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Original Article

Effective use of PI3K inhibitor BKM120 to treat human osteosarcoma

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Abstract: Aberrant activation of the PI3K/AKT pathway is associated with chemoresistance, disease progression and unfavourable outcome in osteosarcoma (OS) patients. Therefore, inhibition of this pathway may improve therapeutic approach in OS. The aim of this study was to evaluate the effects of NVP-BKM120 (BKM120), a selective pan-class I PI3K inhibitor, on OS in vitro and vivo. BKM120 inhibited OS cell proliferation and promoted apoptosis in a dose-dependent manner. In addition, BKM120 decreased P-AKT expression without effecting total AKT in the OS cell lines. Finally, BKM120 also suppressed tumor growth in vivo, reducing the size of OS xenografts. Taken together, the findings indicate that BKM120 has potential as a novel therapeutic agent for the treatment of OS.

Keywords: Osteosarcoma, BKM120, PI3K inhibitor

Introduction

Osteosarcoma (OS) is the most common primary bone malignancy that occurs predominantly in children and adolescents [1]. Pulmonary metastases and local non-resectable recurrence are common in OS patients, and approximately 30% of patients with OS has detectable clinically metastatic disease at the time of diagnosis [2, 3]. Despite recent the development of different regimens of multi-drug chemotherapy combined with surgical resection over the past 30 years, the five-year survival rate of OS patients remains around 60-70% according to published series [4, 5]. Further, constitutive and acquires resistance to current chemotherapy agents also affect prognosis of OS patients [6]. Therefore, this is great interest in researching novel therapeutic agents to further improve the prognosis of OS patients.

The phosphatidylinositol 3-kinase enzyme/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway plays a critical role in maintaining cell growth, proliferation, differentiation, and survival, and is frequently dysregulated in cancer [7]. PI3K-activated Akt can modulate cellular functions such as cell proliferation, differentiation, survival and motility, all of which might seriously influence cancer progression [8, 9]. It is well confirmed that the pathway is one of the most important oncogenic pathways in OS [10, 11]. In addition, our previous study also found that stromal cell derived factor 1 (SDF-1) activates the PI3K/AKT pathway to promote OS cells proliferation and migration [12].

Consequently, inhibition of PI3K/AKT pathway may be useful in inhibiting OS growth. NVP-BKM120 (BKM120) is a synthetic small molecules inhibitor that acts as a potent selective pan-class I PI3K inhibitor and inhibits the catalytic subunit p110α of PI3K by competitive binding of the lipid kinase domain on its ATP binding site [13]. It has been described that BKM120 inhibition of the PI3K pathway leads to anti-proliferative and proapoptotic effects in many cell lines from different tumor types involving PI3K pathway (breast, glioblastoma, ovary, prostate) [14, 15]. In current study, we have examined the effect of BKM120 on SOSP-9607/MG63 proliferation and apoptosis in vitro

and on OS tumour growth in vivo using the SOSP-9607 mouse model.

Materials and methods

Materials

RPMI-1640 medium was purchased from HyClone (Thermo Scientific, Waltham, MA). Immobilon Western reagents, polyvinylidene fluoride membrane and enhanced chemiluminescence reagents were from Millipore Corporation (Billerica, MA). Monoclonal antibodies against p-AKT and AKT were purchased from Signalway Antibody LLC (Israel). BKM120 was purchased from Selleck (Shang Hai, China), and dissolved in DMSO (Sigma-Aldrich, St Louis, MO, USA) at a concentration of 10 mM as a stock solution and used by diluting in RPMI-1640 to give a final concentration. Peroxidase-conjugated goat anti-rabbit immunoglobulin G and monoclonal antibodies against b-actin were purchased from Bioworld Technology (Minnesota, MN).

Cell culture

Human OS cells SOSP-9607 were established and maintained in our laboratory as previously described [16], and they were grown in RPMI 1640 (HyClone, Logan, UT, USA) supplemented with 10% fetalbovine serum, penicillin (100 U/mL), streptomycin(100 lg/mL), and glutamine (2 mM) at 37°C in 5% CO_2 and 95% air. The MG63 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA) and placed in a 5% CO_2 incubator. The cells were harvested for subsequent experiments after incubation for the desired period.

Cell proliferation

Cultured cells were seeded into 96-well plates (Corning, NY) and incubated for 24 h. Then, they were treated with different concentrations (0-10 μM) of BKM120 for 24, 48 and 72 h. Cell viability was examined at the indicated time points using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The absorbance of each well was measured at 492 nm after incubation with 20 μl of MTT for 4 h at 37°C. The curve of cell growth inhibition was then drawn and growth inhibition efficiency was examined. Each experiment was performed in triplicate and the results were given as means ± SD.

Colony formation assay

Cells (200 cells per well) were seeded into 6-well plates and were treated with different concentrations (0, 0.1, 1, and 10 μM) of BKM-120 for 14 days in a 5% CO_2 incubator at 37°C. Colony cells were fixed in methanol for 10 min. Then, colonies were stained with 0.5% crystal violet for 10 min. All visible Colony cells were quantified. The experiment was performed three times independently.

Apoptosis analysis

Cells were cultured in the presence of BKM120 (0, 0.1, 1, and 10 μM) for 72 h and stained with Annexin V-FITC and propidium iodide (Beyotime) for 15 min in darkness. Then, the stained cells were analyzed immediately by flow cytometry (BD Biosciences) as the manufacturer's protocol.

Cell cycle analysis

Cells were cultured in the presence of BKM120 (0, 0.1, 1, and 10 μM) for 72 h and fixed with 70% cold ethanol for 1 h. Then, cells were stained with propidium iodide (PI) as per manufacturer's instructions. The cell cycle distribution was analyzed by flow cytometry (BD Biosciences) at 488 nm.

Western blot analysis

Cellular lysates were prepared using prechilled lysis buffer (1% Triton X-100, 50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L ethylenediaminetetraacetic acid, 150 mmol/L NaCl, 2 mmol/L phenylmethanesulfonylfluoride, and 1 mmol/L sodium orthovanadate). The lysates were clarified by centrifugation at 12,000 rpm for 20 min and resolved by sodium dodecyl sulfate-denatured polyacrylamide gelelectrophoresis. The immunoblotting procedure was performed as previously described [17].

In vivo xenograft study

This study was approved by the Animal Ethics Committee of Hong Hui Hospital, Xi'an Jiaotong University College of Medicine. Four-week-old female BALB/c nude mice were purchased from the Experimental Animal Centre of the Fourth

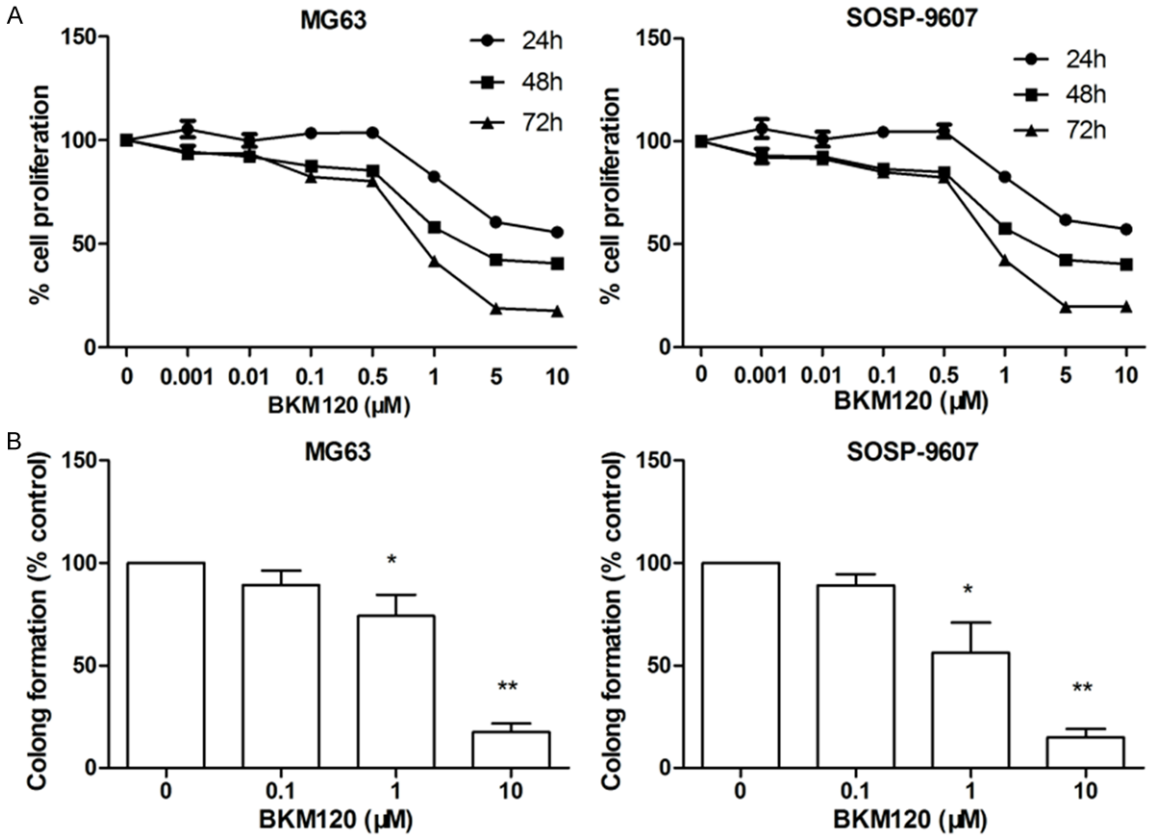

Figure 1. BKM120 inhibits cell proliferation and colony formation of in both MG63 and SOSP-9607 cell lines in a dose-dependent manner. (A) Cell proliferation and (B) colony formation were examined after treatment with different concentrations of BKM120. *P<0.05, **P<0.01 vs. control (untreated group).

Military Medical University (Xi'an, China), and maintained under specific pathogen-free condition.

Mice were implanted subcutaneously with 1×10^6 SOSP-9607 cells in 100 μl. When tumors reached a size of 100 mm^3, the xenografted nude mouse were randomly divided into control (vehicle) and BKM120 (50 mg/kg) groups. BKM120 was intraperitoneally administered three times a week for 6 weeks. The length (L) and width (W) of tumor dimensions were measured once a week with calipers, and the volume (mm^3) was calculated according to the formula: V=(L×W2)/2. The mouse were sacrificed at 42 days after inoculation, and tumors were harvested and weighed. P-Akt and Akt expression levels in the tumors tissues were tested using Western blot. Finally, tumor tissues were fixed with 10% formalin, embedded in paraffin wax and stained with H&E. Histopathological evaluation of OS was performed as previously described [18].

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA) and represented as mean ± standard deviation. Two-tailed Student's t-test was employed for analyzing both in vitro and in vivo data. The AKT and P-AKT Western blot were analyzed with a one-way ANOVA test. Two-way ANOVA was applied to analyze tumor growth. In each case P<0.05 was considered statistically significant. In each case P<0.05 was considered statistically significant.

Results

BKM120 inhibits cell proliferation, colony formation in OS cells

The results of MTT assay showed that BKM120 inhibited the cell proliferation of MG63 cells in a dose-dependent and time-dependent manner, with an IC50 of 0.947 μM BKM120 at day

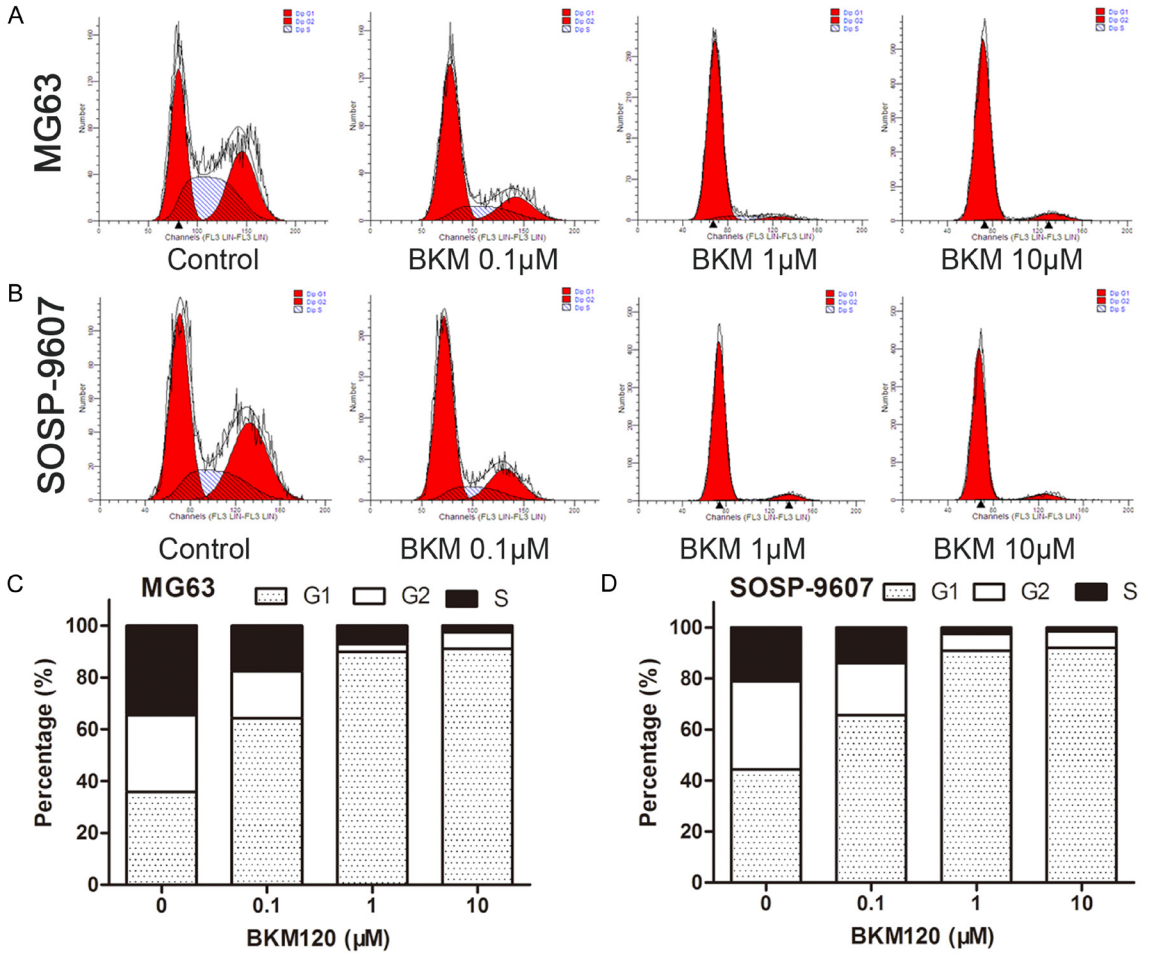

Figure 2. BKM120 inhibits cell cycle progression in both MG63 and SOSP-9607 cells in a dose-dependent manner. Cell cycle distribution of treated MG63 (A and C) and SOSP-9607 (B and D) was determined by flow cytometric analysis.

3 (**Figure 1A**). And, BKM120 inhibited the cell proliferation of SOSP-9607 cells in a dose-dependent and time-dependent manner, with an IC50 of 1.238 μM BKM120 at day 3 (**Figure 1A**). The inhibition ratio of 1 μM BKM120 at day 1, 2, 3 was 17.6%, 42.2% and 58.3% in MG63 cells (**Figure 1A**). The inhibition ratio of 1 μM BKM120 at day 1, 2, 3 was 17.3%, 42.3% and 57.7% in SOSP-9607 cells (**Figure 1A**).

The effects of BKM120 on the cell colony formation of MG63 and SOPS-9607 cells were also determined. The results of colony assay showed that BKM120 obviously decreased the clonogenicity of OS cells (MG63 and SOPS-9607) in a dose-dependent manner ($P<0.05$) (**Figure 1B**).

To investigate the effect of BKM120 on cell cycle distribution, BKM120-treated MG63 and SOPS-9607 cells were stained with PI after 72 h of culture. Flow cytometry investigations revealed that BKM120 increased cell numbers in the G0/G1 phase in a dose-dependent manner (**Figure 2**). These data revealed that BKM120 could inhibit the proliferation in both MG63 and SOPS-9607 cells.

BKM120 induces apoptosis

Apoptosis in the MG63 cells were detected using flow cytometry. The result showed that BKM120 significantly increased apoptosis in the MG63 and SOSP-9607 cells in a dose-dependent manner (**Figure 3**, $P<0.05$).

BKM120 inhibits PI3K signalling in OS cell lines

The regulation of protein levels by BKM120 were detected using Western bolt analysis. To

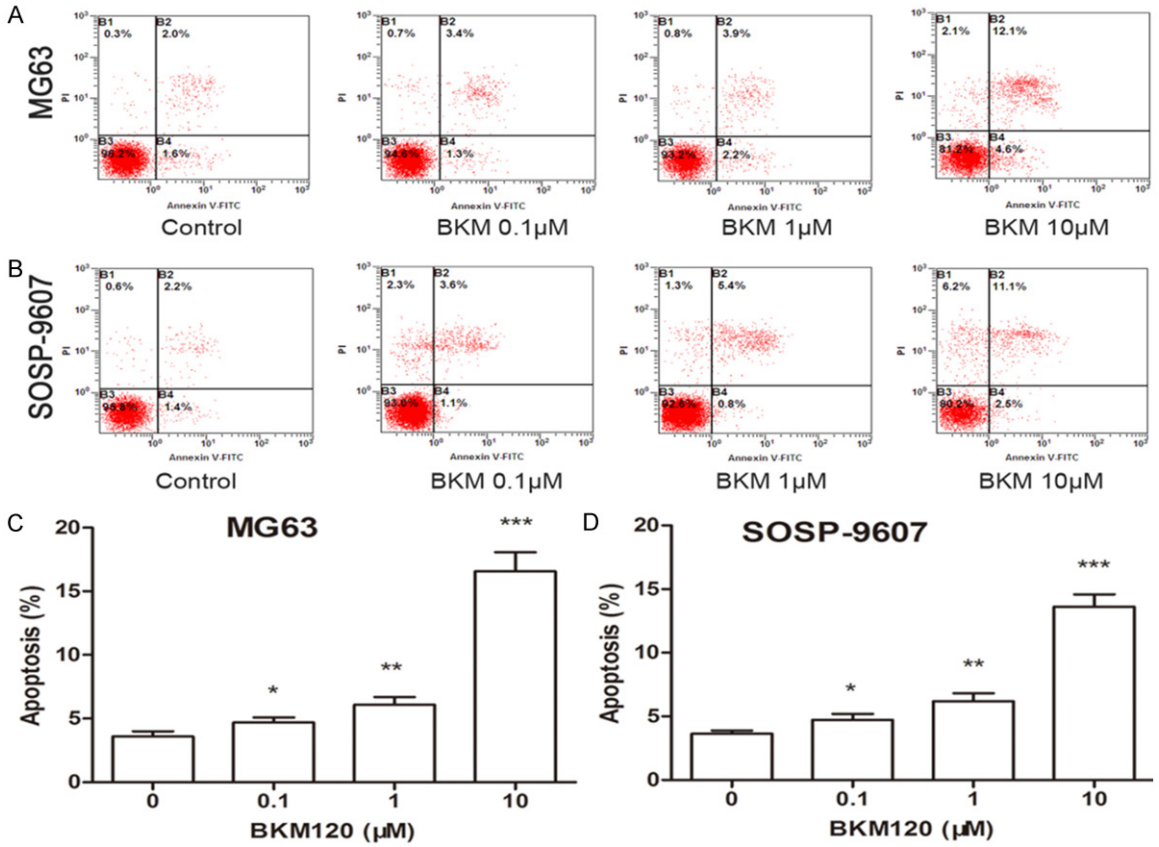

Figure 3. BKM120 induces apoptosis in both MG63 and SOSP-9607 cells in a dose-dependent manner. Cell apoptosis of MG63 (A and C) and SOSP-9607 (B and D) cells was measured using flow cytometry analysis after treatment with various concentrations of BKM120 (0, 0.1, 1 and 10 μM). *P<0.05, **P<0.01, ***P<0.001 vs. control (untreated group).

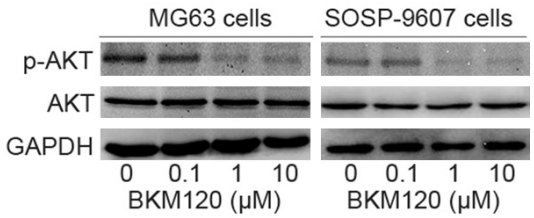

Figure 4. BKM120 affects the PI3K/AKT signaling pathway in both MG63 and SOSP-9607 cells. Expression of AKT and P-AKT proteins in MG63 and SOSP-9607 cells were determined by western blot analysis after treatment with different concentrations of BKM120 (0, 0.1, 1 and 10 μM). GAPDH was used as a control.

detect the dose-dependent effect of BKM120-induced phosphorylation of AKT, MG63 and SOSP-9607 cells were treated with BKM120 at different concentrations (0, 0.1, 1, and 10 μM). This effect showed that BKM120 decreased the phosphorylation of AKT in the MG63 and SOSP-9607 cells in a dose-dependent manner (**Figure 4**).

BKM120 inhibits tumor growth of OS in vivo

The mice were sacrificed 42 days after post-inoculation, and orthotopic tumor tissue was excised. Treatment with BKM120 obviously abolished tumor growth (**Figure 5A** and **5D**). Tumors were confirmed based on histopathological evaluation (**Figure 5B** and **5C**). BKM120 treatment markedly reduced tumor weight compared with the control (P<0.05). The mean tumors weight at study termination were 0.83 ± 0.08 and 1.64 ± 0.12 g in the BKM120 and control group (**Figure 5E**). Tumors in the BKM120 group expressed lower P-Akt levels compared with control group (**Figure 5F**) indicating that BKM120 significantly inhibited the tumor growth in vivo.

Discussion

It is well established that a disordered expression of the PI3K/AKT pathway plays an important role in cancer cell growth, proliferation, differentiation, and survival through regulating

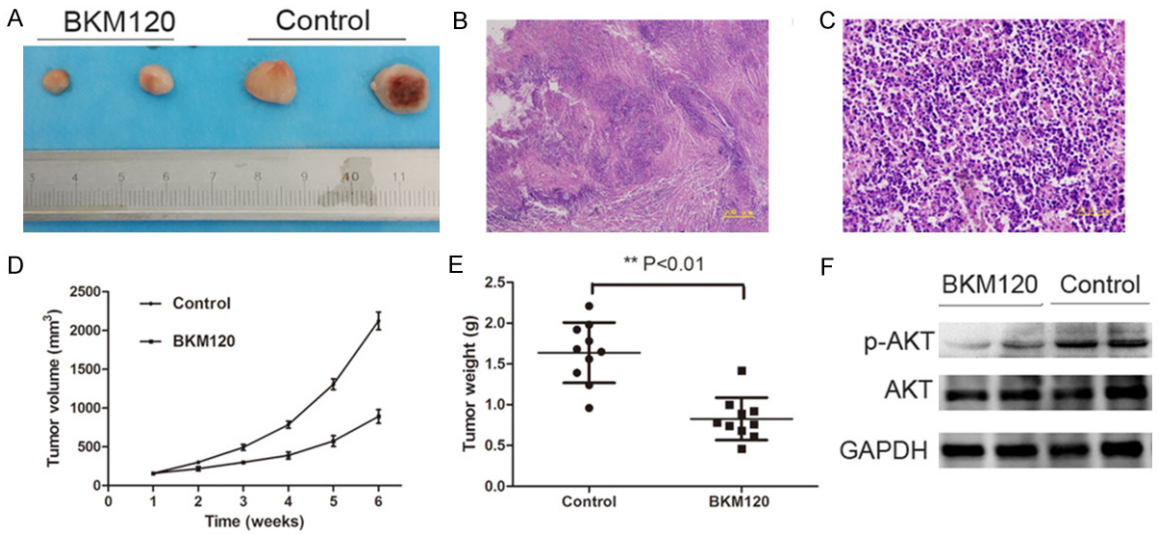

Figure 5. BKM120 inhibits the growth of SOSP-9607 OS in vivo. (A) Representative photomicrographs of tumors from the untreated (control) and BKM120 treatment group. Representative images of H&E-stained tumor tissues at a magnification of ×40 (B) and ×400 (C). (D) Tumor growth curves for SOSP-9607 tumor were measured after the inoculation. The width (W) and length (L) of tumor were measured every 7 days after the inoculation and the volume of tumor was calculated. (E) Tumor weight from untreated (control) and BKM120 treatment group. Data are presented as means ± SD. (F) Expression of AKT and P-AKT proteins in tumor tissues were determined by western blot analysis. GAPDH was used as a control. **P<0.01.

physiologic cell cycle progression, differentiation and growth. In years past, many studies have shown aberrant activation of the PI3K/AKT pathway in different malignant tumor, including OS, contributes to tumorigenesis and development [19-21]. Furthermore, several studies have demonstrated that the activation of this axis plays an important role in poor prognosis and chemoresistance of OS [22].

In the present study, the efficacy of the PI3K inhibitor BKM120 as a potential therapeutic inhibitor of the PI3K/AKT pathway was demonstrated in the human OS. Previous studies in many tumors have shown that BKM120 can inhibit cellular proliferation and cell cycle progression through selectively inhibiting the PI3K/AKT pathway [23, 24]. In the present study, the cytotoxic potential of BKM120 was examined in MG63 and SOSP-9607 OS cells, and the results demonstrated that BKM120 significantly inhibited cellular proliferation and cell cycle progression in a dose-dependent manner. In some previous reports, BKM120 induced cytotoxicity results in a moderate amount of cell cycle arrest in the G2-M phase [25, 26]. However, other studies showed that BKM120 can led to substantial accumulation in the G1 phase of the cell cycle [27]. In our study, we found that BKM120 treatment resulted in increase of cells in the G1 phase of the cell cycle in MG63 and SOSP-9607 cells. These discrepancies may be because 1) these two types of regulation co-exist in cells; 2) a different cell line was used for the study. Accordingly to previous reports, the PI3K/AKT pathway plays important roles in apoptosis [24, 28]. BKM120-induced cell death is mediated through apoptosis which is associated with the cleavage of caspases 3, 8, and 9 [25]. In the present study, we observed that BKM120 promoted cell apoptosis in MG63 and SOSP-9607 cells. To better understanding the precise mechanism of the BKM120 in OS cells, we examined the protein levels of the PI3K/AKT pathway after BKM120 treatment. The results showed that BKM120 can reverse P-AKT levels in a dose-dependent manner. Finally, in vivo administration of BKM120 to a xenotransplant mouse model of OS significantly suppressed OS growth.

So far, a lot of the PI3K/AKT inhibitors have been discovered and developed. Some drugs of targeting the PI3K signaling pathway have entered clinical trials, and recent studies has evaluated the clinical outcomes of the PI3K/AKT inhibitors [29, 30]. Yu et al. showed that

treatment of human glioma cells with NVP-BEZ235, a PI3K/AKT/mTOR inhibitor, resulted in decreases in activity in vitro and down-regulation of PI3K/AKT/mTOR pathway, and combining NVP-BEZ235 with temozolomide caused additive toxicity in vivo [31]. Recently, Alleqretti used BKM120, a selective pan-class I PI3K inhibitor, showed that treatment with BKM120 increased the cytotoxic effect of the glycolitic inhibitor dichloroacetate in acute myeloid leukemia cell lines and primary samples [32]. BKM120 has shown great future in controlling solid tumors in preclinical mouse models and to increase sensitivity to therapy drugs [33, 34]. To our study, there are several limitations to this study. Our study mainly evaluated a selective pan-class I PI3K inhibitor BKM120 alone. Second, we only examined MG63 and SOSP-9607 OS cells. Further, studies with PI3K inhibitor involving multiple cell lines and combining with other drugs are necessary to more accurately manage human OS.

Overall, the present study reveals that the aberrant activation of the PI3K/Akt signaling pathway play an important role in the OS tumorigenesis. Our findings confirm that BKM120 effectively inhibits this signaling pathway and was effective in inhibiting OS cell proliferation, inducing cell cycle arrest and inducing cell apoptosis in vitro. Moreover, the therapy of BKM120 shows an effective way to suppress tumor growth in vivo. These findings suggest that selective the PI3K/Akt signaling pathway inhibitors such as BKM120 may be a potential strategy for the treatment of OS.

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Disclosure of conflict of interest

None.

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