Targeting the CD20 and CXCR4 Pathways in Non-Hodgkin Lymphoma with Rituximab and High-Affinity CXCR4 Antagonist BKT140

Katia Beider1, Elena Ribakovsky1, Michal Abraham3, Hanna Wald3, Lola Weiss2, Evgenia Rosenberg1, Ethan Galun2, Abraham Avigdor1, Orly Eizenberg3, Amnon Peled2,3, and Arnon Nagler1

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 non-Hodgkin lymphoma (NHL) cell lines and primary samples from bone marrow aspirates of patients with NHL. In vivo efficacy was evaluated in xenograft models of localized and disseminated NHL with bone marrow 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 bone marrow stromal cells (BMSC) further increased CXCR4 expression and protected NHL cells from rituximab-induced apoptosis, whereas BKT140 abrogated this protective effect. Furthermore, BKT140 showed efficient antilymphoma activity in vivo in the xenograft model of disseminated NHL with bone marrow involvement. BKT140 treatment inhibited the local tumor progression and significantly reduced the number of NHL cells in the bone marrow. Combined treatment of BKT140 with rituximab further decreased the number of viable lymphoma cells in the bone marrow, 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 lymphoma (NHL) is a heterogeneous group of malignancies of B cells or T cells (1). Approximately 80% to 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 monoclonal antibody (mAb) activity (4).

Stromal cell–derived factor-1 (SDF-1/CXCL12; ref. 5), was initially identified as a pre-B-cell growth-stimulating factor (6). CXCL12 signals through CXCR4, a 7 transmembrane, G-protein–coupled receptor, that is expressed by normal and malignant cells of hematopoietic and nonhematopoietic lineage (7). Data from knockout mice indicate that the CXCR4 receptor plays an important role in hematopoiesis (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-cell chronic lymphocytic leukemia (B-CLL; ref. 14), B-cell but not T-cell acute lymphoblastic
Translational Relevance
B-cell non-Hodgkin's lymphoma (NHL) represents the most common malignant lymphoid neoplasm. Although anti-CD20 antibody rituximab significantly improved the outcome of patients with NHL, 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. The interaction of malignant B cells with stromal cells via CXCR4/CXCL12 signaling may provide chemoresistance. Therefore, blockade of CXCR4 may antagonize the survival and spreading of lymphoma cells and restore their chemosensitivity.

Our results show potent antilymphoma effect of CXCR4-specific high-affinity antagonist BKT140 in vitro and in vivo. The BKT140-mediated antilymphoma 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.

leukemia (15, 16), multiple myeloma (17), and some acute myeloid leukemias (AML; ref. 18). CXCR4 mediates the homing to and engraftment of AML (19) and pre-B acute lymphoid leukemia cells to the bone marrow of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (20). CXCR4/CXCL12 interactions not only protect CLL cells from apoptosis, but also allow the migration of CLL cells beneath bone marrow stromal cells (BMSC), 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 hematologic 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 lymphoma cell lines Raji, Ramos, BL-2, and BJAB, and diffuse large B-cell lymphoma (DLBCL) 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). The cells were maintained in log-phase growth in RPMI-1640 medium (Biological Industries) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1 mmol/L 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 bone marrow of 7 patients with NHL. 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 mAbs (eBioscience). Mouse isotype control antibodies were also purchased from eBioscience. The cells were analyzed by FACS (Becton Dickinson Immunocytometry Systems) using CellQuest software.

Cell viability assay
NHL cells were seeded at 2 x 10^5 viable cells/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) and the percentage of viable PI-negative cells in culture was determined by FACS (Becton Dickinson Immunocytometry Systems).

Apoptosis assays
NHL cells were plated at a density of 2 x 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-fluorescein isothiocyanate (FITC) and 7-aminoactinomycin D (7-AAD; eBioscience) according to manufacturer’s instructions and analyzed by flow cytometry. Caspase-3 enzymatic activity was measured using the CaspGLOW Red Caspase-3 Staining Kit (MBL International), according to the manufacturer’s instructions.

Assessment of mitochondrial membrane potential (ΔΨm)
The cationic lipophilic fluorochrome DiOC6 (Sigma-Aldrich) was used to measure the ΔΨm. Briefly, NHL cells were cultured with different concentrations of BKT140 and rituximab for 12 hours, harvested, resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS with 0.1% FCS and 0.1% NaNO3) containing 200 nmol/L DiOC6, and incubated for 15 minutes in a 37°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. The cells were collected, washed with cold PBS, and then fixed with 4% of paraformaldehyde (PFA) for 30 minutes. Fixed cells were resuspended in staining buffer containing 0.1% saponin (Sigma-Aldrich) and 40 μg/ml RNase and incubated at
Cultured BMSCs were plated at a density of 2 × 10^4 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 2 × 10^5 cells/mL in a medium supplemented with 1% FCS. Following 48 hours of coincubation, nonadherent 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. The cells were counterstained with anti-CD20 antibody rituximab. Primary CD20^+ lymphoma cells from patients with bone marrow involvement were distinguished from BMSCs after gating on CD20^+ lymphoma cells.

**Coculture experiments**

Primary human BMSCs were isolated and expanded from bone marrow aspirates of healthy donors after signed informed consent. The mononuclear cells were plated in Dulbecco’s modified Eagle medium (DMEM) with low glucose (Biological Industries) supplemented with 15% heat-inactivated FCS, 1 mmol/L 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 a density of 2 × 10^4 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 2 × 10^5 cells/mL in a medium supplemented with 1% FCS. Following 48 hours of coincubation, nonadherent 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. The 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 (Jerusalem, Israel). 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 (5 × 10^6/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-embedded sections (10 μmol/L) were initially dewaxed, rehydrated, treated with EDTA buffer, and blocked with CAS-blocking reagent (Zymed Laboratories) for 30 minutes at room temperature. Samples were then incubated overnight at 4°C in a humidified chamber with antihuman CXCR4 mAb, clone 12G5 (R&D Systems) diluted to final concentration of 10 μg/mL. Next, the sections were incubated with secondary anti-mouse horseradish peroxidase–conjugated antibody (DakoCytomation) for 30 minutes at room temperature. 3-Amino-9-ethylcarbazole (AEC) was used for color development, and the sections were counterstained with hematoxylin.

**In situ TUNEL**

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

**Results**

**CXCR4 expression in NHL cell lines and in primary lymphoma cells from patients with bone marrow 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 DLBCL cell lines OCI-Ly7, OCI-Ly19, and SU-DHL-4, and low—in BJAB cells, respectively (Supplementary Fig. S1A). All NHL cell lines expressed high levels of CD20 antigen (Supplementary Fig. S1B), therefore being reliable targets for anti-CD20 antibody rituximab. Primary CD20^+ lymphoma cells in the bone marrow samples from the patients also expressed high levels of CXCR4 (Supplementary Fig. S1C).

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 patients with NHL with bone marrow involvement were allowed to migrate in response to elevated amounts of CXCL12. As shown in Supplementary Fig. S2A, CXCL12 induced the Transwell migration of high CXCR4-expressing cells BL-2 in a dose-dependent manner, whereas low CXCR4-expressing cells BJAB did not respond to CXCL12. Furthermore, primary lymphoma cells showed dose-dependent migratory response to CXCL12. Importantly, CXCL12-induced cell migration of primary lymphoma cells correlated with CXCR4 cell surface expression (Supplementary Fig. S2B).

**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 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 cells BL-2 and Raji showed higher sensitivity to rituximab than low CXCR4-expressing cells BJAB (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 3 NHL cell lines tested. Furthermore, similar effect was observed in primary lymphoma cells obtained from patients with NHL with bone marrow involvement—incubation with rituximab induced surface expression of CXCR4 (Fig. 1B). Taking together, these results show 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 cooperates with rituximab in vitro in antilymphoma activity**

Next, the effect of CXCR4 inhibitor BKT140 on NHL cell viability was assessed. BL-2, BJAB, and Raji cells were treated with different concentrations of BKT140 (4–100 μmol/L) during 48 hours and cell viability was tested by flow cytometry using the PI exclusion method. As shown in Fig. 2A and B, BKT140 significantly reduced the number of viable cells and increased the percentage of dead cells in a dose-dependent manner in all 3 NHL cell lines tested. High CXCR4 BL-2 and Raji cells showed 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 μmol/L, rituximab 10 μg/mL, or combination of both agents during 48 hours, and cell viability was measured using the 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 bone marrow involvement (Fig. 2D). To determine the specificity of BKT140 and rituximab effects, CD20+ CXCR4-positive Jurkat T cells were used, which responded to BKT140 treatment but were not affected by rituximab (Supplementary Fig. S3A).

**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 the 7-AAD staining method. An accumulation of Annexin-V–positive cells was observed following rituximab treatment confirming early-stage apoptosis induction, whereas 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
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 μmol/L) with rituximab (100 μg/mL) significantly increased caspase-3 activation in BL-2 and Raji cells (Fig. 3B and Supplementary Fig. S3B).

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 (ΔΨm) using DiOC6 dye. Combination of BKT140 (8 μmol/L) with rituximab (100 μmol/L) significantly increased the number of apoptotic depolarized cells in all 3 NHL cell lines tested, compared with the effect induced by each agent alone. Importantly, high CXCR4-expressing cells BL-2 and Raji showed higher sensitivity and enhanced rate of apoptosis promoted by the combination of BKT140 and rituximab than low CXCR4-expressing BJAB cells (Fig. 3D and Supplementary Fig. S3C). These results further emphasize the role of CXCR4 in BKT140-induced NHL apoptosis and...
Interaction with 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 bone marrow 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 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 transmigrated through the confluent monolayer of BMSCs, producing so-called “cobble stone” structures. In contrast, low CXCR4-expressing BJAB mostly remained in nonadherent floating fraction (Supplementary Fig. S4A). 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 cells BL-2 and Raji 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 cells BJAB 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...
examined in BL-2, BJAB, and Raji cells, cultured with or without BMSCs support. Low dose of BKT140 (8 μmol/L) effectively targeted floating lymphoma cells. In contrast, lymphoma cells that were in direct contact with stroma cells were protected from low dose of BKT140. However, elevated dose of BKT140 (40 μmol/L) 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 bone marrow involvement

To assess the effect of BKT140 inhibitor on lymphoma development and spread in vivo, xenograft model of disseminated lymphoma with bone marrow 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 bone marrow (days 21–28). All animals inoculated with BL-2 cells developed local tumors and subsequent dissemination to the bone marrow.
Kinetic studies detected massive lymphoma insemination in the bone marrow, reaching 40% to 50% of total mononuclear cells in the murine bone marrow at day 35 following the inoculation. No significant lymphoma dissemination was observed in other hematopoietic organs, such as spleen and liver (Fig. 5A). Importantly, subcutaneous inoculation with low CXCR4-expressing BJAB cells resulted in local tumor development without the bone marrow involvement (Fig. 5A) and in long-term survival: animals survived during 70 to 80 days without any signs of systemic disease and

![Diagram](image-url)

**Figure 5.** In vivo NHL models of localized and disseminated lymphoma with bone marrow involvement. BKT140 reduces bone marrow spread and suppresses local lymphoma tumor growth. BKT140 cooperates with rituximab in bone marrow and blocks rituximab-induced CXCR4 expression in local tumor. A, BL-2 and BJAB cells (5 x 10^6) 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 antihuman 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 carried out using NCSS. C, BL-2 cells (5 x 10^6) 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 5 days, followed by 2 days of no drug and then 5 additional daily injections (total of 10 injections). The number of human CD20^+ cells in the bone marrow of mice was determined using specific staining and FACS analysis. Data are presented as mean ± SD from 5 mice. D, BJAB cells (5 x 10^6) were injected subcutaneously 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 hours before termination). Local tumor growth was measured with caliper and tumor area (length x width) was calculated. Data are 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 and control versus BKT140-treated. Cell nuclei are visualized using 4',6-diamidino-2-phenylindole (DAPI) staining. Magnification x200 and x400.
were sacrificed due to large local tumor volume. In contrast, mice injected with high CXCR4-expressing BL-2 lymphoma cells survived for only 38 to 45 days and succumbed to lymphoma short time after bone marrow disease development (Fig. 5B). Indirectly, these facts may indicate that the spread and subsequent growth of CXCR4-expressing BL-2 cells in the bone marrow 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 bone marrow, 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 2 weeks. Two different treatment regimens were tested: (i) the residual disease regimen, in which BKT140 was administered on day 3 following BL-2 inoculation and discontinued following 2 weeks. In contrast, (ii) in the progressive disease regimen, spread of BL-2 lymphoma to the bone marrow 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 bone marrow. However, early start of BKT140 administration reflecting the residual disease regimen was more effective, remarkably reducing the bone marrow lymphoma disease by 92% (P < 0.0006) comparing with the 62% reduction (P < 0.02) achieved with the late start of BKT140 injections in progressive disease model (Fig. 5C). Animals showed 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 bone marrow 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–allophycocyanin (APC) revealed that BKT140 treatment not only reduces the number of BL-2 cells in the bone marrow but rather promotes lymphoma cell apoptosis in the bone marrow microenvironment (Supplementary Fig. S4).

In addition, the effect of BKT140 on lymphoma in vivo growth was further assessed in nondisseminated 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, top). Furthermore, BKT140 significantly induced apoptosis in vivo in BJAB tumors, as approved by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (Fig. 5E).

**Combination of BKT140 with rituximab effectively targets NHL in the bone marrow microenvironment**

The effect of BKT140 and combinational therapy on the disseminated lymphoma disease was tested. BL-2–innoculated mice with established tumor and bone marrow 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 bone marrow involvement, rituximab treatment alone showed minimal effect on the NHL bone marrow disease, reducing the number of BL-2 cells only by 20% (Fig. 6A). BKT140 effectively reduced the bone marrow tumor burden by 77% compared with the untreated animals (P < 0.01). However, the combination treatment of BKT140 with rituximab further decreased the number of viable lymphoma cells in the bone marrow, achieving 93% reduction (P < 0.001) comparing with the untreated control (Fig. 6A). These results clearly show the effectiveness of the combined treatment against NHL in the bone marrow microenvironment.

Mobilization of tumor cells into the blood by CXCR4 antagonists was previously shown in patients with AML and in models of AML and multiple myeloma (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⁺ BL-2 cells in the circulation was detected by flow cytometry. Interestingly, 5 doses of BKT140 did not significantly affect the number of circulating lymphoma cells, compared with 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 bone marrow involvement, lymphoma cells are accessible to rituximab in the blood but not in the bone marrow. However, BKT140 effectively targets lymphoma cells in the bone marrow niche and in local tumor, whereas the combination with rituximab significantly enhances the antitumor 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 top). 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 bone marrow biopsies express surface CXCR4 (34, 35). Interaction of lymphoma cells with bone marrow-derived stromal cells supports lymphoma cell survival and protects them from rituximab cytotoxicity. We observed that both interaction with primary BMSCs 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 molecules such as AMD3100, AMD11070, and AMD3465 were already shown to reverse the protective effect of stroma and induce chemosensitivity in multiple myeloma, mantle cell lymphoma, AML, ALL, and CIL (22, 25, 34–37).

Recently, it was reported that neutralizing mAbs 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 Epstein-Bar virus (EBV)-transformed B cells injected intraperitoneally into NOD/SCID mice and prolonged animal survival (39). Furthermore, recently published study has shown 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 bone marrow 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 white blood cells (WBC), hematopoietic progenitors, and stem cells and efficiently synergize with granulocyte colony-stimulating factor (G-CSF) in its ability to mobilize WBC and progenitors (43). Furthermore, we have recently shown that BKT140 induces 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 antilymphoma 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 bone marrow microenvironment. The discrepancy between the action of BKT140 versus AMD3100 may be partially explained by relatively low affinity of AMD3100 (1 vs. 84 nmol/L; ref. 45).

To evaluate the in vivo antilymphoma effect of BKT140, we established a novel xenograft model of B-cell lymphoma with bone marrow 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 bone marrow. 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 bone marrow and mediates the stroma-induced protection from immunotherapy. BKT140 treatment inhibited local tumor progression and significantly reduced tumor burden in the bone marrow. Combination treatment of BKT140 with rituximab further decreased the number of viable lymphoma cells in the bone marrow, achieving 93% reduction.

Previous study of Mancuso and colleagues analyzed the presence of lymphoma cells in the blood of patients with NHL with bone marrow involvement and revealed concordance of 95% between bone marrow and blood. Among the discordant cases (i.e., the presence of neoplastic B lymphocytes in the bone marrow, but under the sensitivity 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 bone marrow (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 bone marrow.

The use of mobilizing agents in leukemia has a potential to redistribute the malignant cells and therefore to aggravate the disease course. Notably, in our in vivo model, we did not 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 bone marrow and in peripheral blood may be particularly important for in vivo purging and collection of lymphoma-free bone marrow grafts for autologous transplantation in patients with NHL. Rituximab use during mobilization procedures has been shown to be effective in obtaining lymphoma-free peripheral blood stem cell (PBSC ref. 47). Therefore, the integration of BKT140 in combination with rituximab into sequential mobilization program of patients with NHL may be a rational strategy for in vivo purging and mobilization of uncontaminated grafts.

Taken together, our findings indicate that CXCR4 contributes to NHL progression and show potent antilymphoma effect of CXCR4-specific high-affinity antagonist BKT140 in vitro and in vivo. The BKT140-mediated antilymphoma 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** K. Beider, E. Ribakovsky, E. Galun, A. Peled, A. Nagler

**Development of methodology:** K. Beider, H. Wald, E. Rosenberg, A. Peled, A. Nagler

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** K. Beider, E. Ribakovsky, M. Abraham, I. Weiss, O. Eizenberg, A. Peled, A. Nagler

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K. Beider, O. Eizenberg, A. Peled

**Writing, review, and/or revision of the manuscript:** K. Beider, E. Ribakovsky, A. Avigdor, E. Eizenberg, A. Peled, A. Nagler

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** K. Beider, E. Ribakovsky, E. Galun, A. Peled, A. Nagler

**Study supervision:** M. Abraham, E. Galun, A. Peled, A. Nagler

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Katia Beider, Elena Ribakovsky, Michal Abraham, et al.


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