BI6727, a polo-like kinase 1 inhibitor with promising efficacy on Burkitt lymphoma cells

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Abstract
Objective: BI6727, an ATP-competitive PLK1 inhibitor, has been shown to cause cell death in multi-tumors. This study aimed to investigate the anti-tumor effect and potential molecular mechanism of BI6727 in human Burkitt lymphoma (BL) cell lines.
Methods: We assessed polo-like kinase 1 (PLK1) expression in BL patient tissues and cells, also investigated the cytotoxic effect using CCK8 assay and flow cytometry. In addition, western blotting and real-time polymerase chain reaction (RT-PCR) assays were used to explore the molecular mechanisms of BI6727 in human BL cell lines.
Results: PLK1 was overexpressed in BL cells compared with normal cells. The PLK1 inhibitor BI6727 reduced activated PLK1 expression and caused mitotic arrest in BL cells. Additionally, BI6727 suppressed cellular proliferation and induced apoptosis in BL cell lines. BI6727 treatment also decreased C-MYC protein and mRNA expression, blocked the PI3K/AKT/mTOR signaling pathway, and stabilized the FBXW7 protein.
Conclusions: Our findings explained a potential molecular mechanism of BI6727 in BL cells and suggested that BI6727 might be a new therapeutic agent for BL in the future.

Keywords
Polo-like kinase 1, BI6727, Burkitt lymphoma, FBXW7, C-MYC, PI3K/AKT/mTOR

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Introduction

Burkitt lymphoma (BL) is a highly aggressive B-cell non-Hodgkin lymphoma derived from germinal center B cells and is one of the fastest growing human tumors.1 It is characterized by MYC deregulation that results from chromosomal translocations to the Ig enhancer regions t(8;14)(q24;q32), t(2;8)(p12;q24), and t(8;22)(q24;q11).2 The deregulation of C-MYC is the seminal event in Burkitt lymphomagenesis. However, it requires cooperation with other lesions, including the constitutive activation of PI3K/AKT/mTOR signaling pathway that results from B-cell receptor (BCR) signaling activation, and abnormal modulation of mRNA transcription via miRNAs.3,4 Most BL patients can achieve remission with intensive treatment, but the prognosis is poor in elderly BL patients and in relapsed and refractory patients, and novel treatments are needed for these patients.5

Polo-like kinase 1 (PLK1) is a serine/threonine kinase that plays crucial roles in many stages of cell division, and it is also involved in other important functions such as DNA replication and cell survival.6 Previous studies have shown that overexpressed PLK1 is associated with tumorigenesis and poor prognosis in multi-tumors, such as non-Hodgkin’s lymphoma.7 Moreover, PLK1 is a mediator of the PI3K signaling pathway via phosphorylation of PTEN, and it promotes C-MYC stabilization,8,9 indicating that PLK1 may be a potential therapeutic target for BL treatment.

BI6727 (Volasertib) is an ATP-competitive inhibitor of PLK1 that is reported to have a very low half-maximal inhibitory concentrations (IC50) of 0.87 μmol/L and an improved pharmacokinetic (PK) profile compared with its predecessor BI2536.10 It has been reported to have antitumor efficacy on a wide range of tumors, and it was found to be well tolerated in numerous clinical trials.11,12 Recently, the Food and Drug Administration (FDA) has designated BI6727 as an orphan drug to treat acute myeloid leukemia (AML).13 However, the precise mechanism of PLK1 in BL has not been reported.

In this study, we reported the anticancer activity of BI6727 in human BL cells and its underlying mechanisms.

Materials and methods

Reagents and antibodies

BI6727 was purchased from Selleck Chemicals (Houston, TX, USA). It was dissolved in DMSO at a concentration of 10 mM and stored at −20°C. Primary antibodies against PLK1(4513), p-PLK1(Thr210) (9062), cdc25C (4688), p-cdc25C(Ser198) (9529), β-actin (3700), glyceraldehyde-3-phosphatede dehydrogenase (GAPDH) (5174), PI3K (4249), p-AKT(Ser473) (4060), mTOR (2983), p-mTOR(Ser2448) (5536), p70S6K (2708), p-p70S6K(Thr389) (9234), p-4E-BP1(Thr70) (9455), caspase-3 (9665), caspase-9 (9508), poly ADP-ribose polymerase (PARP) (9532), MCL-1 (4572), and Cyclin E1 (4129) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against FBXW7 (ab171961) and C-MYC (ab32072) were purchased from Abcam (Cambridge, UK).

Cell culture

The human BL cell lines Raji and Namalwa (both of which are EBV-positive) and normal cells (L02 and human umbilical vein endothelial cells [HUVEC]) were obtained from the American Type Culture Collection (Manassas, VA, USA). Two BL cells were cultured in RPMI-1640 culture medium (Corning, NY, USA) that was supplemented with 10% FBS (Gibco-RRL,
Grand Island, NY, USA) at 37°C in a humidified incubator with 5% CO₂.

**Immunohistochemistry staining**

Immunohistochemistry (IHC) staining was performed on clinical BL samples that were obtained from 12 BL patients, and the other experiments were performed on BL cell culture cells. The patients’ BL samples were obtained, and the medical history was collected retrospectively. The patients had provided written informed consent for future research use of the samples and clinical data when the tumor biopsies were taken. Immunohistochemistry was performed to evaluate PLK1 expression in 12 BL samples from BL patients. However, complete information was only available for 10 patients who received the initial treatment. The other two patients were transferred to another hospital after diagnosis and they were lost to follow-up and could not be included in the analysis. We analyzed the 2-year overall survival (OS) rate in the patients. This study protocol was approved by the ethics committee at Clinical Pathology Diagnosis Center of Ningbo City (2017098).

PLK1 expression in BL tissue samples was detected using IHC. Paraffin-embedded BL tissue sections (3 to 5 µm thick) were obtained. Sections were deparaffinized by three incubations in xylene for 7 minutes and hydrated using an ethanol gradient. Then, antigen retrieval was performed with 10-mM citrate buffer (pH 6.0) in a microwave oven for 20 minutes. Next, endogenous peroxidase was blocked by treatment with 3% hydrogen peroxide (20 minutes at ambient temperature) followed by blocking with 10% normal goat serum (30 minutes at ambient temperature). The samples were sequentially incubated with anti-PLK1 antibody (1:50 dilution, CST, #4513) overnight at 4°C in a humid environment, and then with streptavidin-peroxidase-labeled secondary antibodies (18 minutes at ambient temperature). Following incubation with DAB substrate kit, sections were counterstained with hematoxylin for 2 minutes. Finally, sections were dehydrated and mounted with a coverslip using Permount (Fisher Scientific, Pittsburgh, PA, USA).

**CCK8 assay**

Cell viability was determined by Cell Counting Kit 8 (Multi Sciences, Hangzhou, Zhejiang, China). Briefly, cells (1 × 10⁴ cells/well) were seeded into 96-well plates and treated with drugs for the indicated times. After incubation, we added 10 µL of CCK8 solution into each well and incubated the cells for another 3 hours. The absorbance of the reaction was measured using a 96-well plate reader (Bio-Rad, Hercules, CA, USA) at 450 nm. IC50 values (half maximal inhibitory concentration) were calculated.

**Cell apoptosis analysis**

Cell apoptosis was assessed using the Annexin V and 7-7-amino-actinomycin staining (AAD) staining kit (Multi Sciences, Hangzhou, Zhejiang, China). Cells (4 × 10⁵ cells) were collected and washed twice with cold 1× PBS after BI6727 treatment. The cells were resuspended in 100 µL of 1× binding buffer and supplemented with 5 µL of Annexin V-APC and 10 µL of 7-AAD. The cells were incubated for 15 minutes at ambient temperature in the dark, and then 400 µL of 1× binding buffer was added.

**Cell cycle analysis**

Cells (1 × 10⁶ cells) were collected and washed twice with cold 1× PBS after exposure to BI6727 of diverse doses or equal volumes of DMSO for 24 hours. Cell cycle distribution was determined by flow cytometry after fixation and incorporation of cell
Western blot analysis

Cells (1 × 10^6 cells) were collected from each sample, washed twice with 1× PBS and lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA). The samples’ protein concentrations were determined using the BCA Protein Assay Kit (Sangon Biotech, Shanghai, China). Protein lysates were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 2 hours in 5% skimmed milk in TBS-T (10 mM Tris–HCl at pH 8.0, 150 mM NaCl, 0.1%Tween-20) and then incubated with primary antibodies overnight at 4°C. Membranes were then washed three times with TBS-T buffer, incubated with anti-rabbit/mouse immunoglobulin (Ig)G antibody conjugated to horseradish peroxidase (Multi Sciences) for 2 hours at room temperature, and washed three times again with TBS-T buffer. The results were analyzed using the ECL detecting kit (Biological Industries, Cromwell, CT, USA) and ImageJ lab software (US NIH, Bethesda, MA, USA, https://imagej.nih.gov/ij/).

Real-time polymerase chain reaction

Total RNA from the BL cells was extracted using the TRIZOL Reagent (Invitrogen, CA, Carlsbad, USA). RNA quality and purity were assessed before reverse transcription. Real time-polymerase chain reaction (RT-PCR) was conducted using the iQSYBR Green Supermix and iCycler Real-time PCR Detection system (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer’s instructions. The forward primer to amplify C-MYC was 5’-TCCCTCCACTCGGAAGGAC-3’ and reverse primer was 5’-CTGGTGCATTTTCGGTTGTTG-3’. Relative mRNA expression was normalized to GAPDH expression in each treatment and untreated control. Melting curves were analyzed to validate a single PCR product of each primer, and the values for the relative quantification were calculated based on the 2^(-ΔΔct) relative expression formula.

Analysis in GEPIA

The RNA-seq data of diffuse large B cell lymphoma (DLBCL) samples were downloaded from The Cancer Genome Atlas (TCGA) database to analyze the expression of PLK1 in DLBCL based on the gene expression profiling interaction analysis (GEPIA), which is a newly developed interactive web server (http://gepia.cancer-pku.cn/) to profile gene expressions between tumor and normal tissues.

Statistical analysis

All data are presented as the mean ± standard deviation (SD) for at least three independent experiments. The IC50 was calculated using the CalcuSyn Software (Biosoft, San Diego, CA, USA). Comparisons between two groups were performed using Student’s t-test and P < 0.05 was considered to be statistically significant. For the clinical samples, the Kaplan–Meier method was used to estimate OS.

Results

Study participants

Ten patients with BL were included in the analysis of this study. The 2-year OS rate for these ten patients was analyzed. The median follow-up periods were 17.5 months (range, 3–36 months) for
patients, as shown in Table 1. The 10 BL patients included 8 men and 2 women, with a median age of 57 years (range, 16–77 years). Clinically, the common infiltration sites were the abdomen (7/10, 70%) and jaw (3/10, 30%). Based on the staging systems, five patients were at stage I and II (50%), and five patients were at stage III and IV (50%).

PLK1 expression in Burkitt lymphoma cells and clinical Burkitt lymphoma tissues

Previous studies showed that PLK1 expression is upregulated in a variety of human cancers.14 We found that PLK1 expression was significantly up-regulated in DLBCLs compared with normal lymphoid tissue (p < 0.05) based on the results derived from GEPIA (Figure 1), but PLK1 expression remains unclear in BL. To assess PLK1 expression in BL cells, western blot was performed on BL cells (Raji and Namalwa cells), with normal L02 cells and HUVECs as control groups. Among the four cell lines, BL cell lines showed significantly elevated PLK1 protein levels compared with L02 cells (p < 0.001) and HUVECs (p < 0.001), which showed low amounts (Figure 2a, b).

IHC was also performed to evaluate PLK1 expression in clinical BL samples. The results showed that the PLK1 expression rate was 80% (8/10) in BL patients, and the tumor samples exhibited strong nuclear staining for PLK1 (Figure 2c). Because two patients were lost to follow-up, we only collected information about 10 patients who received initial treatment (Table 1). There was a trend for higher 2-year OS rate (100%) in PLK1-negative BL patients compared with an OS rate of 37.5% in the PLK1-positive BL patients. However, more patients are needed to assess the validity of this observation.

BI6727 suppresses cellular proliferation in Burkitt lymphoma cells

The anti-proliferative effect of BI6727 was investigated using Raji and Namalwa cells that were treated with the indicated concentrations of BI6727 for 24 and 48 hours, and they were assessed using the CCK-8 assay. BI6727 inhibited BL cell proliferation in a dose- and time-dependent manner, as
shown in Figure 3a and b. The half maximal inhibitory concentration (IC50) values of BI6727 in Raji and Namalwa cells were 9.485 nM and 5.276 nM at 48 hours, respectively.

**Bi6727 induced G2/M arrest in Burkitt lymphoma cells**

Cell cycle progression is partially responsible for the cell proliferation. To further determine whether BI6727 has an effects on the cell cycle in the BL cells, we analyzed the cell-cycle distribution of Raji and Namalwa cells that were treated with BI6727 using flow cytometry. As shown in Figure 4a and b, BI6727 can dramatically inhibit the cell cycle on the G2/M phase. The arrest rates in Raji and Namalwa cells were 89.77% and 85.6%, respectively, at a concentration of 40 nM for 24 hours. In addition, treatment with BI6727 caused an increase in the sub-G0 phase on Namalwa cells, which indicates apoptosis, but this was not observed for Raji cells, which revealed that Namalwa cells might be more sensitive to BI6727 compared with Raji cells. To further explore the potential molecular mechanism induced by BI6727 on G2/M arrest, we analyzed the expression of PLK1, p-PLK1, and the proteins involved in the G2/M checkpoint, including Cdc25C and p-Cdc25C. Consistent with the results of the cell cycle assay, we detected decreased PLK1 and p-PLK1 expression in treated BL cells at 24 hours and reduced Cdc25C and p-Cdc25C expression at 48 hours (Figure 4c and d). These results suggested that the effects of BI6727 on suppressing human BL cell proliferation are partially dependent on the arrest of G2/M phase transition.

**Bi6727 promotes Burkitt lymphoma cells apoptosis**

To explore the underlying mechanism of BI6727-induced cell death in BL cells, we first investigated the effect of BI6727 on apoptosis induction by Annexin V/7AAD staining. After treatment with various BI6727 concentrations for 48 hours, the proportions of apoptotic cells significantly increased compared with baseline (Figure 5a and b). At 40 nmol/L, BI6727, significantly induced apoptosis in Namalwa cells (P < 0.0001) and in Raji cells (P < 0.001). Additionally, the activation of relative apoptotic proteins, such as caspase-3, caspase-9, and PARP were detected by
western blot and results are shown in Figure 5c. Cleaved caspase-3, caspase-9, and PARP were easily observed with increasing doses after treating BI6727 for 24 hours. All these data confirmed that BI6727 can induce apoptosis in BL cells.

**BI6727 down-regulates PLK1 leading to C-MYC reduction in BL cells**

We then evaluated whether BI6727 influences MYC expression in BL. In our results, exposure of two BL cell lines to BI6727 for 24 hours resulted in a reduction in the protein expression level in Raji cells (P < 0.001) and Namalwa cells (P < 0.0001), and decreased expression observed on mRNA expression level was treated for 48 hours (P < 0.05) (Figure 6).

**BI6727 affects FBXW7 and the PI3K/AKT/mTOR signaling pathway in BL cells**

We next investigated the effects of BI6727 on the PI3K/AKT/mTOR signaling pathways in BL cells. As shown in Figure 7, PI3K, p-AKT(Ser473), mTOR, p-mTOR (Ser2448), p-p70S6K(Thr389), and p-4E-BP1 (Thr70) were downregulated, which suggests that activation of PI3K/AKT/mTOR signaling pathway was intrinsically involved in BI6727-induced BL cell death.
PLK1 also promotes degradation of the E3 ubiquitin-ligase FBXW7, which targets many oncoproteins for ubiquitination and degradation and acts as a tumor suppressor. Thus, we next assessed whether FBXW7 was involved in the BI6727 induced signaling cascade. As we expected, inhibition of PLK1 activity with BI6727 significantly increased the FBXW7 protein expression and the accompanying degradation of FBXW7 downstream targets, such as cyclinE1 and Mcl-1 in Raji and Namalwa cells. mTOR is also targeted for ubiquitination and consequent degradation by directly binding to the FBXW7. Thus, BI6727 might suppress mTOR activity through both the PI3K/AKT and FBXW7 pathways.

**Discussion**

Despite remarkable progress that has been made in the pathobiology and management of BL, it is still incurable, especially for older patients. Therefore, novel therapeutic strategies are required for improved outcomes and reduced therapy-related toxicity.

PLK1 plays a critical role in regulating numerous stages of mitosis, and its overexpression is associated with cancer progression in many tumors including non-Hodgkin’s lymphoma. We analyzed the PLK1 expression level in DLBCL and normal lymphoid tissues by referring to the RNA-seq data that were available from the TCGA database, and we found that PLK1 expression is significantly upregulated in DLBCL, which is consistent with previous reports. In our study, 8 out of 10 BL samples showed high PLK1 expression by IHC and elevated PLK1 levels were observed in the two BL cell lines compared with normal cells. In future studies, we will obtain cells from more BL patients and their data for retrospective analysis.

BI6727 is an ATP-competitive kinase inhibitor of PLK1 that has shown clinical efficacy in multiple tumor types as a monotherapy or in combination with other anticancer therapies. Here, we researched the role of the PLK1 inhibitor BI6727 in BL cells for the first time. We confirmed that BI6727 suppressed cellular proliferation in two BL cell lines in a dose and time-dependent manner.
The G2/M transition was strongly regulated by the Cyclin B1/CDK1 complex, which is necessary for cell-cycle progression. The complex is kept inactive through phosphorylation of CDK1 (Tyr-15), which triggers G2/M cell cycle arrest. Recent studies have demonstrated that active PLK1 phosphorylates Cdc25C on Ser198 during G2/M phase, which is required to promote nuclear translocation of Cdc25C during prophase and control cyclin-dependent kinase 1 (CDK1).

Figure 4. G2/M cell cycle arrest in BL cells after BI 6727 treatment. (a and b) Raji and Namalwa cells were treated with the indicated doses of BI6727 for 24 hours and flow cytometry was performed to examine the cell cycle distribution. Percentages of cells in each cell cycle stage are presented as the averages of triplicate experiments. (c) The expression of PLK1 and phospho-Thr210 is detected by western blotting analyses after exposed to BI6727 for 24 hours in Raji cells and Namalwa cells. (d) Cdc25C and phospho-Ser198 expression was detected by western blotting analyses after exposed to BI6727 for 48 hours in Raji cells and Namalwa cells. Data represent an independent experiment from three repeated tests with similar results. BL, Burkitt lymphoma; PLK1, polo-like kinase 1.
In our study, treatment of BL cell lines with BI6727 increased the percentage of cells that are arrested in the G2/M phase. Significant decreases in p-PLK1(Thr210), cdc25C, and p-cdc25C (Ser198) were also observed after treatment with BI6727 for 48 hours. These data indicated that BI6727 resulted in G2/M cell cycle arrest in BL cells. In addition, we also demonstrated that BI6727 induced cell apoptosis in BL cells upon BI6727 treatment. (a and b) Raji and Namalwa cells were treated with increasing doses of BI6727 for 48 hours and flow cytometry was performed to examine the apoptotic cells. (c) Raji and Namalwa cells treated with BI6727 at the indicated doses for 24 hours were analyzed by western blotting with antibodies against caspase-3, caspase-9, and poly ADP-ribose polymerase (PARP). β-actin was used as a loading control. Data represent an independent experiment from three repeated tests with similar results.

BL, Burkitt lymphoma.
BL cell line apoptosis in a dose-dependent manner. An increase in cleavage of caspase-3 and -9 and cleavage of PARP after treatment with BI6727 were detected by western blot. Thus, BI6727 induced BL apoptosis via caspase-dependent pathways.

The C-MYC gene usually acts as an oncogenic transcription factor that regulates multiple cellular processes that are involved in cell growth, proliferation, apoptosis, and cellular metabolism. Deregulated C-MYC expression could drive the BL pathobiology. Numerous studies have shown that C-MYC inhibition exerts antitumor activity in BL and, thus, C-MYC is regarded as a promising target for the treatment of BL.

Figure 6. BI6727 inhibits C-MYC in both protein and mRNA levels. (a) Western blot indicated the protein expression of C-MYC in BL cells after treatment with BI6727 for 24 hours. GAPDH was used as a loading control. (b) Relative expression level of C-MYC protein in BL cells upon BI6727 treatment. (c) qRT-PCR was performed to evaluate C-MYC mRNA expression in BL cells upon BI6727 treatment for 48 hours. Data represent an independent experiment from three repeated tests with similar results. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
BL, Burkitt lymphoma; GAPDH, glyceraldehyde-3-phosphatedehydrogenase; qRT-PCR, quantitative realtime-polymerase chain reaction.
therapeutic target. In our study, we found that BI6727 could decrease C-MYC mRNA and protein expression in BL cell lines. However, deregulation of C-MYC alone is not sufficient for BL pathogenesis.

Recently, Sander et al. showed the synergy between PI3K signaling and MYC in Burkitt lymphomagenesis, and the PI3K/AKT/mTOR pathway is thought to play a role in BL pathogenesis. In this study, PI3K-subunit p110α is inhibited in BL cell lines after treatment with BI6727.

mTOR is a crucial downstream protein in the PI3K/AKT pathway, and it is a central regulator of cell survival, proliferation, and metabolism. It acts in two distinct complexes, mTORC1 and mTORC2. mTORC2 activation compensates for mTORC1 inhibition via Akt phosphorylation at Ser473. Our study showed that BI6727 led to dephosphorylation of mTOR on Ser2448, S6 on Thr389 and 4E-BP1 on Thr70, suggesting that BI6727 decreased mTORC1 activity. Moreover, BI6727 also successfully inhibited phosphorylation of AKT at Ser473, a direct substrate of mTORC2, indicating that BI6727 could suppress mTORC2-AKT signaling to prevent further activation of mTORC1.

mTOR is regulated by the PI3K/AKT pathway, and it is targeted for FBXW7-mediated ubiquitination and degradation. As an important recognition factor of the ubiquitin–proteasome system, FBXW7 acts as an important tumor suppressor protein and controls proteasome-mediated degradation of oncoproteins such as mTOR, Cyclin E, and Mcl-1. Additionally, a previous study has established that FBXW7 is associated with prognosis on T-cell lymphoma. It has been shown that PLK1 plays a critical role in the negative regulation of FBXW7 stability. Based on the results of this study, we observed an increase in FBXW7 protein expression in two BL cell lines that were treated with BI6727. Consistent with this result, BI6727 caused potent suppression of Cyclin E and Mcl-1, as the downstream targets of FBXW7. Mcl-1 is a pro-survival member of the BCL-2 family that regulates cell apoptosis besides the caspase family, and human BL is highly dependent upon Mcl-1 for survival. Cyclin E is frequently overexpressed in malignant cells, and its deregulation leads to genomic instability and cancer. Our results indicated that FBXW7 upregulation might be an important step in treatment with BI6727 in BL cells.

In conclusion, our findings show that BI6727 suppresses cellular proliferation, blocks the cell cycle in G2/M, and induces apoptosis in two BL cells. Our results indicate that BI6727 exerts antitumor activity in BL cells by inhibiting C-MYC and PI3K/AKT/mTOR signaling pathways and by enhancing FBXW7 activity. These data suggest that BI6727 might be a promising therapeutic agent in BL.
Data availability
Original data files are available upon a reasonable request.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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