4-Chlorobenzoyl berbamine induces apoptosis and G2/M cell cycle arrest through the PI3K/Akt and NF-κB signal pathway in lymphoma cells

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Abstract. Berbamine is an herbal compound derived from Berberis amurensis, which is used in Chinese traditional medicine. However, few studies have investigated this anti-tumor effect or the underlying mechanisms of berbamine on lymphoma cells. We investigate the effect, as well as the mechanism of action, of 4-chlorobenzoyl berbamine (BBD9) on Raji, L428, Namalwa and Jurkat lymphoma cell lines. Our findings show that BBD9 inhibits cell proliferation and induces cell apoptosis in lymphoma cell lines as well as G2/M cell cycle arrest through PI3K/Akt and NF-κB signaling pathways in a caspase-dependent manner. These results may provide new insights into the treatment of lymphoma.

Introduction

Lymphoma, including both Hodgkin’s and non-Hodgkin’s lymphoma (NHL), is a common hematological malignancy. The overall cure rate is unsatisfactory and new therapeutic strategies are urgently needed. Cyclophosphamide, daunorubicin, vincristine and prednisone comprise the classical chemotherapy treatment, known as the CHOP regimen. However, recent studies revealed many new therapies for this disease. Rituximab, an anti-CD20 antibody, when used alone or in combination with systemic chemotherapy is expanding the therapeutic options for patients with B-cell lymphoma (1). New monoclonal antibodies, such as galiximab, an anti-CD80 antibody, have significant anti-tumor activity as a single agent or in combination with rituximab against various B-cell lymphoma cell lines in vitro and in vivo (2). Proteasome inhibitors and immunomodulatory drugs (IMiDs), such as bortezomib and lenalidomide, have demonstrated clinical potential for NHL (3). Additionally, autologous stem cell transplantation may cure a portion of patients (4). However, a significant number of patients either do not respond to initial therapy or relapse thereafter, which stresses the need to identify new treatment options. Therefore, advances in the treatment of lymphoma require the identification of novel therapeutic targets. Many studies have revealed that some abnormally activated signal pathways correlate with the pathogenesis or survival of lymphoma cells. Polgar et al reported that the chromosomal translocation t(2;5) (p23;q35) was associated with anaplastic large cell lymphoma (ALCL), a common non-Hodgkin’s lymphoma. When the fused gene is expressed, the 80 kDa chimeric protein activates the ‘survival’ kinase phosphatidylinositol 3-kinase (PI3K) (5). Since the PI3K/AKT pathway has been implicated in the growth and survival of hematological malignancies, inhibiting this pathway is considered a useful therapeutic approach (6). The t(14;18) is present in 85-90% of follicular lymphomas. It results in an overexpression of Bcl-2, which inhibits apoptosis and plays a role in lymphomagenesis (7). The Bcl-2 proto-oncogene was first cloned as the t(14;18) translocation breakpoint from human follicular B-cell lymphoma, and it has become apparent that many cell types express Bcl-2 as the result of transcriptional regulation. Lymphoma cells with the t(14;18) translocation also show high protein levels of nuclear factor-κB (NF-κB), which can activate Bcl-2 expression as well (8). Therefore, targeting apoptotic pathways as well as these signaling pathways could be a novel strategy for lymphoma therapy.

Berbamine is an herbal compound derived from Berberis amurensis, which is used in Chinese traditional
and NF-κB lines as well as G2/M cell cycle arrest through PI3K/Akt proliferation and induces cell apoptosis in lymphoma cells. 4-Chlorobenzoyl berbamine (BBD9) is a new derivative of berbamine synthesized for its potent anti-tumor activity. Its structure is shown in Fig. 1A. It was dissolved in dimethyl sulfoxide (DMSO) and diluted to their final desired concentrations in each culture dish.

In this study, we have investigated the effect of BBD9 on lymphoma cells. The results show that BBD9 inhibits cell proliferation and induces cell apoptosis in lymphoma cell lines as well as G2/M cell cycle arrest through PI3K/Akt and NF-κB signaling pathways in a caspase-dependent manner.

Materials and methods

Chemicals. BBD9 was a generous gift from Professor Rong-Zhen Xu (Oncology Institute of the Second Affiliated Hospital, Zhejiang University School of Medicine). The chemical structure is shown in Fig. 1A. It was dissolved in dimethyl sulfoxide (DMSO) and diluted to their final desired concentrations in each culture dish.

Cell culture. The lymphoma cell lines Raji, L428, Namalwa and Jurkat were obtained from the Institute of Cell Biology (Shanghai, China). Cell lines were cultured in RPMI-1640 (Gibco BRL, USA), 10% fetal bovine serum (FBS; Gibco BRL), 100 μg/ml streptomycin, 100 IU/ml penicillin and 2 mM glutamine (Gibco BRL) in a humidified atmosphere of 5% CO2 at 37˚C.

Cell proliferation assay. In vitro, the inhibition of cell proliferation by BBD9 was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plates (200 μl/well) at a density of ~1.5-2x10^5/ml. After exposure to various concentrations of BBD9 (~0.5-4 μg/ml) for 24, 48, and 96 h, 20 μl of MTT (5 mg/ml) was added to each well and incubated for an additional 4 h. The supernatants were discarded, 200 μl of DMSO was added to dissolve the formazan crystal and the absorbance was measured at 490 nm by a microplate reader (Model 550, Bio-Rad, USA). The experiments were run in triplicate.

Cell apoptosis assay. To quantitatively assess the rate of apoptosis, the Annexin V-FITC apoptosis detection kit (Biovision, USA) was used. Briefly, 1x10^6 Raji cells were seeded in 24-well plates and treated with a gradient of BBD9 (0, 1, 2, 4 μg/ml) for 24 h. Then, the cells were washed twice with cold PBS and resuspended in 500 μl of binding buffer containing 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI, Biovision Kit) for 15 min at room temperature in the dark. Samples were analyzed by FACScan flow cytometry (FACScan, BD, USA) and obtained data were analyzed with CellQuest software.

Cell cycle analysis. Cell cycle analysis was performed by staining the DNA with PI. About 1x10^6 cells were incubated with 2 μg/ml of BBD9 for various times (0, 4, 8, 12 and 24 h). Cells were then collected and washed with PBS and suspended in 70% ice-cold ethanol and kept overnight at 4˚C. Then, the samples were washed with PBS and incubated with 1 U/ml of RNaseA (DNase-free) followed by staining with 10 μg/ml PI for 15 min in the dark. The percentage of cells in the different phases of the cell cycle was analyzed by FACScan flow cytometry.

Western blotting. Cells were harvested and extracted with lysis buffer (Cell Signaling, USA) and incubated on ice for 30 min. The lysates were centrifuged at 12,000 x g for 10 min, after which the supernatant contained the cellular lysate. Nuclear and cytoplasmic extracts were achieved using a nuclear extraction kit (Chemicon, USA), and the procedure was done according to the manufacturer’s instructions. The cytoplasmic and the nuclear portions of the cell lysate were collected in fresh tubes respectively and stored at -20˚C.

The protein concentration in the supernatant, as well as the cytoplasmic and the nuclear portions, was determined with the classical Bradford method (14). Samples containing equivalent proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and followed by Western blotting. The gel was transferred to a PVDF membrane (Millipore, USA), blocked with TBS Tween-20 buffer containing 5% non-fat dry milk and incubated with primary antibodies overnight at 4˚C. Then, the membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibody. Protein antibody complexes were detected by enhanced chemiluminescence (ECL, Santa Cruz, USA) according to the manufacturer’s recommendations and exposed on X-ray film. Antibodies against caspase-3, caspase-8, caspase-9, the cleaved poly (ADP-ribose) polymerase (PARP), the Bcl-2 family, the IAP family, Akt and phospho-Akt (pAkt) were purchased from Cell Signaling Technology Inc., USA, β-actin and lamin B (Santa Cruz Inc., USA) were used as loading controls.

RNA extraction and quantitative real-time PCR. After exposure to BBD9 for 8 h, the cells were harvested and washed twice with PBS. Total cell RNA was extracted by TRizol (Invitrogen, USA). After adding chloroform, the mixture was centrifuged at 12,000 x g for 15 min. The supernatant containing RNA was collected and isopropanol was added for precipitating RNA. After centrifugation, the pellet containing RNA was washed by 75% ethanol and resuspended in 0.1% (v/v) DEPC water. Then 1 μg of RNA was taken and reverse transcribed to cDNA with an Invitrogen RT kit and an oligo (dT) primer. Real-time PCR was then conducted in a volume of 25 μl, which contained 1 μl of primers, 2 μl of cDNA sample, 12.5 μl of 2X SYBR pre-mix EX Taq (Takara, Japan) and 9.5 μl of double-distilled water. Samples were amplified in the IQ5™ real-time PCR system (Bio-Rad) for 40 cycles under the following conditions: denaturation for 15 sec at 95˚C, annealing and extension for 60 sec at 60˚C. The primers used for real-time PCR were: phosphatase and tensin homolog deleted on chromosome 10 (PTEN); forward 5’-GGATCAGACTGTTATGATAATG-3’, reverse 5’-TCTCCTACTGGT-3’.
TTT GTG TAC AGC-3'; c-abl (an internal control): forward 5'-CCG CTG ACC ATC AAT AAG GAA-3', reverse: 5'-GAT GTA GTT GCT TGG GAC CCA-3'. All primers were purchased from Sangon (Shanghai, China). A good linear relationship was obtained from the standard curves. ΔCt = Ct (PTEN) - Ct (c-abl). ΔCT indicates the difference in mRNA levels between the target gene and the internal control. Higher ΔCT values indicate that the mRNA level is lower.

Immunofluorescence. Cells were first harvested and suspended in PBS after 24 h of exposure to various concentrations (0-3 μg/ml) of BBD9 and then plated on glass slides for fixation by 4% paraformaldehyde for 30 min at room temperature. After several washes with PBS, samples were incubated with PBS including 0.5% Triton X-100 for 30-60 min. Then, they were washed again three times with PBS for 5-10 min each time. The slides were first incubated with 5% BSA for 30 min to block the non-specific binding. Then they were incubated with an antibody to the NF-κB P65 subunit (at a 1:50 dilution in PBS containing 1% BSA) at 4°C overnight. After being washed three times in PBS, slides were incubated with Texas Red-conjugated goat anti-rabbit antibody to detect the NF-κB antibody for 30 min in the dark at room temperature. Secondary antibody was subsequently
removed by PBS and the samples were protected from light exposure and stained with 300 nM DAPI (40-6-diamidino-2-phenylindole) for 2-5 min. After three washes in PBS, the samples were mounted with coverslips and observed using a fluorescence microscope (Axiovert 200, Carl Zeiss, Microimaging, Inc., USA).

Statistical analysis. All results are presented as the mean ± SD. Statistical significance at P<0.05 was assessed by Student’s t-test.

Results

BBD9 inhibits cell growth and induces cell apoptosis. Raji, L428, Namalwa and Jurkat lymphoma cell lines were exposed to BBD9 with concentrations ranging from 0.5-4 μg/ml for 24, 48 and 72 h. All of these lymphoma cell lines were sensitive to BBD9, and the IC50 of Raji, L428, Namalwa, Jurkat cells exposed to BBD9 for 24 h was ~1.5, 1.89, 1.83 and 1.59 μg/ml, respectively (Fig. 1B). Furthermore BBD9 inhibited cell proliferation in a dose- and time-dependent manner (Fig. 1C-E).

To study the mechanism of growth inhibition, we analyzed the percentage of apoptotic cells after 24 h treatment of BBD9 in Raji and L428 cell lines by AV-PI staining (Fig. 2A). The percentage of early apoptotic cells (AV+PI) was from 2.87±0.35 to 46.96±3.58 in Raji cells and 4.45±0.97 to 35.87±4.44 in L428 cells, which represented a statistically significant increase correlated to the increasing BBD9 concentrations compared to the control group (P<0.05). Moreover, PARP and caspase-3, caspase-8 and caspase-9 cleavage were detected in BBD9-treated Raji and L428 cells by Western blot analysis (Fig. 2B). These results suggest that BBD9 inhibits cell growth in Raji and L428 cell lines through a mechanism involving apoptosis.

The Bcl-2 family and the inhibitor of apoptosis (IAP) family were involved in BBD9-induced apoptosis. Members of the Bcl-2 family and the inhibitor of apoptosis (IAP) family regulate apoptotic conditions. Thus, the notion of whether BBD9-induced apoptosis is modulated by the expression of these pro- and anti-apoptotic proteins needed to be investigated. After a 24 h exposure to BBD9, there was a slight decrease in the level of Bcl-2 and a dose-dependent increase in the level of Bax protein (Fig. 3A), which activates caspase-9 by decreasing the Bcl-2/Bax ratio. Bak, Bim, phospho-Bad (pBad) and other regulatory proteins of the Bcl-2 family seemed to be increased, while Mcl-1 and Bcl-xl decreased after exposure...
to BBD9 (Fig. 3A). The down-regulation of members of the IAP family including XIAP, CIAP1, CIAP2 and Survivin was also observed in BBD9-treated cells (Fig. 3B).

**BBD9 induced G2/M cell cycle arrest involving cell cycle-associated proteins CDK1, cyclin A and cyclin B.** Many anti-cancer agents arrest the cell cycle at one specific cycle phase and then induce apoptosis. Fig. 4A illustrates the effect of BBD9 on the Raji cell line. The percentage of cells in the G2/M phase increased rapidly from 8.41% at 0 h to 25.22% at 24 h after treatment with 2 μg/ml BBD9 (Fig. 4A). These results demonstrate that BBD9 inhibited cell growth via inducing G2/M phase arrest.

The cell cycle is regulated by cyclin and cyclin-dependent kinase (CDK) and the activity of Cdk1-cyclin B and Cdk1-cyclin A complexes is required for entry into mitosis. In this study, the levels of the cell cycle-associated proteins were determined by Western blotting. As shown in Fig. 4B, BBD9 decreased the protein levels of CDK1, cyclin A and cyclin B in a dose-dependent manner.

**BBD9 inactivated the NF-κB pathway by inhibiting both phosphorylation of IκB (pIκB) and NF-κB nuclear translocation in Raji Cells.** Since NF-κB is associated with inflammation, proliferation, carcinogenesis and apoptosis, we postulated that BBD9 might mediate NF-κB activation. To explore this hypothesis, we examined NF-κB (P65) nuclear translocation in Raji cells. IκB associates with NF-κB in the cytoplasm; however, upon IκB phosphorylation, this association is relieved and NF-κB is released, thereby releasing it to translocate to the nucleus. Our results revealed that, following BBD9 treatment, total IκB levels in the cytoplasm remained unchanged, but the level of pIκB was decreased. The nuclear content of NF-κB was decreased after BBD9 treatment (Fig. 5A). These results suggest that BBD9 inhibited NF-κB nuclear translocation by blocking pIκB. Immunofluorescent staining revealed the intracellular distribution of the p65 subunit of NF-κB. In the treatment group with 3 μg/ml BBD9, this subunit was localized in the cytoplasm, while in the control group, it was present within the nucleus (Fig. 6).

**Discussion**

Lymphoma is a refractory malignant tumor associated with poor treatment outcomes, despite an array of available chemotherapy drugs. Although the management of lymphoma remains challenging, new therapeutic options are forthcoming. Recently, 4-chlorobenzoyl berbamine (BBD9), a novel
synthetic medicine derived from berbamine, was demonstrated to have potent anti-tumor activity (11-13). However, the effect of berbamine and especially the mechanism of action are not well understood.

In this study, we found that BBD9 inhibits cell proliferation in lymphoma cell lines in a dose- and time-dependent manner and induces G2/M cell cycle arrest. The Raji, L428, Namalwa and Jurkat lymphoma cell lines were all sensitive to BBD9 and showed the corresponding inhibition of cell proliferation. Their IC50 values were very low, which may prove beneficial for establishing an effective concentration in vivo.

Many anti-tumor agents arrest the cell cycle at a certain cycle phase, either G1/S or G2/M, and then induce apoptosis. Cell cycle check points may ensure that the cells have time to repair DNA. The entry of eukaryotic cells into mitosis is regulated by CDK activation, a process controlled at several steps including cyclin binding and CDK phosphorylation. The activated CDK1-cyclin B and CDK1-cyclin A complexes translocate into the nucleus at the G2/M checkpoint and trigger the G2/M transition so as to undergo cell proliferative processes (15). The formation of an active nuclear cyclin B1-CDK1 complex is a highly intricate procedure requiring many different levels of regulation including phosphorylation, ubiquitination and degradation, allowing for the stoichiometric regulation of cell cycle events (16). Thus, this regulatory step represents a potential target for controlling cell proliferation. Our cell cycle analysis revealed a prominent time-dependent G2/M arrest in Raji cells after their exposure to BBD9. Furthermore, Western blotting demonstrated decreases in cyclin A, cyclin B and CDK1 levels following BBD9 treatment.

Apoptosis is the most common way that anti-tumor medicine induces cell death. Our studies demonstrated that BBD9 induces apoptosis in the human lymphoma cell line,
Raji. After exposure to various concentrations of BBD9 for 24 h, the percentage of early apoptosis evidenced by AV-PI staining was gradually increased. Furthermore, incubation of lymphoma cells with 1-4 μg/ml BBD9 for 24 h resulted in caspase-3 cleavage and the appearance of PARP. Caspase-3 is the ultimate executor of the caspase family that is associated with apoptosis. Activation of caspase-3 will induce PARP cleavage, chromosomal DNA strand breakage and finally apoptosis (17). Therefore, the appearance of cleaved PARP as well as the activation of the caspase family serve as indicators that apoptosis has occurred. This means that BBD9 inhibits the proliferation of lymphoma cells at least partly via a caspase-3-dependent apoptotic mechanism. Importantly, this is consistent with previously reported data (11).

The most common apoptotic pathway is associated with the mitochondrial-mediated pathway, which was regulated by the Bcl-2 family. The proteins of the Bcl-2 family are important regulators of apoptosis and are subcategorized as either proapoptotic (Bax, pBad, Bim, Bak) or anti-apoptotic (Bcl-2, Bcl-xl, Mcl-1) proteins (18). The IAP family, which includes various members such as CIAP-1, CIAP-2, XIAP, and Survivin, may bind caspases and inhibit their activity (17). BBD9 was found to down-regulate anti-apoptotic proteins and up-regulate proapoptotic proteins. These data shed light on the proapoptotic properties of BBD9 and hence implicate this novel drug as a promising agent for the effective treatment of lymphoma.

The PI3K signaling pathway initiates a cascade of signaling events primarily via the induction of specific protein-serine/threonine kinases and leads to the suppression of apoptosis (19). Persistent activation of the PI3K/Akt pathway has been observed to be involved in multidrug resistance (MDR) in lymphoma cell lines, mantle lymphoma, diffuse large B-cell lymphoma (DLBCL) and T-cell lymphoma (20-23). Akt has been postulated to phosphorylate >9000 proteins (24). The activated Akt can transduce an anti-apoptotic signal by multiple mechanisms, including phosphorylation of downstream target proteins involved in the regulation of cell growth such as the Bcl-2 family (Bim, Bad, p21Cip1), NF-κB and the modulation of the IAP protein family such as the Foxo3a transcription factor (25,26). The PI3K/Akt pathway may also associate with the IκB kinase (IKK), which phosphorylates the inhibitory element IκB, thereby releasing NF-κB to translocate to the nucleus. Therefore, inhibition of PI3K/Akt correlates with down-regulation of NF-κB activity (20). Classical NF-κB (p50/p65 dimer) is usually kept in an inactive form in the cytoplasm by stable association with IκB. Activation of the IKK complex causes phosphorylation of the IκB, subsequent ubiquitination and proteasome degradation. This leads to the liberation and nuclear translocation of classical NF-κB followed by target gene activation (17). The constitutive activation of NF-κB, which can cause cell proliferation and resistance to apoptosis, is observed in Hodgkin/Reed-Sternberg (H-RS) cells and T-cell leukemic/lymphoma cells (27,28). In our studies, BBD9 reduced the phosphorylated IκB content, which led to IκB stabilization and consequent inhibition of NF-κB translocation. Therefore, BBD9 inhibited the proliferation of lymphoma cells, suggesting that the NF-κB signaling pathway might be a novel target for the treatment of lymphoma.

The tumor suppressor PTEN is a tumor suppressor protein whose loss of lipid phosphatase activity is associated with lymphomagenesis (29). Mutations and hemizygous deletions of PTEN have been frequently detected in non-Hodgkin's lymphoma (NHL) (30). Fridberg et al reported that loss of nuclear PTEN correlated with poor survival in cases of DLBCL (31). Many different genetic mechanisms can result in functional inactivation or silencing of PTEN (26). PTEN has been associated with apoptosis through inhibiting PI3K/Akt signaling (32). The constitutive activation of Akt correlates with the expression of the phosphorylated, inactive form of PTEN (33). We discovered increased PTEN both in protein and mRNA levels after exposure to BBD9 in Raji cell. This suggests that BBD9 may inhibit Raji cell proliferation via the PTEN/PI3K/AKT pathway.

In short, this study demonstrates that BBD9 induces significant cytotoxicity to lymphoma cell lines. The mechanisms are considered to induce apoptosis and G2/M cycle arrest. These mechanisms may be regulated by the PI3K/Akt survival pathway involving the prevention of NF-κB nuclear translocation, down-regulation of Bcl-2/Bax as well as the IAP family, and up-regulation of PTEN, triggering the activation of caspase cascades. However, understanding the potency of this effect of BBD9 on lymphoma cell lines, the underlying mechanisms involved and the implications for lymphoma patients necessitates further study.

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References


