Targeting the CD20 and CXCR4 Pathways in Non Hodgkin Lymphoma

with Rituximab and high affinity CXCR4 antagonist BKT140

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TRANSLATIONAL RELEVANCE

B-cell Non Hodgkin lymphoma (NHL) represents the most common malignant lymphoid neoplasm. Although anti-CD20 antibody rituximab significantly improved the outcome of NHL patients, the relapsed/refractory rates are still high. Chemokine receptor CXCR4 and its ligand CXCL12 are critically involved in the survival and trafficking of normal and malignant B lymphocytes. Taking together with the fact that interaction of malignant B cells with stromal cells via CXCR4/CXCL12 signaling may provide chemo-resistance, blockade of CXCR4 may antagonize the survival and spreading of lymphoma cells and restore their chemo-sensitivity.

Our results demonstrate potent anti-lymphoma effect of CXCR4-specific high-affinity antagonist BKT140 in vitro and in vivo. The BKT140-mediated anti-lymphoma effect synergizes with that of rituximab. Moreover, BKT140 effectively targets lymphoma cells in the bone marrow microenvironment, overcoming the stroma-induced resistance to rituximab.

These findings suggest the possible interaction between CD20 and CXCR4 pathways in NHL, and provide the scientific basis for the development of novel combined CXCR4-targeted therapies for refractory NHL.
ABSTRACT

**Purpose:** Chemokine axis CXCR4/CXCL12 is critically involved in the survival and trafficking of normal and malignant B lymphocytes. Here, we investigated the effect of high affinity CXCR4 antagonist BKT140 on lymphoma cell growth and rituximab-induced cytotoxicity in vitro and in vivo.

**Experimental Design:** In vitro efficacy of BKT140 alone or in combination with rituximab was determined in NHL cell lines and primary samples from BM aspirates of NHL patients. In vivo efficacy was evaluated in xenograft models of localized and disseminated NHL with BM involvement.

**Results:** Antagonizing CXCR4 with BKT140 resulted in significant inhibition of CD20+ lymphoma cell growth and in the induction of cell death, respectively. Combination of BKT140 with rituximab significantly enhanced the apoptosis against the lymphoma cells in a dose-dependent manner. Moreover, rituximab induced CXCR4 expression in lymphoma cell lines and primary lymphoma cells, suggesting the possible interaction between CD20 and CXCR4 pathways in NHL. Primary BMSCs further increased CXCR4 expression and protected NHL cells from rituximab-induced apoptosis, while BKT140 abrogated this protective effect. Furthermore, BKT140 demonstrated efficient anti-lymphoma activity in vivo in the xenograft model of disseminated NHL with BM involvement. BKT140 treatment inhibited the local tumor progression and significantly reduced the number of NHL cells in the BM. Combined treatment of BKT140 with rituximab further decreased the number of viable lymphoma cells in the BM, achieving 93% reduction.

**Conclusions:** These findings suggest the possible role of CXCR4 in NHL progression and response to rituximab, and provide the scientific basis for the development of novel CXCR4-targeted therapies for refractory NHL.
INTRODUCTION

Non-Hodgkin’s lymphoma (NHL) is a heterogeneous group of malignancies of B cells or T cells (1). Approximately 80–85% of NHL are B-cell malignancies in origin and more than 95% of these express surface CD20.

Combining chimeric monoclonal anti-CD20 antibody rituximab (2) with standard chemotherapy regimens is associated with higher response rates, and improved survival in a subset of patients. Unfortunately, a significant percentage of patients who initially respond to rituximab, eventually relapse (3). Scientific efforts are increasingly being focused in developing new strategies to improve mAb activity (4).

Stromal cell-derived factor-1 (SDF-1/CXCL12) (5), was initially identified as a pre-B-cell growth-stimulating factor (6). CXCL12 signals through CXCR4, a seven transmembrane, G-protein coupled receptor, that is expressed by normal and malignant cells of hematopoietic and non-hematopoietic lineage (7). Data from knockout mice indicate that the CXCR4 receptor plays an important role in haematopoiesis (8-11). CXCR4–CXCL12 axis is particularly important in the homing and retention of hematopoietic progenitor cells in the marrow microenvironment (12, 13).

There is growing evidence that CXCR4 expression and function in hematopoietic malignancies have a major impact on disease progression. CXCR4 levels are significantly elevated in B-CLL (14), B-cell but not T-cell acute lymphoblastic leukemia (15, 16), multiple myeloma (17), and some AMLs (18). CXCR4 mediates the homing to and engraftment of AML (19) and pre-B acute lymphoid leukemia cells to the bone marrow (BM) of NOD/SCID mice (20). CXCR4/CXCL12 interactions not only protect CLL cells from apoptosis but also allow the migration of CLL cells
beneath BM stromal cells, suggesting that CLL cells use this mechanism to infiltrate the marrow (21).

Microenvironment-mediated chemoresistance, which involves CXCR4/CXCL12 axis, is now well recognized in different hematological malignancies, including ALL and CLL (7). However, its role in NHL is less defined. Further studies are thus required to elucidate the critical determinants of CXCR4-mediated resistance of malignant B cell disorders. The knowledge of such mechanisms will guide identification of molecular targets for therapeutic interventions overcoming rituximab resistance.

**MATERIALS AND METHODS**

*Cells and Cell Lines*

The following human NHL cell lines were used: Burkitt's lymphoma cell lines Raji, Ramos, BL-2 and BJAB, and diffuse large B-cell lymphoma cell lines OCI-LY7, OCI-LY19 and SUDHL-4. The cells were kindly provided by laboratory of Prof. Dina Ben-Yehuda (Hadassah Hebrew University Hospital, Israel). Cells were maintained in log-phase growth in RPMI 1640 medium (Biological Industries, Kibbutz Beth Haemek, Israel) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1mM L-glutamine, 100 U/ml penicillin, and 0.01 mg/ml streptomycin (Biological Industries) in a humidified atmosphere of 5% CO₂ at 37°C.

After informed consent, primary NHL cells were collected from the BM of 7 NHL patients. Diagnosis was diffuse large B cell NHL.

*Measurement of surface CD20, CD19 and CXCR4 expression*

The expression of CD20, CD19 and CXCR4 (clone 12G5) on the surface of NHL cell lines and primary NHL cells was evaluated using specific monoclonal antibodies
Mouse isotype control antibodies were also purchased from eBioscience. The cells were analyzed by FACScalibur (Becton Dickinson Immunocytometry Systems), using CellQuest software.

**Cell viability assay**

NHL cells were seeded at 2×10^5 viable cells/1 ml per well into a 24-well plate in triplicates in a medium supplemented with 1% FCS and incubated with different concentrations of 4F-benzoyl-TN14003 (BKT140) (Biokine Therapeutics Ltd.) or Rituximab (Roche, purchased from Sheba Medical Centre pharmacy) for 48 hours. Following the incubation, the cells were stained with propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) and percent of viable PI-negative cells in culture was determined by FACScalibur.

**Apoptosis assays**

NHL cells were plated at density of 2×10^5 viable cells/ml in a medium supplemented with 1% FCS and cultured with different concentrations of BKT140 and rituximab for 48 hours. Apoptosis was determined by staining with Annexin V-FITC and 7-amino-actinomycin D (7-AAD) (eBioscience) according to manufacturer’s instructions and analyzed by flow cytometry. Caspase-3 enzymatic activity was measured using CaspGLOW Red Caspase-3 Staining Kit (MBL International, Woburn, MA), according to the manufacturer’s instructions.

**Assessment of mitochondrial membrane potential (∆Ψm)**

The cationic lipophilic fluorochrome DiOC6 (Sigma-Aldrich) was used to measure the ∆Ψm. Briefly, NHL cells were cultured with different concentrations of BKT140
and rituximab for 12 hours, harvested, resuspended in FACS buffer (PBS with 0.1% fetal calf serum and 0.1% NaN3) containing 200 nM DiOC6, and incubated for 15 minutes in a 37\(^\circ\)C. The cells were then analyzed by FACS for the loss of DiOC6 fluorescence.

Cell Cycle Analysis

NHL cells were exposed in vitro to different concentrations of BKT140 and rituximab for 48 hours. Cells were collected, washed with cold PBS, then fixed with 4% of paraformaldehyde (PFA) for 30 min. Fixed cells were resuspended in staining buffer containing 0.1% saponin (Sigma-Aldrich) and 40 µg/ml RNase and incubated at 37\(^\circ\)C for 15 min. Then cells were then stained with 10 µg/ml 7-AAD in the dark for 30 min. The DNA content was detected using FACS.

Cell migration assay

Migration assay was performed in triplicate using 5-µm pore size Transwells (Costar, Cambridge, MA). The lower compartment was filled with 600 µl of 1% FCS RPMI 1640 medium containing different (50 ng/ml, 250 ng/ml, 500 ng/ml) concentrations of CXCL12 (PeproTech EC, London, UK), and 5x10\(^5\) cells in 100 µl of 1% FCS RPMI 1640 medium were applied to the upper compartment. The amount of cells migrated within 4 hours to the lower compartment was determined by FACS and expressed as a percentage of the input.

Co-culture experiments

Primary human BM stomal cells (BMSCs) were isolated and expanded from BM aspirates of healthy donors after signed informed consent. The mononuclear cells
were plated in DMEM medium with low glucose (Biological Industries) supplemented with 15% heat-inactivated FCS, 1mM L-glutamine, 100 U/ml penicillin, and 0.01 mg/ml streptomycin (Biological Industries) in a humidified atmosphere of 5% CO₂ at 37°C. Medium was refreshed once a week and adherent cells were cultured. BMSCs were plated at density of 2x10⁴ cells per well in 24-well plates and incubated overnight. The following day NHL cells were seeded on top of stromal cells, alone or in combination with BKT140 and rituximab, at a density of 2x10⁵ cells/ml in a medium supplemented with 1% FCS. Following 48 hours of co-incubation, non-adherent cells were collected and adherent cell fraction was harvested with trypsin/EDTA. The cells were washed with PBS and analyzed by FACS for viability (using PI exclusion) and CXCR4 expression. Cells were counterstained with anti-CD20 and NHL cells were distinguished from BMSCs after gating on CD20+ lymphoma cells.

**Murine xenograft models of human NHL**

NOD/SCID mice were maintained under defined flora conditions at the Hebrew University Pathogen-Free Animal Facility. All experiments were approved by the Animal Care Committee of the Hebrew University. NOD/SCID mice were injected subcutaneously with BJAB or BL-2 cells (5x10⁶/mouse) into the right flank and developed clearly palpable tumors. To investigate the therapeutic potential of CXCR4 antagonist BKT140 on NHL dissemination and growth, mice injected with BL-2 cells were treated with subcutaneous injections of 300 µg BKT140 or control saline, in a site different to tumor injection. For the combination therapy studies, BL-2 bearing mice were treated with 300 µg of BKT140, 500 µg of rituximab, or combination of both agents, administered by separate subcutaneous injections.
**CXCR4 immunohistochemistry**

Subcutaneous xenograft tumors generated by BL-2 cells injected into NOD/SCID mice were harvested and fixed in 4% PFA. Paraffin-embed sections (10 μM) were initially dewaxed, rehydrated, treated with EDTA buffer and blocked with CAS blocking reagent (Zymed Laboratories, San Francisco, CA, USA) for 30 minutes in room temperature. Samples were then incubated overnight at 4°C in a humidified chamber with anti-human CXCR4 monoclonal antibody, clone 12G5 (R&D Systems, Minneapolis, MN) diluted to final concentration 10 μg/ml. Next, the sections were incubated with secondary anti-mouse horseradish peroxidase-conjugated antibody (DakoCytomation, Glostrup, Denmark) for 30 minutes at room temperature. 3-amino-9-ethylcarbazole (AEC) was used for color development, and sections were counterstained with hematoxylin.

**In situ TdT-mediated dUTP nick end labeling (TUNEL)**

Apoptotic nuclei in control and BKT140-treated BL-2 or BJAB xenograft tumor sections were visualized using Fluorescein In Situ Cell Death Detection Kit according to the manufacturer’s instructions (Roche Diagnostic, Mannheim, Germany). The slides were then mounted with Permount Mounting Medium (Fisher Scientific, Barrington, IL, USA) and analyzed under a fluorescent microscope.
RESULTS

CXCR4 expression in NHL cell lines and in primary lymphoma cells from patients with BM involvement

First, we characterized the surface expression of CXCR4 in a panel of human NHL cell lines (n=7). Strong CXCR4 expression was found in Burkitt lymphoma BL-2, Raji, and Ramos cells, intermediate CXCR4 - in diffuse large B cell lymphoma (DLBCL) lines OCI-LY7, OCI-LY19 and SU-DHL-4, and low - in BJAB cells, respectively (Supp 1A). All NHL cell lines expressed high levels of CD20 antigen (Supp 1B), therefore being reliable targets for anti-CD20 antibody rituximab. Primary CD20-positive lymphoma cells in the BM samples from the patients also expressed high levels of CXCR4 (Supp 1C).

To assess the functionality of CXCR4 receptor expressed by lymphoma cells, we tested the in vitro chemotaxis of NHL cells. Cell lines BL-2 and BJAB and primary lymphoma cells from NHL patients with BM involvement were allowed to migrate in response to elevated amounts of CXCL12. As shown in supplementary figure 2A, CXCL12 induced the trans-well migration of high CXCR4-expressing BL-2 cells in a dose-dependent manner, while low-CXCR4 BJAB cells did not respond to CXCL12. Furthermore, primary lymphoma cells demonstrated dose-dependent migratory response to CXCL12. Importantly, CXCL12-induced cell migration of primary lymphoma cells correlated with CXCR4 cell-surface expression (Supp 2B).

Rituximab inhibits the cell growth and induces CXCR4 surface expression in NHL cells

Next, the effect of rituximab on lymphoma cell growth in vitro was examined, exposing BL-2, BJAB and Raji cells to the elevated concentrations (10 µg/ml and 100
µg/ml) of rituximab in vitro. Rituximab directly inhibited the cell growth and significantly diminished the number of viable cells in culture. The inhibitory effect was dose-dependent. High CXCR4-expressing BL-2 and Raji cells demonstrated higher sensitivity to rituximab than low-CXCR4 expressing BJAB cells (Fig. 1A). Subsequently, the effect of rituximab treatment on CXCR4 expression levels in BL-2, BJAB and Raji cells was tested. Interestingly, statistically significant increase in cell-surface expression of CXCR4 was induced by rituximab in all three NHL cell lines tested. Furthermore, similar effect was observed in primary lymphoma cells obtained from NHL patient with BM involvement – incubation with rituximab induced surface expression of CXCR4 (Fig. 1B). Taking together, these results demonstrate CXCR4-modulating effect of rituximab in NHL, showing that anti-CD20 antibody rituximab decreases the viability, but increases CXCR4 expression in NHL cells.

**CXCR4 inhibitor BKT140 induces apoptosis of NHL cells and co-operates with rituximab in vitro in anti-lymphoma activity**

Next, the effect of CXCR4 inhibitor BKT140 on NHL cell viability was assessed. BL-2, BJAB and Raji cells were incubated with different concentrations of BKT140 (4-100 μM) during 48 hours and cell viability was tested by flow cytometry using PI exclusion method. As demonstrated in Fig. 2A-B, BKT140 significantly reduced the number of viable cells and increased the percent of dead cells in a dose-dependent manner in all three NHL cell lines tested. High CXCR4 BL-2 and Raji cells demonstrated higher sensitivity to BKT140-induced cell death than low-CXCR4 BJAB cells. Next, the effect of combination of BKT140 with rituximab on NHL cell viability was evaluated. The cells were treated either with BKT140 8 μM, rituximab 10 µg/ml or combination of both agents during 48 hours, and cell viability was
measured using PI exclusion method. The combined treatment with both compounds significantly reduced the number of viable cells in culture (Fig. 2C), suggesting that BKT140 enhances the anti-NHL effect of rituximab. Similar effect of BKT140-induced cytotoxicity was observed in additional NHL lines tested (Ramos and OCI-LY19) (data not shown) and primary NHL cells from patients with BM involvement (Fig. 2D). To determine the specificity of BKT140 and rituximab effects, were used CD20-negative CXCR4-positive Jurkat T cells, which responded to BKT140 treatment but were not affected by rituximab (Supp 3A).

**BKT140 induces apoptotic cell death of NHL**

To analyze the mechanism of BKT140-induced cytotoxicity in NHL cells, we next examined phosphatidylserine exposure, a hallmark of apoptosis, using Annexin V combined with 7-AAD staining method. An accumulation of Annexin-V positive cells was observed following rituximab treatment confirming early stage apoptosis induction, while BKT140 increased the number of both early apoptotic (Annexin V+/7-AAD-) and late apoptotic/dead (Annexin V+/7-AAD+) cells. Furthermore, combination of rituximab with BKT140 significantly increased the number of Annexin V/7-AAD double positive cells, indicating subsequent cell death (Fig. 3A). To examine the apoptotic pathways activation, we assessed the cleavage of caspase 3 in NHL cells treated with BKT140 and rituximab. We first found that BKT140 treatment induced caspase 3 activation in NHL cells in a dose-dependent manner. Moreover, combination of BKT140 (8 µM) with rituximab (100 µg/ml) significantly increased caspase 3 activation in BL-2 and Raji cells (Fig. 3B, Supp 3B).

The effect of BKT140 and rituximab on cell cycle progression and DNA distribution in NHL cells was then studied. Rituximab slightly increased the BL-2 cells population
in G2/M phase, therefore promoting G2/M cell arrest. In contrast, BKT140 treatment resulted in the accumulation of the cells at sub G0/G1 phase, indicating the DNA damage consistent with apoptosis. Combination of BKT140 with rituximab reversed the G2/M arrest and further increased the population in sub G0/G1 phase, up to 54% in BL-2 and to 45% in BJAB cells, respectively, therefore further promoting apoptotic DNA fragmentation (Fig. 3C).

To further elucidate the mechanism by which BKT140 enhances rituximab-induced apoptosis in NHL cells, we examined the possible involvement of mitochondria testing mitochondrial transmembrane potential ($\Delta\Psi_m$) using DiOC6 dye. Combination of BKT140 (8 µM) with rituximab (100 µM) significantly increased the number of apoptotic depolarized cells in all three NHL cell lines tested, compared to the effect induced by each agent alone. Importantly, high CXCR4-expressing cells BL-2 and Raji demonstrated higher sensitivity and enhanced rate of apoptosis promoted by the combination of BKT140 and rituximab than low CXCR4-expressing BJAB cells (Fig 3D, Supp 3C). These results further emphasize the role of CXCR4 in BKT140-induced NHL apoptosis and strength the rational for CXCR4 inhibition combined with rituximab for the treatment of CXCR4-expressing B cell lymphomas.

**Interaction with BM stromal cells (BMSCs) elevates surface CXCR4 expression on NHL cells and supports their survival and proliferation**

Previous reports have shown that stromal cells in bone marrow microenvironment support CLL survival and protect the cells from chemotherapy-induced apoptosis (22, 23). To investigate the interaction between NHL cells and BM microenvironment in vitro and its effect on rituximab sensitivity, lymphoma cell lines BL-2, Raji and BJAB
were treated with rituximab in the absence or presence of primary BM stromal cells (BMSCs). Floating and adherent fractions of NHL cells were analyzed differentially. First, the incorporation of lymphoma cell lines into the monolayer of stromal cells was examined. Interestingly, high CXCR4-expressing BL-2 and intermediate CXCR4-expressing Raji cells effectively adhered and trans-migrated through the confluent monolayer of BMSCs, producing so-called "cobble stone" structures. In contrast, low CXCR4-expressing BJAB cells mostly remained in non-adherent floating fraction (Supp 4A). Then, the effect of stromal cells on NHL cell survival and proliferation in serum-reduced conditions was evaluated. Consistent with microscopic observations, BMSCs effectively supported the survival and proliferation of CXCR4-expressing BL-2 and Raji cells that were in close contact with stroma. Relative increase in lymphoma proliferation was in correlation with CXCR4 expression levels. In contrast, the proliferation of low CXCR4-expressing BJAB cells was not significantly affected by the interaction with BMSC (Fig. 4A).

Next, the effect of BMSCs on CXCR4 expression by lymphoma cells was evaluated. Primary BMSCs significantly increased the surface expression of CXCR4 by BL-2 and Raji cells. The effect was more profound in adherent cell fraction. Rituximab treatment further elevated the CXCR4 levels on lymphoma cells (Fig. 4B, Supp 4B). These data suggest that primary BMSCs protect NHL B cells from spontaneous apoptosis, in agreement with previously published findings. Moreover, the enhancing effect of primary stromal cells on CXCR4 expression by NHL cells was hereby demonstrated for the first time, emphasizing the role of BM microenvironment in CXCR4-mediated lymphoma cell survival.
BKT140 reverses the protective effect of BMSCs and enhances rituximab-induced cell death

Subsequently, the ability of primary stromal cells to confer the resistance of NHL cells to rituximab was examined. Viability of lymphoma cell lines treated with rituximab for 48 hours in the absence or presence of primary BMSCs was tested. BMSCs were able to protect lymphoma cells against rituximab-induced cytotoxicity (Fig. 4C).

The ability of BKT140 antagonist to overcome the stroma-mediated resistance of NHL cells was tested next. Initially, the effects of BKT140 in combination with rituximab were examined in BL-2, BJAB and Raji cells, cultured with or without BMSCs support. Low dose of BKT140 (8 µM) effectively targeted floating lymphoma cells. In contrast, lymphoma cells that were in direct contact with stroma cells were protected from lose dose of BKT140. However, elevated dose of BKT140 (40 µM) significantly (p<0.01) induced cell death in both floating and adherent fractions of CXCR4-expressing lymphoma cells cultured with BMSCs, abrogating the protective effect of stromal cells. Combination of BKT140 with rituximab further increased the rituximab-mediated cell death in the presence of BMSCs (Fig. 4D).

Establishment of in vivo model of disseminated NHL with BM involvement

To assess the effect of BKT140 inhibitor on lymphoma development and spread in vivo, xenograft model of disseminated lymphoma with BM involvement in mice was established. Human BL-2 cells were injected subcutaneously into NOD/SCID mice, developed invasive local tumors (at days 12-14 following the cell injection) and then specifically spread to the BM (days 21-28). All animals inoculated with BL-2 cells developed local tumors and subsequent dissemination to the BM. Kinetic studies
detected massive lymphoma insemination in the BM, reaching 40-50% of total mononuclear cells in the murine BM at day 35 following the inoculation. No significant lymphoma dissemination was observed in other hematopoietic organs, such as spleen and liver (Figure 5A). Importantly, subcutaneous inoculation with low CXCR4-expressing BJAB cells resulted in local tumor development without BM involvement (Fig. 5A) and in long-term survival: animals survived during 70-80 days without any signs of systemic disease and were sacrificed due to large local tumor volume. In contrast, mice injected with high CXCR4 expressing BL-2 lymphoma cells survived for only 38-45 days and succumbed to lymphoma short time after BM disease development (Fig. 5B). Indirectly, these facts may indicate that the spread and subsequent growth of CXCR4-expressing BL-2 cells in the BM are the major causes of the animal’s mortality.

**BKT140 inhibits the spread of NHL to the BM**

To examine the in vivo effect of BKT140 on the lymphoma dissemination to the BM, NOD/SCID mice were subcutaneously inoculated with BL-2 cells and treated with daily subcutaneous injections of BKT140 (300 µg/injection), 5 days a week, during two weeks. Two different treatment regimens were tested: 1) the residual disease regimen, in which BKT140 was administered on day 3 following BL-2 inoculation and discontinued following two weeks. In contrast, 2) in the progressive disease regimen, spread of BL-2 lymphoma to the BM was first allowed to occur, and then BKT140 treatment was started on day 28 after the BL-2 cell inoculation. Both regimens significantly reduced the number of human CD20+ cells in the murine BM. However, early start of BKT140 administration reflecting the residual disease regimen was more effective, remarkably reducing the BM lymphoma disease by 92%
(p<0.0006) comparing to the 62% reduction (p<0.02) achieved with the late start of BKT140 injections in progressive disease model (Fig. 5C). Animals demonstrated good tolerability to the treatment regimens with BKT140, no significant modifications of body weight or side effects were observed.

To further elucidate the mechanism by which BKT140 targets NHL in the BM microenvironment, we evaluated the potential effect of BKT140 on apoptosis induction in vivo in the CD20+ BL-2 cells. Flow cytometry analysis with Annexin V-APC revealed that BKT140 treatment not only reduces the number of BL-2 cells in the BM but rather promotes lymphoma cell apoptosis in the BM microenvironment (Supp 4).

In addition, the effect of BKT140 on lymphoma in vivo growth was further assessed in non-disseminated lymphoma model initiated by BJAB cells. NOD/SCID mice subcutaneously inoculated with BJAB cells were treated daily with BKT140, starting from day 3 after cell injection, 5 days a week, during 2 weeks, and local tumor growth was monitored. Significant suppression of tumor growth was observed in BKT140-treated animals. The median growth of BJAB tumors was delayed by 78% on day 55 and by 60% on day 60 as compared with vehicle control (Fig. 5D). BJAB-generated tumors expressed low but detectable levels of CXCR4, whereas BKT140 treatment totally blocked CXCR4 staining in local tumor tissues (Fig. 5E, upper panel). Furthermore, BKT140 significantly induced apoptosis in vivo in BJAB tumors, as approved by TUNEL staining (Fig. 5E).
**Combination of BKT140 with rituximab effectively targets NHL in the BM microenvironment**

The effect of BKT140 and combinational therapy on the disseminated lymphoma disease was tested. BL-2-innoculated mice with established tumor and BM dissemination were subcutaneously injected with BKT140, rituximab or combination of both therapeutic agents, starting from day 28. Importantly, under conditions of large primary tumor with established BM involvement, rituximab treatment alone demonstrated minimal effect on the NHL BM disease, reducing the number of BL-2 cells only by 20% (Fig. 6A). BKT140 effectively reduced the BM tumor burden by 77% compared to the untreated animals (p<0.01). However, the combination treatment of BKT140 with rituximab further decreased the number of viable lymphoma cells in the BM, achieving 93% reduction (p<0.001) comparing to the untreated control (Fig. 6A). These results clearly demonstrate the effectiveness of the combined treatment against NHL in the BM microenvironment.

Mobilization of tumor cells into the blood by CXCR4 antagonists was previously shown in AML patients and in models of AML and MM (24, 25). To evaluate the effect of CXCR4 blockade on lymphoma cell release to the circulation, the blood of tumor-bearing animals was collected 24 hours after the last injection of BKT140 or rituximab at the experiment termination. Small number of human CD20-positive BL-2 cells in the circulation was detected by flow cytometry. Interestingly, five doses of BKT140 did not significantly affect the number of circulating lymphoma cells, compared to untreated control mice. In contrast to BKT140, rituximab notably reduced the number of BL-2 cells in the blood (Fig. 6B). Furthermore, combination of BKT140 with rituximab powerfully reduced the local tumor mass of BL-2-generated diffused tumors, as indicated by body weight gain of BL-2-inoculated animals (Fig.
6C). These data suggest that in progressive lymphoma disease with bulk tumor and BM involvement, lymphoma cells are accessible to rituximab in the blood but not in the BM. However, BKT140 effectively targets lymphoma cells in the BM niche and in local tumor, whereas the combination with rituximab significantly enhances the anti-tumor effect of BKT140 in vivo.

Next, the CXCR4 expression in local BL-2-produced tumors was assessed. In accordance with in vitro results, rituximab-treated BL-2 tumors exhibited increased CXCR4 expression, tested by immunohistochemical analysis. In contrast, CXCR4 staining was significantly reduced in BL-2 tumors treated with BKT140, alone or in combination with rituximab (Fig. 6D upper panel). Furthermore, in vivo apoptosis induction was detected by TUNEL in BL-2-produced local tumors following BKT140 or rituximab treatment. However, the combinational treatment resulted in profoundly increased apoptosis rates (Fig. 6D). These results further confirm an increasing effect of rituximab and blocking effect of BKT140 on CXCR4 expression in NHL cells in vivo.

**DISCUSSION**

CXCR4/CXCL12 axis has been implicated in the development and progression of many hematopoietic and solid tumors (26-31). In accordance with previous reports (32, 33), our data indicate that lymphoma cell lines as well as primary lymphoma cells from patient BM biopsies express surface CXCR4 (34, 35). Interaction of lymphoma cells with BM-derived stromal cells supports lymphoma cell survival and protects them from rituximab cytotoxicity. We observed that both interaction with primary BM stromal cells as well as rituximab treatment further increased CXCR4 cell surface
levels on lymphoma cells. This may endow tumor cells with increase direct and indirect survival signals.

CXCR4 neutralization with small molecule such as AMD3100, AMD11070 and AMD3465 were already shown to reverse the protective effect of stroma and induce chemo-sensitivity in MM, mantle cell lymphoma, AML, ALL and CLL (22, 25, 34-37).

Recently it was reported that neutralizing monoclonal antibodies for CXCR4 significantly delayed tumor growth of lymphoma Namalwa cells in NOD/SCID mice (38). Moreover, antagonizing CXCR4 and CXCL12 significantly inhibited the growth of EBV-transformed B cells injected intraperitoneally into NOD/SCID mice, and prolonged animal survival (39). Furthermore, recently published study demonstrated the efficacy of cell-penetrating lipopeptide CXCR4 antagonists, called pepducins, to enhance rituximab-induced apoptosis of lymphoma cells in vitro and in vivo (40).

Small molecule CXCR4 inhibitors mentioned previously were able to abrogate stroma-mediated resistance but lack direct cytotoxic or chemotherapy-increasing activity in the absence of stromal cells. Here we evaluated the effect of the high affinity CXCR4 antagonist BKT140 on NHL cell viability, interaction with BM microenvironment and rituximab responsiveness. BKT140 originally designed as a human immunodeficiency virus (HIV) entry inhibitor through specific binding to CXCR4 (41). BKT140 inhibits CXCR4-mediated adhesion and migration of cells from hematopoietic origin (42). BKT140 was found to induce mobilization of mature WBCs, hematopoietic progenitors and stem cells and efficiently synergize with G-CSF in its ability to mobilize WBC and progenitors (43). Furthermore, we have recently shown that BKT140 induce a CXCR4-dependent selective apoptotic cell
death of hematopoietic malignant cells such as multiple myeloma and leukemia cells (44).

In the present study we observed that CXCR4 antagonist BKT140 alone directly induced the apoptosis of NHL cell lines and primary NHL cells. Combination of BKT140 with rituximab significantly increased the cytotoxic anti-lymphoma effect, synergistically inducing mitochondrial damage, caspase-3 activation and subsequent apoptosis of lymphoma cells. Moreover, our data indicate that disrupting the CXCR4/CXCL12 axis using CXCR4 antagonist BKT140 is an effective way to abrogate BMSC-mediated resistance of NHL to rituximab and to target NHL in BM microenvironment. The discrepancy between the action of BKT140 versus AMD3100 may be partially explained by relatively low affinity of AMD3100 (1nM versus 84 nM) (45).

To evaluate the in vivo anti-lymphoma effect of BKT140 we established a novel xenograft model of B-cell lymphoma with BM involvement in mice. Human CXCR4-expressing B NHL cell line, BL-2, was subcutaneously implanted into NOD/SCID mice, resulting in the development of aggressive local tumors which specifically spread to the BM. This model enables us to investigate the role of CXCR4 in different aspects of lymphoma progression.

We found that CXCR4/CXCL12 drives lymphoma specific migration to the BM and mediates the stroma-induced protection from immunotherapy. BKT140 treatment inhibited local tumor progression and significantly reduced tumor burden in the BM. Combination treatment of BKT140 with rituximab further decreased the number of viable lymphoma cells in the BM, achieving 93% reduction.

Previous study of Patrizia Mancuso and colleagues analyzed the presence of lymphoma cells in the blood of NHL patients with BM involvement and revealed
concordance of 95% between BM and blood. Among the discordant cases (i.e., presence of neoplastic B lymphocytes in the BM, but under the sensibility of the technique in the blood), 62% of samples were collected after rituximab treatment alone or in association with chemotherapy. The authors suggested that during treatment with rituximab, neoplastic cells may be depleted from the blood, but are still present in the BM (46).

These observations are supported by our current results in a disseminated NHL model, showing the ability of rituximab to decrease the number of circulating lymphoma cells in the blood, albeit an inability to target NHL cells in the BM.

The use of mobilizing agents in leukemia has a potential to re-distribute the malignant cells and therefore to aggravate the disease course. Notably, in our in vivo model we didn’t detect the mobilization of lymphoma cells to the blood following BKT140 treatment. In contrast, the numbers of viable circulating lymphoma cells in the blood were reduced with BKT140. This ability to diminish the numbers of viable NHL cells in BM and in peripheral blood may be particularly important for in vivo purging and collection of lymphoma-free BM grafts for autologous transplantation in NHL patients. Rituximab use during mobilization procedures has been shown to be effective in obtaining lymphoma-free PBSC (47). Therefore, the integration of BKT140 in combination with Rituximab into sequential mobilization program of NHL patients may be rational strategy for in vivo purging and mobilization of uncontaminated grafts.

Taken together, our findings indicate that CXCR4 contribute to NHL progression and demonstrate potent anti-lymphoma effect of CXCR4-specific high affinity antagonist BKT140 in vitro and in vivo. The BKT140-mediated anti-lymphoma effect synergizes with that of rituximab. Moreover, BKT140 effectively targets lymphoma cells in the
BM microenvironment, overcoming the stroma-induced resistance to rituximab. These findings suggest the possible interaction between CD20 and CXCR4 pathways in NHL, and provide the scientific basis for the development of novel combined CXCR4-targeted therapies for refractory NHL.

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FIGURE LEGENDS

Figure 1. Effect of rituximab on NHL cell viability and CXCR4 expression.

(A) Viability of NHL cell lines and primary lymphoma cells from BM aspirates of NHL patients, treated with elevated doses of rituximab (10 µg/ml or 100 µg/ml) during 48 hours, was analyzed using PI exclusion method. (B) NHL cell lines and primary NHL cells were incubated in serum-reduced conditions (1% FCS) in the absence or presence of rituximab (20 µg/ml) during 48 hours, and CXCR4 cell-surface expression was evaluated and quantified by FACS. Data are represented as mean of triplicates ± standard deviation (SD). Probability values of t-test are presented (**p<0.01). Experiment was repeated twice.

Figure 2. BKT140 dose-dependently decreases the viability of NHL cells and co-operates with rituximab in vitro.

NHL cell lines BL-2, BJAB and Raji treated with elevated concentrations of BKT140 (4, 8, 20 and 40 µM) during 48 hours. Viability was determined using PI exclusion method using FACS analysis, and percentages of viable (A) versus dead (B) cells were detected. (C) The indicated NHL cell lines were co-incubated in the absence or presence of 8 µM BKT140, 10 or 100 µg/ml rituximab or combination of both agents for 48 hours. (D) Primary lymphoma cells from BM aspirates of NHL patients with BM involvement were incubated in the absence or presence of 8 µM BKT140, 20 µg/ml rituximab or combination of both agents for 48 hours. Data are represented as mean of triplicates ± SD. Probability values of t-test are presented (**p<0.01).

Figure 3. BKT140 and rituximab synergistically induce NHL cell apoptosis with mitochondrial involvement and promote caspase 3 activation.

(A) BL-2 cells were incubated in the absence or presence of 8 µM BKT140, 100 µg/ml rituximab or combination of both agents for 48 hours. Apoptosis was detected
using Annexin V-FITC and 7-AAD co-staining. The percentage of early apoptotic (Annexin V+/7-AAD-) and late apoptotic/dead (Annexin V+/7-AAD+) is displayed. 

(B) Caspase-3 cleavage was determined by fluorescent labeling of activated caspase 3 in BL-2 and Raji cells treated with 8 µM BKT140, 100 µg/ml rituximab or combination of both agents for 48 hours. (C) Cell cycle analysis by 7-AAD staining was performed on BL-2 and BJAB cells incubated in the absence or presence of 8 µM BKT140, 100 µg/ml rituximab or combination of both for 48 hours. The subdyploid DNA peak (subG0/G1) represents apoptotic cell fraction. (D) Mitochondrial membrane potential (ΔΨm) was determined by flow cytometry using DiOC6 staining in BL-2, BJAB and Raji cells incubated in the absence or presence of 8 µM BKT140, 100 µg/ml rituximab or combination of both for 12 hours. Apoptotic cells showed a decrease in staining with DiOC6. Data is presented as mean of triplicates ± SD (**p< 0.01).

**Figure 4. Interaction with BMSC promotes NHL growth, elevates CXCR4 on NHL cells and protects them from rituximab. BKT140 treatment overcomes the protective effect of stromal cells.**

(A) NHL cells (2x10^7/ml) were co-cultured in serum-reduced conditions (1% FCS) in the absence or presence of BMSCs for 24 and 48 hours. Viable NHL CD20+ cells in floating and adherent fractions were determined by FACS using PI exclusion. (B) BL-2 cells were cultured with stromal cells in the presence or absence of rituximab (20 µg/ml) for 48 hours. CXCR4 surface expression on NHL was analyzed by FACS. (C) NHL BL-2, BJAB and Raji cells were cultured with stromal cells in the presence or absence of rituximab (20 µg/ml) for 48 hours. Viability was determined using XTT method. (D) BL-2 cells were cultured with stromal cells in the presence or absence of rituximab (10 µg/ml), BKT140 (8 and 40 µM) and combination of both agents for 48
hours. Viability of floating and adherent NHL cells was determined by FACS using PI discrimination. Data is presented as mean ± SD from triplicates (\(*\*p< 0.01\)).

**Figure 5. In vivo NHL models of localized and disseminated lymphoma with BM involvement.** BKT140 reduces BM spread and suppresses local lymphoma tumor growth. BKT140 co-operates with rituximab in BM and blocks rituximab-induced CXCR4 expression in local tumor.

(A) BL-2 and BJAB cells (5x10^6) were subcutaneously injected into the flank of NOD/SCID mice. On day 35 following the local injection the presence of human lymphoma cells in the murine bone marrow (BM) and spleen was assessed by FACS using specific anti-human CD20 antibody (representative FACS plots are displayed).

(B) Kaplan-Meier survival curve of animals subcutaneously injected with BL-2 or BJAB cells, followed during 60 days, 5 mice per group. Calculation was performed using NCSS. (C) BL-2 cells (5x10^6) were subcutaneously injected into the flank of NOD/SCID mice. According to the regimen for the residual disease (early start), mice were treated with subcutaneous injections of 300 µg BKT140 or control saline in a volume of 200 µl, starting from day 3 following the NHL inoculation. According to the progressive disease regimen (late start), BKT140 injections were started on day 28. Treatment schedule for both regimens was daily injections for five days, followed by two days of no drug and then five additional daily injections (total of 10 injections). Number of human CD20+ cells in the BM of mice was determined using specific staining and FACS analysis. Data is presented as mean ± SD from 5 mice.

(D) BJAB cells (5x10^6) were injected s.c. into NOD/SCID mice, and animals were treated with BKT140 injections according to residual disease regimen (10 daily injections on days 2-15 and 1 injection 24 prior to termination). Local tumor growth was measured with caliper and tumor area (length x width) was calculated. Data is
shown as mean, ± SD, 5 mice per group (**p< 0.01 when compared with control group). (E) Representative slides of CXCR4-stained and TUNEL-stained BJAB local tumor sections, control vs. BKT140-treated. Cell nuclei are visualized using 4′,6-diamidino-2-phenylindole (DAPI) staining, magnification ×200 and x400.

Figure 6. BKT140 co-operates with rituximab in vivo, reduces BM spread and blocks rituximab-induced CXCR4 expression in local tumor.

Combinational schedule of BKT140 and rituximab for established BM disease. BL-2-bearing mice were treated starting from day 28 after lymphoma inoculation with subcutaneous injections of BKT140 (300 µg/m), rituximab (500 µg/m), or both agents, administered by separate injections. BKT140 was administered on days 28, 29, 30, 32 and 33 (total of 5 injections). Rituximab was administered on days 28, 29 and 32 (total of 3 injections). Number of human CD20+ cells in the BM (A) and blood (B) and body weight gain of mice (C) on day 35 after BL-2 cell inoculation. Control indicates the group of age matched mice without BL-2 injection or treatments. Data is presented as mean ± SD of 7 mice per group. (D) CXCR4 expression and TUNEL staining in BL-2-produced local xenografts, treated with BKT140, rituximab or both agents. Cell nuclei are visualized with DAPI staining. Magnifications of x100, x200 and x400 (small images) are shown.
Figure 1.

A

BL-2

BJAB

Raji

NHL P1

NHL P2

Live cells (% of control)

Rituximab (µg/ml) - 10 100

B

BL-2

BJAB

Raji

NHL P1

NHL P2

CXCR4

Rituximab (20µg/ml) - +

CXCR4 (MFI)

Rituximab (20µg/ml) - +
Figure 2.

A

B

C

D

Live cells ( % of control )

Dead cells ( % of control )

BL-2

BJAB

Raji

BL-2

BJAB

Raji

BL-2

BJAB

Raji

BL-2

BJAB

Raji

BL-2

BJAB

Raji

BKT140 (µM)

Rituximab (µg/ml)

Rituximab (µg/ml)

BKT140 (µM)

BM NHL P1

BM NHL P2

Live CD19+ cells ( % of control )

Live CD19+ cells ( % of control )

Rituximab (µg/ml)

Rituximab (µg/ml)

BKT140 (µM)

BKT140 (µM)
Figure 3.

A

![Bar chart showing percentage of apoptosis](image)

Rituximab (µg/ml) - 100 - 100
BKT140 (µM) - 8 - 8

B

![Bar chart showing caspase 3 activation](image)

Rituximab (µg/ml) - 100 - 100
BKT140 (µM) - 8 - 8

C

![Bar chart showing cell distribution](image)

BL-2
Sub G0/G1 - 100 - 100
G2/M - 8 - 8

BJAB
Sub G0/G1 - 100 - 100
G2/M - 8 - 8

D

![Bar chart showing % of DIOG-apoptotic cells](image)

BL-2
% of DIOG-apoptotic cells - 100 - 100

BJAB
% of DIOG-apoptotic cells - 8 - 8

Raji
% of DIOG-apoptotic cells - 100 - 100
**Figure 4.**

(A) Graph showing the percentage increase in live cells over time for BL-2, BJAB, and Raji cell lines. The graph compares the effects of BMSC floating and adherent cells with and without Rituximab treatment, indicating a significant difference (p<0.01).

(B) Graph showing the MFI of CXCR4 for BL-2, BJAB, and Raji cell lines with and without BMSC floating and adherent cells. The graph includes Rituximab treatment, with a significant increase in MFI (p<0.01).

(C) Bar graph showing the percentage of live cells (% of ctrl) for BL-2, BJAB, and Raji cell lines with and without BMSC floating and adherent cells with Rituximab treatment. The graph indicates a significant difference (p<0.01).

(D) Bar graph showing the percentage of live cells (% of ctrl) for BL-2, BJAB, and Raji cell lines with and without BMSC floating and adherent cells with different concentrations of BKT140. The graph includes Rituximab treatment, with a significant increase at 10 µg/ml (p<0.01).
Figure 5.

A. Human CD20

B. Survival

C. CD20+ cell number

D. Tumor size

E. CXCR4 expression and DAPI staining

- Early (s.c. BKT140)
- Late (control)

CD20+ cell number:
- 62% reduction (p<0.02)
- 92% reduction (p<0.0006)
Figure 6.

A  

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D  

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Targeting the CD20 and CXCR4 Pathways in Non Hodgkin Lymphoma with Rituximab and high affinity CXCR4 antagonist BKT140

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