CXCR4 antagonist 4F-benzoyl-TN14003 inhibits leukemia and multiple myeloma tumor growth

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Objective. The chemokine receptor CXCR4 and its ligand CXCL12 are involved in the progression and dissemination of a diverse number of solid and hematological malignancies. Binding CXCL12 to CXCR4 activates a variety of intracellular signal transduction pathways that regulate cell chemotaxis, adhesion, survival, proliferation, and apoptosis.

Materials and Methods. Here, we demonstrate that the CXCR4 antagonist, 4F-benzoyl-TN14003 (BKT140), but not AMD3100, exhibits a CXCR4-dependent preferential cytotoxicity toward malignant cells of hematopoietic origin. BKT140 significantly and preferentially stimulated multiple myeloma apoptotic cell death. BKT140 treatment induced morphological changes, phosphatidylserine externalization, decreased mitochondrial membrane potential, caspase-3 activation, sub-G1 arrest, and DNA double-stranded breaks.

Results. In vivo, subcutaneous injections of BKT140 significantly reduced, in a dose-dependent manner, the growth of human acute myeloid leukemia and multiple myeloma xenografts. Tumors from animals treated with BKT140 were smaller in size and weights, had larger necrotic areas and high apoptotic scores.

Conclusions. Taken together, these results suggest a potential therapeutic use for BKT140 in multiple myeloma and leukemia patients.

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Chemokines are a superfamily of small cytokines, named for chemoattractant activities [1,2]. The homeostatic chemokine CXCL12 and its receptor CXCR4 are critically involved in cancer progression and metastasis [3–7]. High surface expression levels of CXCR4 in acute myeloid leukemia (AML) cells correlate with reduced patient survival and a high probability of relapse [8–10]. The CXCR4-CXCL12 axis is also responsible for the retention of AML cells in the bone marrow and for increased AML cell survival and anticancer drug resistance [11,12]. CXCR4 and CXCL12 are involved in many aspects of multiple myeloma (MM) biology, including interaction and adhesion of MM to the bone marrow stromal cells and extracellular matrix components, proliferation, and migration of MM cells [11,13]. CXCR4 may facilitate the homing of malignant MM cells to the bone marrow environment that supports MM cell survival and proliferation and protects them from drug-induced apoptosis [14].

However, CXCR4 signaling can activate a variety of intracellular effector molecules, resulting in multiple biological effects including cell death [15]. During the last several years, a large body of evidence indicates that the HIV-1 envelope, glycoprotein gp120, induces CXCR4-dependent apoptosis of several uninfected cell types, including CD4$^+$ and CD8$^+$ T cells, peripheral blood mononuclear cells (PBMC), neuronal cells, and endothelial cells [16,17].

There are several known CXCR4 antagonists; 4F-benzoyl-TN14003 (BKT140) is a 14-residue polypeptide downsized and modified from a naturally occurring horseshoe crab protein. It is a highly selective and unique CXCR4 antagonist [18]. BKT140 binds CXCR4 with high affinity (1 nM) [19] and inhibits migration and adhesion of cells from hematopoietic origin [20]. It was demonstrated
that BKT140 can overcome stromal cell adhesion-mediated drug resistance of malignant cells [21]. Furthermore, it was recently shown that BKT140 effectively inhibits CXCL12-mediated osteoclast activity stimulated by the myeloma cell line RPMI8226 [22].

AMD3100 (Plerixafor), which is a byciclam, is a selective CXCR4 antagonist as well. Interestingly, Trent et al. [23] found that the structural basis for the interaction of BKT140 and AMD3100 with CXCR4 confirms that the mechanisms used by these agents are different. Furthermore, it was suggested that BKT140 acts as an inverse agonist, and AMD3100 acts as a weak partial agonist when the coupling of CXCR4 to Gz subunits in mammalian cells was assayed by [35S]GTPγS binding [24].

In the current study, we examined the effect of BKT140 on the survival and proliferation of human AML and MM cells in vitro and in vivo.

Materials and methods

Cell lines and CXCR4 antagonists

The following human cell lines were used in the study: acute promyelocytic leukemia cells NB4, HL-60 (CCL-240), acute T-cell leukemia cells Jurkat (TIB-152), MM cells U266 (TIB-196), RPMI8226 (CCL-155), NCI-H929 (CRL-9068), and ARH77 (CRL-1621). All cell lines were purchased from ATCC (Manassas, VA, USA) and cultured as described previously [25].

4F-benzoyl-TN14003 (BKT140) was kindly provided by Bio-Kine Therapeutics, Ness Ziona, Israel. AMD3100 (Plerixafor) was purchased from Sigma-Aldrich (St Louis, MO, USA).

Samples from human patients

Peripheral blood samples from patients with AML and bone marrow samples from patients with MM were collected during routine diagnostic procedures after informed consent was obtained in accordance with regulations of Chaim Sheba Medical Center (Tel-Aviv, Israel). Mononuclear cells were separated by Ficoll-Histopaque (Sigma-Aldrich) density-gradient centrifugation. CD34+ hematopoietic stem cells were purified using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cell viability and apoptosis assay

Hematopoietic cancer cell lines or normal PBMC or CD34+ cells were seeded at 2 × 10^5 viable cells/1 mL per well in a 24-well plate in triplicates in a medium supplemented with 0.1% fetal calf serum or 1% fetal calf serum and incubated with different concentrations of BKT140 or AMD3100 for 24 hours. BKT140 was treated with 1 M hydrochloric acid (HCl) to achieve a pH of 2.7 to 3 at room temperature for 30 minutes and the pH was adjusted to 7 using concentrated NaOH. Proteinase K (DakoCytomation, Glostrup, Denmark) was added to BKT140 at a final concentration of 100 µg/mL, incubated at 37°C for 1 hour, and inactivated by heat treatment (65°C for 30 minutes). After incubation, cells were stained with propidium iodide (PI; Sigma-Aldrich) and the percent of viable PI-negative cells in culture was determined by FACSscalibur (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA), using CellQuest software. Studies with a combination of PI-negative cells in culture was determined by FACScalibur (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA), using CellQuest software. Studies with a combination of PI-negative cells in culture was determined by FACScalibur (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA), using CellQuest software. Studies with a combination of PI-negative cells in culture was determined by FACScalibur (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA), using CellQuest software. Studies with a combination of PI-negative cells in culture was determined by FACScalibur (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA), using CellQuest software.
Flow cytometry analysis of caspase-3 activation
In order to detect the increase in caspase-3 cleavage and activation, BKT140-treated and untreated NB4 and RPMI8226 cells were harvested, fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, incubated with specific primary anticleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA, USA), followed by incubation with secondary fluorescein-conjugated antibody, and analyzed by flow cytometry.

Flow cytometric analysis
Cells were stained with human-specific antibodies and analyzed by FACSCalibur (Becton Dickinson) using the CellQuest software. For CXCR4 expression analysis, antihuman CXCR4 polyclonal anti–N-terminus antibody (Chemicon International, Temecula, CA, USA) was used. For CD138 or CD34 expression characterization, fluorescein-conjugated antihuman monoclonal antibodies were purchased from IQ Products (Groningen, The Netherlands).

Tumor xenografts and antitumor activity of BKT140
Severe combined immune-deficient (SCID)/beige mice (C.B-17/IcrHsd-SCID-bg) 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. NB4 cells resuspended in PBS were injected subcutaneously into the flanks of the mice (200 μL per mouse containing 5 × 10⁶ cells). Tumor growth was monitored daily, and mice were randomized to drug-treated or control PBS-treated groups (10 mice per group) when the tumor size (width × length) reached 0.04 cm². BKT140 was administered subcutaneously at a dose of 200 μg per mouse each day for 5 days.

RPMI8226 cells were mixed with Matrigel (Sigma-Aldrich) and were injected subcutaneously into the flanks of the mice (200 μL per mouse containing 10 × 10⁶ cells). According to the residual disease protocol, 24 hours after cell injection, animals were randomized to drug-treated or control PBS-treated groups (10 mice per group) and started to get treatment with different doses of BKT140 (100 μg per mouse or 300 μg per mouse, injected subcutaneously). BKT140 was administered each day for 5 days, followed by 2 days without drug and then 5 additional daily injections (total of 10 injections). Once palpable, tumors were measured using a vernier caliper and tumor size (width × length) was calculated. For the therapeutic protocol, once the RPMI8226 tumors reached 1 cm² in size, mice were randomized and treated with daily subcutaneous injections of BKT140, 300 μg per injection, for 5 days. At the end of the experiments, animals were sacrificed, tumors were harvested, measured, and weighed.

Statistical analysis
Data are presented as mean ± standard deviation or standard error. Statistical comparison of means was performed by a two-tailed unpaired Student’s t-test. Differences with a p < 0.05 were determined as statistically significant.

Results
BKT140 displays selective toxicity toward AML and MM cells
To determine the effect of CXCR4 antagonists on the survival and of leukemia and MM cell lines, cells were treated with BKT140 or AMD3100, and 24 hours later, cell viability was assayed. Treatment of MM cells RPMI8226, U266, NCI-H929, and ARH77 with BKT140, but not AMD3100, stimulated cell death with an IC₅₀ ranging from 4 to 8 μM (Fig. 1A). BKT140-dependent cell death of acute leukemia cells NB4, HL-60, U937, and Jurkat was less significant with an IC₅₀ ranging from 20 to 40 μM (Fig. 1B, data not shown). Similarly, BKT140 but not AMD3100-dependent cell death was observed in all bone marrow–derived MM and acute leukemia cells tested (Fig. 1C). Treatment of human keratinocytes, CD34⁺ hematopoietic stem cells and PBMC with BKT140 did not significantly alter their viability (Fig. 2A). These data indicate that BKT140 selectively induces cytotoxicity in MM and AML cells.

BKT140 overcomes the protective effect of interleukin-6 (IL-6) on MM cells
Previously, it was shown that the bone marrow niche provides proliferative and antiapoptotic signals to MM cells [26]. IL-6 is an important component of the bone marrow milieu that regulates growth and resistance of MM cells to chemotherapy. Therefore, we next examined whether BKT140 can overcome the protective effects of exogenously added IL-6. ARH77 were cultured in the presence of IL-6 (50 ng/mL) and BKT140. We found that treatment with BKT140 can overcome IL-6–dependent proliferation and survival of ARH77 MM cells (Fig. 2B).

BKT140 augments the anti-MM activity of a novel antimyeloma agent bortezomib
Combination therapy is a useful strategy for the treatment of MM patients. We next examined the effect of BKT140 treatment in combination with bortezomib, a reversible proteasome inhibitor with significant cytotoxic activity against myeloma cells. Treatment of ARH77 and U266 MM cells with BKT140 (8 μM) in combination with bortezomib (5 nM) significantly enhanced bortezomib-mediated cytotoxicity. In contrast, the combination of AMD3100 with bortezomib did not increase the cytotoxic effect of the drug (Fig. 2C).

BKT140 specifically triggers CXCR4-dependent cell death in leukemia and MM cells
To address the specificity of BKT140 in BKT140-induced cell death, the peptide was destroyed with HCL or proteinase K treatment before culturing with MM cells. HCL treatment causes denaturation and hydrolysis of the peptide, whereas treatment with the enzyme, Proteinase K, digests the peptide by proteolysis. As shown in Figure 3A, pretreatment of BKT140 with either HCL or Proteinase K totally inhibited the cytotoxic activity of BKT140 against RPMI8226 MM cells, suggesting that MM cell death is dependent on the presence of intact BKT140.

In order to verify the involvement of CXCR4 in hematopoietic malignant cell destruction by BKT140, we investigated the protective effect of CXCR4 blocking against BKT140-induced
cell death. High cell-surface expression and messenger RNA of CXCR4 were found in all leukemia and MM cell lines and primary cells (Fig. 3B). Preincubation of the leukemia cells NB4 and HL60, but not the MM cells RPMI8226, with the CXCR4 antagonist AMD3100 completely inhibited BKT140-dependent cell death (Fig. 3C). BKT140-induced cell death is associated with CXCR4 expression and is blocked by AMD3100 in a cell-type-dependent manner. Similarly, CXCR4-gp120–induced apoptosis blocked by AMD3100 is also cell-type-dependent [27,28].

**BKT140 stimulates apoptotic cell death in leukemia and MM cells**

Several lines of evidence suggest that BKT140 stimulates cell death through apoptosis. Treatment of leukemia (NB4) and MM (RPMI8226) cells with BKT140 induced a reduction in cell size and increased granularity (data not shown). Furthermore, BKT140 treatment increased the exposure of phosphatidylserine, tested by Annexin-V/7-AAD double staining, on the surface of leukemia (NB4) and MM (RPMI8226) cells (Fig. 4A).

To confirm the results obtained with Annexin-V staining and to determine the signaling pathway mediating the proapoptotic effect of BKT140, we measured the effects of BKT140 on mitochondrial membrane potential ($\Delta \Psi_m$) and caspase-3 activity. Mitochondrial membrane permeabilization is an important marker of intrinsic and extrinsic pathways of apoptosis induction [29]. Corresponding with Annexin-V–detected apoptosis, BKT140 reduced mitochondrial membrane potential ($\Delta \Psi_m$) in a dose-dependent manner in both NB4 and RPMI8226 cells (Fig. 4B). Caspase-3 activation plays an essential role in apoptosis [30]. BKT140

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**Figure 1.** BKT140 exerts potent specific cytotoxicity against human leukemia and MM cells in a dose-dependent manner. (A) MM cell lines RPMI8226, ARH77, U266, and NCI-H929; (B) AML cell lines NB4 and HL60, and (C) primary blood-isolated cells from patients with AML (AML patient 1 and AML patient 2) and bone marrow-isolated cells from patients with MM (MM patient 1 and MM patient 2) were treated with BKT140 or AMD3100 for 24 hours at the indicated concentrations and the viability was measured by flow cytometry using PI exclusion. Numbers of viable cells are presented as percents of untreated control. Each dot represents the mean ± standard deviation of triplicates from individual experiments ($**p < 0.005$).
treatment induced the degradation of procaspase-3 in a dose-dependent manner in both NB4 and RPMI8226 cells (Fig. 5A). Increased levels of active cleaved caspase-3 were only seen at the higher dose probably due to the low sensitivity of the FACS assay (Fig. 5B).

Treatment of leukemia and MM cells with BKT140 also increased the number of hypoploid cells (subG1 cells). Hypoploid cells are apoptotic cells that undergo degradation and subsequent leakage of nuclear DNA (Fig. 5C). Indeed, apoptosis-specific DNA fragmentation, detected by TUNEL staining and flow cytometry, was evident in both cell types (Fig. 5D). These data suggest that BKT140-induced mitochondrial damage and caspase-3 activation may result in the subsequent downstream DNA damage and the onset of apoptosis in AML and MM cells.

**BKT140 inhibits leukemia and MM tumor growth in vivo**

To investigate the potential of BKT140 to inhibit leukemia and MM tumor growth in vivo, we tested the effect of BKT140 on NB4 and RPMI8226 cells growing as a subcutaneous xenograft in immune-compromised mice. NB4 cells (5 × 10⁶ per mouse) were resuspended in PBS and injected subcutaneously into the flanks of SCID/beige mice. After 5 days of BKT140 administration, mean tumor size (width × length) of the treatment group was significantly lower than that of the control group (p < 0.008) (Fig. 6A, I). A significant reduction in mean tumor weight was also observed in the BKT140-treated group compared to the control group (53% growth inhibition; p < 0.02) (Fig. 6A, II). Moreover, TUNEL staining of NB4 tumor sections revealed massive apoptosis in the BKT140-treated group (Fig. 6A, III).

In order to establish myeloma xenografts, RPMI8226 cells (10 × 10⁶ per mouse) were suspended in Matrigel and injected subcutaneously into the flanks of SCID/beige mice. Two different treatment regimens were used. In the residual disease regime, mice were randomized 1 day after
the cell challenge to receive subcutaneous injections of BKT140 at 100 μg per mouse, 300 μg per mouse or a vehicle alone. BKT140 was administered each day for 5 days, followed by 2 days of rest and then five additional daily injections. As shown in Figure 6B, BKT140 treatment significantly inhibited subsequent myeloma tumor growth in a dose-dependent manner. On day 40 of the experiment, the 100-μg—treated group demonstrated a 37% inhibition of tumor growth ($p < 0.05$), whereas in the 300-μg—treated group a 78% inhibition of tumor growth was observed ($p < 0.0005$) (Fig. 6B, II). In the therapeutic mode, mice bearing 100-mm$^2$ tumors were split into two groups. The first group was treated with subcutaneous injections of BKT140 (300 μg per mouse) and the second group treated with vehicle only for 5 consecutive days. Tumor growth was significantly inhibited after treatment with BKT140 (Fig. 6C; $p < 0.01$). Moreover, TUNEL staining of tumor sections revealed massive apoptosis in the BKT140-treated group (Fig. 6B, III). Furthermore, treatment with BKT140 promoted extensive necrotic tissue damage in treated tumors (Fig. 6D). It is important to note that no major adverse effects of BKT140 injections were observed in treated animals.

Discussion

The CXCR4/CXCL12 pathway is a pivotal factor in cancer disease progression and thus a potential therapeutic target for cancer. Selective inhibition of CXCR4 using neutralizing antibody against CXCR4, small interfering RNA or specific CXCR4 antagonists, suppresses CXCL12-induced tumor growth, migration of cancer cells, invasion, neoangiogenesis, and metastases [3,31–35].

In this study, we examined the effect of the CXCR4 antagonists, AMD3100 and BKT140, on the survival of cancer cells of different origin. We found that BKT140 in
the range of 4 to 8 μm, but not AMD3100, exhibited preferential cytotoxicity toward malignant cells of hematopoietic origin, as compared to primary normal cells or solid prostate and breast tumor cells. Preliminary experimental data showed that micromolar concentrations of BKT140 in the blood of rats, dogs, and humans are achievable and accompanied with minor toxicity (data not shown).

MM is an incurable plasma-cell malignancy characterized by extensive lytic bone disease and microenvironmental changes of the bone marrow contributing to the persistence of the tumor and its resistance to the drugs [36]. The CXCR4/CXCL12 axis is critical for the homing of MM cells to the protective bone marrow niche [37]; it induces the proliferation of MM cells and protects them from drug-induced apoptosis [13]. AML is a heterogeneous group of diseases characterized by the uncontrolled proliferation of myeloid blasts with a reduced capacity to proliferate into mature cells [38]. Despite its sensitivity to chemotherapy, a majority of patients eventually relapse from minimal residual disease [21]. The bone marrow is the major site for minimal residual disease, where adhesion of AML and MM cells to bone marrow components may provide protection from the drugs [38]. CXCR4 is involved in the cross-talk between MM and leukemia cells and the BM microenvironment. It has been shown that CXCR4 regulates homing of AML cells to the BM [39], and adhesion of AML cells to the stromal fibronectin, which in turn protects the cells from spontaneous and drug-induced apoptosis [12].

Thus, the CXCR4/CXCL12 axis plays a crucial role in the homing and maintenance of MM and leukemia cells in the BM microenvironment. Previous studies provided evidence that targeting CXCR4 with the antagonist, AMD3100, increased the sensitivity of leukemia cells to chemotherapy [40,41]. Another recent study demonstrated that AMD3100 disrupted the interaction of MM cells with the bone marrow and sensitized them to therapeutic agents [42]. In addition to AMD3100, the effect of other CXCR4 antagonists, analogues of BKT140, were shown to enhance the antiproliferative and cytotoxic effects of anticancer agents in chronic lymphocytic leukemia [43].

Here we first demonstrated the direct and specific dose-dependent antileukemia and anti-MM cytotoxic effect of the CXCR4 antagonist BKT140. Although both BKT140 and AMD3100 are CXCR4-blocking agents, their properties and CXCR4 interaction and signaling mechanisms are different. AMD3100 demonstrates a weak partial agonist activity. In contrast, BKT140 has the characteristic

Figure 4. BKT140 induces cell apoptosis in leukemia and myeloma cells. NB4 and RPMI8226 cells were treated with BKT140 at concentrations of 8 μM and 40 μM for 24 hours. (A) Flow cytometric analysis of apoptosis with Annexin-V/7-AAD staining was performed. (B) Mitochondrial membrane potential (ΔΨm) was determined by flow cytometry using DiOC6 and PI staining. Viable cells stained only green; early apoptotic cells showed a decrease in staining with DiOC6 and late necrotic or secondary apoptotic cells stained fluorescent red. Data is presented as mean ± standard deviation from triplicates (**p < 0.01).
of an inverse agonist [24]. Having partial agonistic activities upon its binding to CXCR4, BKT140 may introduce its unique cell-specific proapoptotic signal.

CD34+ normal progenitors express a similar level of CXCR4 as NB4 or HL-60. Nevertheless, treatment with BKT140 selectively triggers an apoptotic pathway in leukemia and MM cells by disrupting mitochondrial membrane integrity, activating caspase-3, and promoting DNA damage. Selective CXCR4-promoted cell apoptosis is a well-established phenomenon in HIV-induced programmed cell death of uninfected cells. In confirmation with our results, others have shown that immune and endothelial cells undergo apoptosis via gp120-CXCR4 interaction. The apoptotic pathway, activated by gp120 binding, involves mitochondrial transmembrane depolarization and caspase-3 activation [44,45]. The mechanism of the recently described BKT140-induced apoptosis in leukemia and myeloma cells is similar to the previously characterized HIV-induced apoptosis.

CXCR4 can activate a variety of intracellular signal transduction pathways and effector molecules, resulting in different biological activities, such as cell chemotaxis, adhesion, survival, proliferation, and apoptosis. CXCR4 signaling differs between cell types and between malignant and normal counterparts and is dependent on multiple factors [46]. A unique combination of type of ligand, ligand concentration, CXCR4 expression level, cell-dependent membrane components, and available intracellular signaling machinery may determine a unique and specific cellular response. This fact can explain the observed strict selectivity of BKT140 cytotoxic effect against malignant cells of hematopoietic origin, as well as differential sensitivity of various hematologic neoplasms to the agent. MM cell lines demonstrated the highest sensitivity to BKT140-induced killing, whereas all tested leukemia cell lines responded to BKT140 treatment with a lower cell death percentage. In contrast, primary malignant leukemia and myeloma cells demonstrated a similar sensitivity pattern to that of cell lines. Furthermore, the antileukemia and myeloma tumor effects of BKT140 in vivo are similar, suggesting that, in addition to killing cells, BKT140 may inhibit other processes involved in the establishment of tumor growth. We have recently demonstrated that BKT140 stimulates normal hematopoiesis and induces a powerful mobilization of immune cells, such as T cells, monocytes

Figure 5. BKT140 induces release of cytochrome C, caspase-3 cleavage, and DNA fragmentation. NB4 and RPMI8226 cells were treated with BKT140 at concentrations of 8 μM and 40 μM for 24 hours. (A) Following treatment, whole-cell lysates were assayed by Western blots and levels of pro-caspase 3 were detected. β-actin detection was used to confirm equal protein loading. (B) The levels of cleaved caspase-3 were detected by intracellular staining using anti-cleaved caspase-3 antibody and analyzed by flow cytometry. Purple line represents the untreated cells; green line represents the cells treated with 8 μM BKT140 and pink line represents the cells treated with 40 μM. (C) DNA distribution in the cells was analyzed after 7-AAD staining. Each bar represents the mean ± standard deviation of triplicates from individual experiments (**p < 0.005). (D) DNA fragmentation was detected using TUNEL staining and flow cytometry analysis. Purple line represents the untreated cells; green line represents the cells treated with 8 μM BKT140 and pink line represents the cells treated with 40 μM.
and neutrophils\[47,48\]. These cells, in turn, may change the tumor microenvironment and support the antitumor effect of BKT140. CXCR4 plays an important role in the angiogenic process; BKT140 may thus inhibit this process and further inhibit tumor growth\[25\]. Importantly, normal human CD34\(^+\) hematopoietic stem cells exhibited relative resistance to BKT140 treatment in vitro.

IL-6 is an important component of the bone marrow microenvironment that acts as an antiapoptotic factor for MM and provides drug resistance within the marrow milieu. Importantly, BKT140 induced the cytotoxic effect in MM cells in the presence of exogenous IL-6, suggesting that BKT140 is able to overcome the protective effect of this cytokine. A combination of novel drugs is a useful strategy for the treatment of MM patients, to achieve enhanced antitumor efficacy. Thus, we combined BKT140 with the novel antimyeloma agent, bortezomib. Our data demonstrate that the combination of BKT140 with bortezomib results in an augmented antimyeloma effect in vitro, indicating the potential effectiveness of BKT140 with novel antimyeloma therapies.

Figure 6. BKT140 inhibits tumor growth in a mouse xenograft model. (A) NB4 (5 \(\times\) 10\(^6\)) cells were injected subcutaneously (SC) in the right flank of SCID/beige mice. Once the tumor sizes reached 0.04 cm\(^2\), BKT140 was administered subcutaneously at a dose of 200 \(\mu\)g per injection daily for the 5 days. (I) Mean \pm standard error (SE) tumor size (width \times length). Arrow on x-axis denotes the days of BKT140 injections. (II) Mean \pm SE tumor weight shown in grams of tumors harvested on day 31 (**\(p < 0.02\)), n = 5 for each group. (III) Representative slides of TUNEL-stained NB4 xenograft sections control vs. BKT140-treated. Cell nuclei are visualized using 4',6-diamidino-2-phenylindole (DAPI) staining, magnification \(\times 200\). (B) RPMI8226 (10 \(\times\) 10\(^6\)) cells mixed with Matrigel were injected SC in the right flank of SCID/beige mice. According to the residual disease regimen, mice received various doses of BKT140 (100 \(\mu\)g per injection or 300 \(\mu\)g per injection) subcutaneously, starting 24 hours after the cell injection, daily during 5 days, followed by 2 days without drug and then 5 additional daily injections (total of 10 injections). (I) Mean \pm standard error (SE) tumor size (width \times length). Arrows on x-axis denote the days of BKT140 injections. (II) Mean \pm SE tumor weight shown in grams of tumors harvested on day 58 (*\(p < 0.05\) and **\(p < 0.0005\)), n = 6 for each group. (III) Representative slides of TUNEL-stained NB4 xenograft sections control vs. BKT140-treated. Cell nuclei are visualized using DAPI staining, magnification \(\times 400\). (C) Therapeutic regimen. Once the RPMI8226 xenografts reached 1 cm\(^2\) in size, mice were randomized and treated with daily subcutaneous injections of BKT140, 300 \(\mu\)g per injection, during 5 days. Arrow on x-axis denotes the days of BKT140 injections. (I) Mean \pm SE tumor size (width \times length). (II) Mean \pm SE tumor weight shown in grams of tumors harvested on day 58 (**\(p < 0.01\)), n = 3 for each group. (D) Representative photo of RPMI8226-challenged mice, untreated vs. BKT140-treated at dose of 300 \(\mu\)g per injection.
Together with previous reports, our data support the notion that CXCR4 can initiate cell apoptosis. Moreover, for the first time, we demonstrate a unique proapoptotic effect of the CXCR4 antagonist BKT140, selectively directed against myeloma cells. Future studies need to fully understand the signaling mechanism that is involved in BKT140-induced apoptosis. Together, our findings provide a basis for the future clinical development of BKT140 as a potential novel therapeutic agent in hematological malignancies.

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Conflict of interest disclosure
Orly Eizenberg is an employee and shareholder of Biokine Therapeutics Ltd., Amnon Peled is a founder and shareholder of Biokine Therapeutics Ltd. (Ness Ziona, Israel). This work was supported in part by a grant from Biokine Therapeutics Ltd. (Ness Ziona, Israel).

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