorigenesis. In the present study, NVP-BEZ235, a dual PI3K/mTOR inhibitor, was revealed to be effective in inhibiting proliferation and inducing cell cycle arrest in MKL-1 cells. Additional investigations revealed that NVP-BEZ235 attenuated PI3K/Akt/mTOR signaling and upregulated the levels of the cell cycle inhibitors p21 and p27. Overall, the present results possess considerable implications for future development of dual PI3K/mTOR inhibitor as potential agents in the management of MCC.

Introduction

Merkel cell carcinoma (MCC) is a rare, but highly aggressive, cutaneous neoplasm (1). Although MCC primarily affects individuals that are elderly or immune suppressed, data from the Surveillance, Epidemiology, and End Results Program of the National Cancer Institute indicate that the incidence of MCC tripled between 1986 and 2001, with 0.15 cases per 100,000 individuals in 1986 and 0.44 cases per 100,000 individuals in 2001 (2). Notably, one-half of patients with MCC present with metastatic disease and exhibit a five-year disease-associated mortality rate of 46% (3). Merkel cell polyoma virus (MCV) was previously identified in MCC, but little is known of the additional oncogenic events involved in the development of this cancer (4). In addition, no chemotherapeutic regimen has

been reported to be effective in the treatment of MCC, and drug combinations in clinical use are frequently based on the histological similarity of MCC to small-cell lung carcinoma (5).

Aberrant activation of the phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is found in numerous types of cancer, and therefore, plays a major role in cancer cell proliferation and anti-cancer drug resistance (6). Components of the PI3K/Akt/mTOR pathway are frequently abnormal in a variety of tumors, making them an attractive target for anti-cancer therapy. Previously, Akt hyperphosphorylation has been found in MCV-independent MCC (7,8). In addition, chronic mTOR activation has been found to promote cell survival in MCC (9). Therefore, the PI3K/Akt/mTOR pathway is a promising target for the management of MCC.

NVP-BEZ235 is an orally bioavailable imidazoquinoline that inhibits the activity of PI3K and mTOR kinase by binding to the adenosine triphosphate binding domains (10). NVP-BEZ235 has been reported to inhibit tumor growth in numerous preclinical models, including prostate, breast and pancreatic carcinoma and glioblastoma, and at present, this agent is in phase I testing in patients with solid tumors (11-13). In the current study, the effect of NVP-BEZ235 on human MCC MKL-1 cells and its underlying mechanism were investigated in order to provide a potential therapeutic option in the management of MCC.

Materials and methods

Patient samples. In accordance with institutional approvals for human study protocols, a total of 22 MCC tumor samples were collected between January 2005 and December 2011 at the Union Hospital, Huazhong University of Science and Technology (Wuhan, China). Written informed consent was obtained from each patient.

Cell lines and reagents. MCC MKL-1 cells (Sigma-Aldrich, St. Louis, MO, USA) were grown in RPMI-1640 supplemented (Gibco Life Technologies, Carlsbad, CA, USA) with 10% fetal calf serum, penicillin and streptomycin (Sigma-Aldrich) at 37°C in a humidified 5% CO₂ atmosphere. NVP-BEZ235 was provided by Novartis (Basel, Switzerland) and dissolved

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in dimethyl sulfoxide (Sigma-Aldrich) to a concentration of 10 mmol/l, stored at -20°C, and further diluted to final concentrations of 50 nM, 100 nM and 200 nM in Dulbecco's modified Eagle's medium at the time of use. Rabbit polyclonal antibodies against phosphorylated mTOR (p-mTOR; Ser2448; cat. no. 2971), phosphorylated Akt (p-Akt; Ser473; cat. no. 4058) and cleaved caspase-3 (cat. no. 9661) used at a dilution of 1:1,000 were purchased from Cell Signaling Technologies (Danvers, MA, USA). Rabbit polyclonal antibodies against p21 (cat. no. sc-397), p27 (cat. no. M-197), p57 (cat. no. C-20) and cyclinD1 (cat. no. sc-753) used at a dilution of 1:250 and mouse monoclonal antibody against tubulin (cat. no. TU-02; dilution, 1:5,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Additional reagents used in the present study consisted of TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison, WI, USA), puromycin (Sigma-Aldrich), polybrene (Sigma-Aldrich), fibronectin (Sigma-Aldrich), RIPA buffer (Sigma-Aldrich) and enhanced chemiluminescence (ECL) detection reagent (EMD Millipore, Billerica, MD, USA).

Western blotting. The cultured cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in 1X RIPA buffer containing 1 mM DTT and Complete Mini EDTA-free protease inhibitor cocktail. Following incubation on ice for 30 min, the cell lysates were clarified by centrifugation at 16,000 x g for 15 min at 4°C. In total, 20 µg of protein was resolved on 8 or 12% sodium SDS-PAGE gels and transferred electrophoretically onto polyvinylidene difluoride membranes by a semi-dry system (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked in 5% fat-free milk in Tris-buffered saline with 0.1% Tween 20 (Sigma-Aldrich) for 1 h, and incubated with primary antibodies at 4°C overnight, followed by incubation with monoclonal goat anti-mouse (1:15,000; cat. no. SC-2055) or goat anti-rabbit (1:25,000; cat. no. SC-2054) secondary antibodies conjugated with horseradish peroxidase. Subsequent to rinsing three times, the membrane was subjected to western blot analysis with ECL detection reagent.

Determination of cell viability. Cell survival was assessed using cell counting kit-8 (CCK-8; Sigma-Aldrich) according to the manufacturer's instructions. In brief, the cells were plated at a density of 10,000 cells per well in a 96-well plate and exposed to serial dilutions of NVP-BEZ235 for 12, 24, 48 and 72 h, respectively. CCK-8 (10 µl) was added to each well at the end of each time point (12, 24, 48 and 72 h) and incubated at 37°C for 4 h. The absorbance at 450 nm was measured using a microplate spectrophotometer (Bio-Rad Model 680; Bio-Rad Laboratories, Inc.).

Determination of apoptosis and cell cycle distribution. Cell death was detected by flow cytometry for Annexin V-fluorescein isothiocyanate, according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Briefly, 1x10⁶ cells were plated in six-well plates for 12 h followed by treatment with NVP-BEZ235 for 24 h prior to Annexin V and propidium iodide (PI) staining (FACSAria; BD Biosciences). For each dye, appropriate electronic compensation of the FACSAria sorter was performed to avoid overlapping of the two emission spectra. For cell cycle analysis, 1x10⁶ cells were seeded in six-well plates for 12 h, followed by treatment with NVP-BEZ235, and

were then labeled with 10 µM bromodeoxyuridine (BrdU; BD Biosciences) for 2 h. BrdU incorporation was detected using Alexa Fluor 488-conjugated mouse anti-BrdU antibody (BD Biosciences), followed by 7-aminoactinomycin D staining for cell cycle analysis, according to the manufacturer's instructions.

Lentiviral transduction of short hairpin (sh)RNA. Lentivectors directing expression of shRNA specific to p21 (construct no., TRCN0000040123) and p27 (construct no., TRCN0000009856) were purchased from Sigma-Aldrich, and non-targeting pLKO.1 scramble shRNA was obtained from Addgene (plasmid no., 1864). To generate recombinant lentiviruses, 293T/17 cells were co-transfected with gene transfer vectors and virus packaging vectors, ΔH8.2 and VSVG using TransIT-LT1 transfection reagent. Virus supernatants were collected 48 h following transfection. MKL-1 cells were transduced with virus supernatant for 48 h in fibronectin-coated six-well plates in the presence of 8 µg/ml polybrene, subsequent to spinoculation at 800 x g for 30 min, at 32°C. Since the lentiviral shRNA particles also encode a puromycin resistance gene for transduction selection, the cells were then washed and grown in culture media containing 2 µg/ml puromycin dihydrochloride (Sigma-Aldrich) for an additional 72 h. The cells were left to recover and proliferate for at least 2 days prior to undergoing any experimental procedure.

Results

Activation of PI3K/Akt/mTOR signaling in MCC. Common tumor suppressor genes and oncogenes, including p53, PTEN, Rb, Ras and B-RAF, are less frequently mutated in MCC in comparison with other skin tumors (14). Growth receptor pathways, such as c-kit, vascular endothelial growth factor and platelet-derived growth factor, are also not highly activated in MCC (14). Previously, mTOR upregulation was found in MCC expressing small T antigens (15). Therefore, the activation of PI3K/Akt/mTOR signaling was investigated in 22 primary human MCC tissue samples prior to examining the effect of the dual PI3K/mTOR inhibitor in MCC cells. Out of the 22 MCC samples tested, 20 out of 22 (90%) were positive for p-mTOR Ser2448, and 19 out of 22 revealed positive staining of p-Akt (Ser473). The findings of the immunohistochemical analysis from a representative sample are revealed in Fig. 1A. In addition, the activation of PI3K/Akt/mTOR signaling was investigated in 5 primary human MCC tissue samples randomly selected by immunoblotting (Fig. 1B), and also confirmed the upregulation of p-mTOR and p-Akt in MCC.

NVP-BEZ235 inhibits cell proliferation and attenuates PI3K/Akt/mTOR signaling in MKL-1 cells. NVP-BEZ235 is an orally bioavailable imidazoquinoline that inhibits PI3K and mTOR kinase activity and has been revealed to inhibit tumor growth in numerous preclinical models. To investigate the *in vitro* effect of NVP-BEZ235 on the growth of MCC, MKL-1 cells were treated with increasing concentrations of NVP-BEZ235 for 12, 24, 48 and 72 h, respectively, and cell viability was analyzed using the CCK-8 assay. As shown in Fig. 2A, treatment with NVP-BEZ235 reduced the viability of MKL-1 cells in a time- and concentration-dependent manner. The ability of NVP-BEZ235 to modulate the signaling pathway

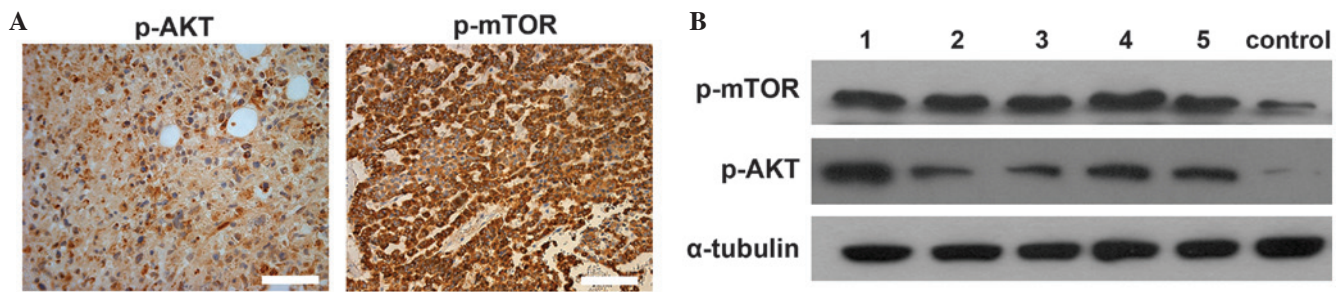


Figure 1. Activation of PI3K/Akt/mTOR signaling in MCC tissues (A) Representative positive immunohistochemical staining of p-AKT and p-mTOR in sections from MCC tissues. Scale bar, 50 μ m. (B) Western blotting revealing the increased expression of p-Akt and p-mTOR of MCC tissue randomly selected compared with the expression in normal skin tissue. Tubulin was used as a loading control. MCC, Merkel cell carcinoma; PI3K, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin.

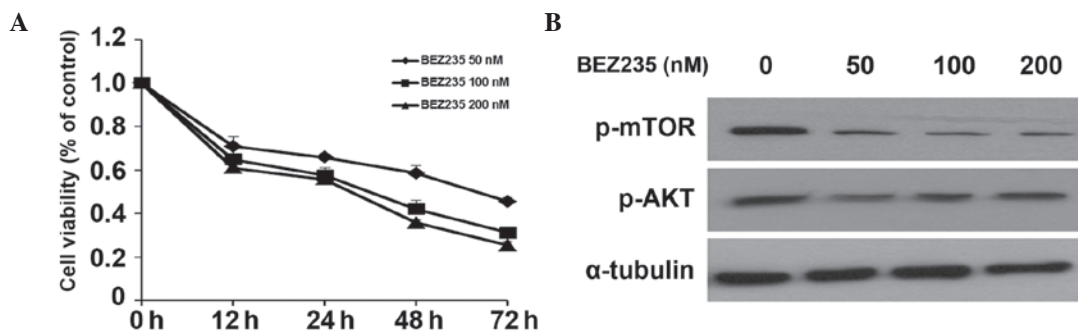


Figure 2. NVP-BEZ235 treatment inhibits cell proliferation and attenuates the activity of the PI3K/Akt/mTOR signaling pathway. (A) Findings of the cell counting kit-8 assay revealing the sensitivity of MKL-1 cells to NVP-BEZ235. The cells were cultured with NVP-BEZ235 (0-200 nM) for 12, 24, 48 and 72 h at each concentration. The average proliferation values of the control untreated samples were considered to be 100%. The data points represent the mean of triplicate results. (B) Western blot analysis revealing the decreased expression of p-mTOR and p-Akt of MKL-1 cells treated with NVP-BEZ235 for 24 h at the indicated concentrations. Tubulin was used as a loading control. PI3K, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin.

in MKL-1 cells was then assessed by western blotting. As expected, NVP-BEZ235 markedly decreased the levels of Akt and mTOR phosphorylation in a dose-dependent manner in the assessed MKL-1 cells (Fig. 2B), confirming the effect of NVP-BEZ235 on MCC.

NVP-BEZ235 induces cell cycle arrest, but not apoptosis. To further gain insight into the mechanisms of growth inhibition exerted by NVP-BEZ235, the effect of this agent on the cell cycle and apoptosis in MKL-1 cells was analyzed by flow cytometry. Consistent with the anti-proliferative effects of NVP-BEZ235, a pronounced decrease of cells in the S phase and a concomitant increase in cells in the G0/G1 phase were observed in the treated groups compared with the control group (Fig. 3A), indicating cell cycle arrest in the G0/G1 phase. Notably, NVP-BEZ235 did not induce apoptosis in MKL-1 cells, which was further confirmed by the absence of caspase-3 cleavage and activation (Fig. 3B and C). Collectively, these data indicated that NVP-BEZ235 induced G0/G1 cell cycle arrest, but not apoptosis, in MKL-1 cells.

Cell cycle arrest induced by NVP-BEZ235 is mainly dependent on p21 and p27 upregulation. To explore whether cell cycle regulatory proteins were involved in the cell cycle arrest of MKL-1 cells, the cells were treated with various concentrations of NVP-BEZ235 and cell lysates were subjected to western blot analysis. As revealed in Fig. 3D, downregulation of the cell cycle promoter cyclin D1 and upregulation of

the negative cell cycle regulators p21 and p27 were detected subsequent to NVP-BEZ235 treatment in MKL-1 cells. The MKL-1 cells were therefore transfected with shRNA targeting either p21 or p27 (Fig. 4A). As shown in Fig. 4B, knockdown of p21 or p27 expression partially rescued NVP-BEZ235-induced cell cycle arrest to a similar degree, which indicates that NVP-BEZ235-induced suppression of proliferation mainly results from the upregulation of p21 and p27.

Discussion

In the present study, the efficacy of NVP-BEZ235 as a potential therapeutic inhibitor of the PI3K/Akt/mTOR pathway was demonstrated in the human MCC MKL-1 cells. The results of the present analysis demonstrated that NVP-BEZ235 was effective in inhibiting proliferation and inducing cell cycle arrest in MKL-1 cells. Additional investigation revealed that NVP-BEZ235 attenuated PI3K/Akt/mTOR signaling and upregulated the expression of p21 and p27. Overall, these results have significant implications for the future development of dual PI3K/mTOR inhibitors as potential agents to treat human MCC.

Deregulation of the PI3K/Akt/mTOR pathway is a common feature of numerous human cancers and contributes to cancer cell survival, promotes resistance to chemotherapy and radiotherapy through the disruption of apoptosis, and initiates cap-dependent translation of mRNA, which is essential for cell cycle progression, differentiation and growth (16-18). By employing the human MCC samples, the present study also

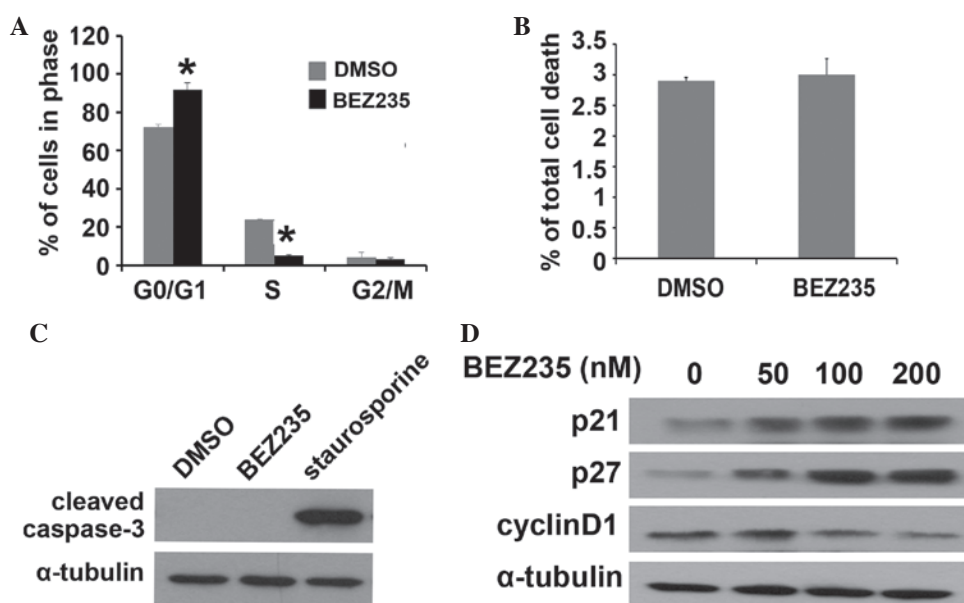


Figure 3. NVP-BEZ235 induces cell cycle arrest, but not apoptosis, in MKL-1 cells. (A) The proportion cells in each cell cycle phase in MKL-1 cells. The cells were treated with 100 nM NVP-BEZ235 for 24 h and stained with bromodeoxyuridine and 7-aminoactinomycin D followed by flow cytometry analysis. (B) Total proportion of Annexin V-positive cells subsequent to NVP-BEZ235 treatment. The cells were treated with 100 nM NVP-BEZ235 for 24 h and then stained with propidium iodide and Annexin V followed by flow analysis. (C) Western blot analysis revealing the negative expression of cleaved caspase-3 in MKL-1 cells treated with NVP-BEZ235. Staurosporine was used as a positive control. (D) Western blot analysis revealing the decreased expression of cyclin D1 and increased expression of p21 and p27 in MKL-1 cells treated with NVP-BEZ235 for 24 h at the indicated concentrations. The data are presented as the mean \pm standard error of mean of triplicate experiments. * $P < 0.05$ compared with DMSO-treated cells. DMSO, dimethyl sulfoxide.

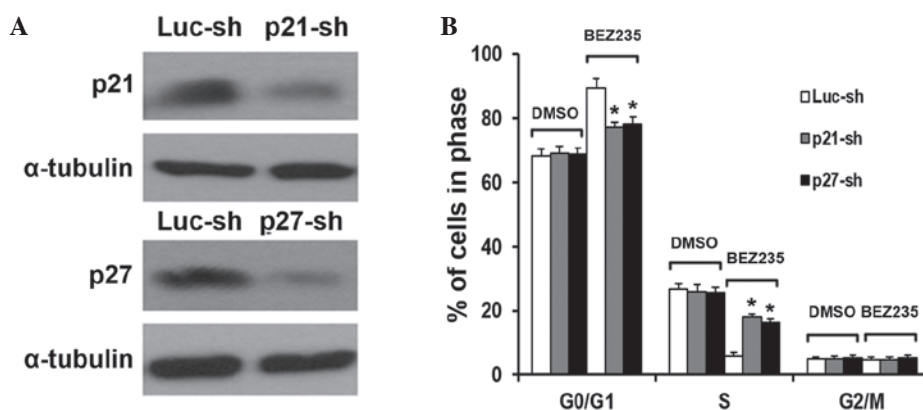


Figure 4. NVP-BEZ235-induced cell cycle arrest depends on the upregulation of p21 and p27. (A) Western blotting revealing the successful knockdown of p21 and p27 expression by shRNA in transfected MKL-1 cells. (B) Flow cytometry revealed a significant reduction in G0/G1 cycle arrest when cells were transfected with p21-targeting shRNA or p27-targeting shRNA compared with control shRNA (* $P < 0.05$). The data are presented as the mean \pm standard error of mean of triplicate experiments. * $P < 0.05$ compared with DMSO-treated control shRNA transfected cells. shRNA, short hairpin RNA; DMSO, dimethyl sulfoxide.

confirmed the activation of PI3K/Akt/mTOR signaling in MCC, which was consistent with previous results (8). Consequently, it is reasonable to assume that targeting PI3K/Akt/mTOR may effectively contribute to the treatment of MCC through the hyperactivation of PI3K/Akt/mTOR signaling.

NVP-BEZ235 is a dual class I PI3K/mTOR small molecule inhibitor that has exhibited promising anti-proliferative activity in a variety of tumor cell lines and several solid cancers (10). In contrast to the limited response to single PI3K and mTOR inhibitors, NVP-BEZ235 has been demonstrated as effective even in tumors harboring abnormal PI3K signaling (19). The present *in vitro* study revealed that NVP-BEZ235 not only downregulated mTOR activity, as indicated by the

dephosphorylation of Ser2448 p-mTOR, but also decreased Akt activity, as indicated by the decreased phosphorylation of Ser473 p-Akt. Depending on the tumor cell type, inhibitors of PI3K/Akt/mTOR signaling have been revealed to function as inhibitors of cell proliferation, by leading to cell cycle arrest, and also as cytotoxic agents, by inducing apoptosis (20-22). Using the human MCC MKL-1 cell line, it was revealed that NVP-BEZ235 demonstrated cytostatic action and led to the accumulation of the cells almost exclusively in the G1 phase. By contrast, no apoptosis was identified through the assessment of cleaved caspase 3 in the MKL-1 cell model.

Previous studies have demonstrated that dual PI3K/mTOR inhibitors are active in preclinical models of leukemia,

lymphoma and solid tumors (23-25). Although the induction of cell cycle arrest by these dual PI3K/mTOR inhibitors has been reported, the mechanism underlying this effect remains unclear. Cell cycle arrest is associated with decreased Rb phosphorylation and subsequent E2F-associated transcription (26). During cell cycle progression, Rb is phosphorylated by cyclin/cyclin-dependent kinase (Cdk) complexes, such as the cyclin D1-3/Cdk4/6 and cyclin E/Cdk2 complexes (27,28). The administration of NVP-BEZ235 in the present study suppressed the expression of cyclin D1 in the MKL-1 cells. By contrast, NVP-BEZ235 treatment increased the protein levels of the cell cycle inhibitors p21 and p27. As a PI3K/mTOR inhibitor, NVP-BEZ235 strongly blocks Akt kinase activity, which results in the aforementioned Akt function becoming inefficient, and thereby induces cell cycle arrest in the G1 phase (10). In addition, NVP-BEZ235 has also been associated with the positive regulation of mid- and late-G1 phase cyclin/Cdk activity through the phosphorylation and inactivation of the Cdk inhibitor p21 in MKL-1 cells (29,30). Overall, the decrease in cyclin D1 and increase in p21 and p27 levels may contribute to the G1 cell cycle arrest observed in NVP-BEZ235-treated MKL-1 cells.

In summary, the present results provide the first demonstration of the anti-proliferative effects and ability to arrest the cell cycle of a dual PI3K/mTOR inhibitor in an established MCC cell line. The present study established the critical role of p21 and p27 upregulation in the mechanism of cell cycle arrest in this class of drugs. The dual PI3K/mTOR inhibitor appears to require additional preclinical and possible clinical assessment for the treatment of MCC.

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