Combination of Imatinib with CXCR4 antagonist BKT140 overcomes the protective effect of stroma and targets CML in vitro and in vivo

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ABSTRACT

Functional role of CXCR4 in CML progression was evaluated. Elevated CXCR4 significantly increased the in vitro survival and proliferation in response to CXCL12. CXCR4 stimulation resulted in activation of Erk1/2, Akt, S6K, STAT3 and STAT5 pro-survival signaling pathways. In accordance, we found that in vitro treatment with CXCR4 antagonist BKT140 directly inhibited the cell growth and induced cell death of CML cells. Combination of BKT140 with sub-optimal concentrations of imatinib significantly increased the anti-CML effect. BKT140 induced apoptotic cell death, decreasing the levels of HSP70 and HSP90 chaperons and anti-apoptotic proteins BCL-2 and BCL-XL, subsequently promoting the release of mitochondrial factors cytochrome c and SMAC/Diablo. BM stromal cells markedly increased the proliferation of CML cells and protected them from imatinib-induced apoptosis. Furthermore, BM stromal cells elevated proto-oncogene BCL6 expression in the CML cells in response to imatinib treatment, suggesting the possible role of BCL6 in stroma-mediated TKI resistance. BKT140 reversed the protective effect of the stroma, effectively promoted apoptosis and decreased BCL6 levels in CML cells co-cultured with BMSCs. BKT140 administration in vivo effectively reduced the growth of subcutaneous K562-produced xenografts. Moreover, the combination of BKT140 with low-dose imatinib markedly inhibited tumor growth, achieving 95% suppression.

Taken together, our data indicate the importance of CXCR4/CXCL12 axis in CML growth and CML-BM stroma interaction. CXCR4 inhibition with BKT140 antagonist efficiently cooperated with imatinib in vitro and in vivo. These results provide the rational basis for CXCR4-targeted therapy in combination with TKI to override drug resistance and suppress residual disease.
INTRODUCTION

Chronic myeloid leukemia (CML) is myeloproliferative disease of hematopoietic stem cells that is driven by the constitutively active oncogenic tyrosine kinase BCR-ABL (1, 2). Inhibition of BCR-ABL activity by tyrosine kinase inhibitors (TKIs) such as imatinib mesylate, revolutionized the treatment of CML and remains a major therapeutic strategy for CML patients (3). However, existence of quiescent leukemic stem cells (LSCs) that are resistant to TKIs and may be responsible for CML resistance and recurrence continues to pose challenges for CML cure (4, 5). Thus, novel therapies that eradicate LSCs are in need.

The bone marrow (BM) microenvironment is believed to have a role in protecting LSCs and CML cells from TKIs-induced apoptosis (6). CXCL12, also called stromal cell-derived factor-1 (SDF-1), a chemokine produced by BM stromal cells, signals through the cognate chemokine receptor CXCR4, which is broadly expressed by normal and malignant cells of haematopoietic and non-haematopoietic origin (7). Data from knockout mice indicate that the CXCR4 receptor plays an important role in haematopoiesis, development, and organization of the immune system (8-11). CXCR4 and its ligand CXCL12 have a key role in the trafficking and retention of normal hematopoietic as well as LSCs in the BM niche (12-14). Furthermore, stroma-derived CXCL12 supports the proliferation of hematopoietic stem cells (HSCs) and prevents their terminal differentiation (15). In CML, leukemia-stroma interactions are impaired. CML adhesion to ECM components and BM stroma was shown to mediate malignant phenotype and protect CML cells from TKI treatment through various molecular mechanisms (16-18). Our previous work showed that the CXCR4 dependent migration of immature CD34 + CML cells is impaired and that the integrin-dependent migration and adhesion in response to
CXCL12 is decreased (19). Others have found that the BCR-ABL oncoprotein interferes with CXCR4-mediated signaling, and suppresses the CXCL12-induced chemotactic response of CML cells (20, 21). Importantly, recent studies demonstrated that CXCR4 can be elevated by TKI treatment and may induce stroma-promoted chemo-resistance (22, 23).

Elucidating the role of the BM milieu and the CXCR4/CXCL12 axis in the response to TKIs and in disease progression may provide a rational basis for the development of novel therapies for CML eradication. A potential strategy to overcome the stroma-provided drug-resistance is to interrupt the CML-stroma cross-talk utilizing CXCR4 neutralization methods. We hypothesized that CXCR4 blockade with specific antagonists can inhibit the survival and spread of CML and LSC cells and may restore their sensitivity to TKIs in the BM microenvironment context.

The present work demonstrates that CXCR4 over-expression in the CML cell line K562 does not increase the migratory potential of CML cells, but rather supports their survival and growth, therefore confirming the pro-survival role of CXCR4 in CML.

Previously, we have shown that the high affinity CXCR4 antagonist BKT140 demonstrates an effective anti-tumor effect both in vitro and in vivo in various hematological malignancies and enhances the pro-apoptotic effect of anti-leukemic compounds (24). In the current study we evaluated the effect of BKT140 on CML cell viability and sensitivity to imatinib in the presence of BM stromal cells. Our results indicate that CML cells are protected by the BM stroma from imatinib-induced apoptosis, and significantly up-regulate expression of the anti-apoptotic factor BCL6 in response to imatinib treatment. BKT140 enhances imatinib activity and disrupts the stroma-mediated protection. Moreover, BKT140 demonstrates a potent anti-CML activity in vivo in a xenograft model.
MATERIALS AND METHODS

Cell lines and CML patient samples

CML cell lines K562 (obtained from ATCC, Rockville, MD) and LAMA-84 (obtained from DSMZ, Braunschweig, Germany), and NK cell line YTS (kindly provided by Prof. Ofer Mandelboim, Hebrew University, Jerusalem, Israel) were maintained at log growth in RPMI1640 medium (Gibco Laboratories) containing 10% fetal calf serum (FCS), sodium pyruvate, 1mM L-glutamine, 100 U/mL penicillin, and 0.01 mg/mL streptomycin (Biological Industries). K562 and LAMA-84 cells were authenticated by STR DNA fingerprinting using AmpFISTR Identifier Kit (Applied Biosystems). YTS cells were not authenticated.

BM samples from informed-consented newly diagnosed CML patients were obtained in accordance with institutional guidelines. Mononuclear cells were collected after standard separation on Ficoll-Paque (Pharmacia Biotech).

Inhibitors

BKT140, a CXCR4 antagonistic peptide, was provided by Biokine Therapeutics and dissolved in sterile saline. Imatinib was purchased from Cayman and dissolved in sterile dimethyl sulfoxide (DMSO). Final concentration of DMSO in cultured cells was less than 0.001%.

Cell line transduction

The retroviral vector pLNC/Luc was prepared by introducing the firefly luciferase gene (luc) downstream of the CMV promoter in the plasmid vector pLNC carrying the Neomycin resistance gene (25). K562 cells were stably transduced with a retroviral vector, using transfer vector- pLNC/Luc and the packaging plasmid- pCL. K562L cells were then transduced again with lentiviral vectors to produce K562 cells co-expressing the luc and the GFP genes (K562LG) or the luc and the bicistronic...
CXCR4-GFP genes (K562LG-CXCR4). This was done using a three-plasmid system: transfer vector- pHRE-CMV-GFP-WPRE or pHRE-CMV-CXCR4-IRES-GFP-WPRE; envelope coding plasmid- VSV-G and a packaging construct- CMVDR8.91. The NK cell line, YTS was similarly transduced and YTSG and YTSG-CXCR4 cells were generated to be used as a negative (Ph-) control.

**Analysis of positively transduced cells**

Cells were analyzed for GFP on a FACSCalibur (Beckton Dickinson) using CellQuest software. Luciferase activity was evaluated after positive selection by luminometer using the Luciferase assay system according to the manufacturer’s instructions (Promega Corp.).

**Flow cytometry analysis of CXCR4 and CXCR7 expression**

Expression levels of the chemokine receptor CXCR4 and CXCR7 by immunostaining with PE-conjugated anti-CXCR4 monoclonal antibody (12G5 clone) (R&D Systems) and PE-conjugated anti-CXCR7 antibody (eBioscience). In primary CML samples, counterstaining with anti-CD34 fluorescein-conjugated antibody was performed.

**Cell migration assay**

Migration assay was performed in triplicates using 5-µm pore size Transwells (Costar). The lower compartment was filled with 600 µl of 1% FCS RPMI 1640 medium containing CXCL12 (500 ng/ml) (PeproTech EC), and 5x10^5 cells in 100 µl of 1% FCS RPMI1640 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.

**RT-PCR analysis**

Total RNA from cells was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Subsequently, cDNA was generated from 1 µg of total
RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). CXCR4 and BCL-6 mRNA levels were evaluated using SYBR Green quantitative PCR. The qPCR reaction contained 100 ng of total RNA-derived cDNAs, forward and reverse primers (300 nM) and PerfeCta SYBR Green FastMix (Quanta Biosciences), and was performed using StepOnePlus Real Time PCR system (Applied Biosystems). Changes in expression levels were normalized to control β2-microglobulin using \( \Delta \Delta C_T \) method of relative quantification using StepOne Software v2.2. Primer sequences are presented in supplementary table 1.

**Survival assay**

K562 or BM-derived primary CML cells were cultured 2x10^5 cells/ml, in triplicates, in 0.1% FCS, in the presence or absence of CXCL12. Cultured cells were collected after 48 hours incubation and the number of cells was enumerated by FACS. Events were acquired during 30 seconds. Dead cells were eliminated by staining with PI. Relative number of viable cells in each sample was determined. To confirm the normalized flow rate and ensure accurate cell count, fixed cell concentration was counted prior to the experiment.

**Apoptosis assay**

Apoptosis was determined by staining with Annexin V-FITC and PI according to manufacturer’s instructions and analyzed by flow cytometry. The percentage of early apoptotic (Annexin V^+/PI^-) and late apoptotic/dead (Annexin V^+/PI^+) cells was quantified.

**Assessment of mitochondrial membrane potential (\( \Delta \Psi_m \))**

The cationic lipophilic fluorochrome DiOC6 (Sigma-Aldrich) was used to measure the \( \Delta \Psi_m \). Briefly, CML cells were cultured with different concentrations of BKT140 and imatinib 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 37°C. The cells were then analyzed by FACS for the loss of DiOC6 fluorescence.

**Cell Cycle Analysis**

CML cells were exposed in vitro to increasing concentrations of BKT140 and imatinib for 48 hours. Cells were collected, washed with cold PBS, and 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°C for 15 min. Cells were then stained with 10 µg/ml 7-amino-actinomycin D (7-AAD) (eBioscience) in dark for 30 min. DNA content was detected using FACS.

**Colony growth inhibition**

K562 cells pre-treated with BKT140 (20 µM), imatinib (0.5 µM) or combination of both agents for 24 hours, were harvested, suspended in culture medium and plated in soft agar in 24-well plates. Following 2 weeks of incubation, colonies were counted and photographed.

**Co-culture experiments**

Primary human bone marrow stromal cells (BMSCs) were isolated and expanded from bone marrow aspirates of healthy donors after signed informed consent as previously described (26). CML cells were labeled with CFSE (5 µM) and seeded on the top of stromal cells, alone or in combination with BKT140 and /or imatinib, 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 (including BMSCs and CML cells) was harvested with trypsin/EDTA. The cells were washed with PBS and analyzed by FACS for viability (using PI exclusion), proliferation. CFSE-unlabeled BMSCs were gated out.
**BrdU incorporation assay**

To evaluate the effect of BMSCs on CML cell proliferation, CFSE-labeled K562 and LAMA-84 cells were cultured in the absence or presence of BMSCs monolayer in a medium supplemented with 1% FCS. Following 36 hours of co-culture, the cells were pulsed with BrdU for additional 12 hours and analyzed for BrdU incorporation using APC BrdU Flow Kit (BD Biosciences) according to the manufacturer's instructions. CFSE-unlabeled BMSCs were gated out. Percent of proliferating BrdU+ CML cells was determined.

**XTT viability assay**

K562 and LAMA-84 cells (2x10⁴ per 100 µl per well) were plated in 1% FCS-containing medium in 96-well flat plates in quadruplicate samples, with different concentration of BKT140, imatinib or a combination of both, in the absence or presence of BMSCs for 48 hours. Cell viability was assessed using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) assay (Biological Industries).

**ELISA**

CXCL12 levels in culture medium were quantified using ELISA (Quantikine Human CXCL12/SDF-1 immunoassay, R&D Systems) according to manufacturer's instructions.

**Immunoblot analysis and proteome arrays**

For immunoblotting total protein lysates (50-70 µg) were resolved by electrophoresis on 10% SDS-PAGE and transferred onto PVDF membranes. Blots were subjected to a standard immuno-detection procedure using specific antibodies and the ECL substrate (Biological Industries). Signal was detected using a Bio-Rad image analyzer (Bio-Rad). Primary antibodies were as follows: pErk1/2 (Sigma-Aldrich), pAKT
(R&D Systems), pS6 and BCL-XL (Cell Signaling Technology), BCL-2 (Dako), HSP70 and HSP90 (Stressgen Biotechnologies Corp.), β-actin (Sigma-Aldrich).

Human Phospho-Kinase Array Kit (R&D Systems) was used to measure the activation of signal transduction in CML cells in response to CXCL12 stimulation. The human Apoptosis Array Kit (R&D Systems) was used to measure the level of expression of pro- and anti-apoptotic proteins before and after the addition of BKT140. Relative expression of proteins was determined following quantification by the Image-Pro plus program.

**Cytochrome C release**

Release of mitochondrial cytochrome c to the cytosol of BKT140- and imatinib-treated CML cells was measured by flow cytometry using the FlowCellect Cytochrome C kit with direct FITC-conjugated anti-cytochrome C antibody (Merck Millipore), according to the manufacturer's instructions.

**Quantification of intracellular phospho-STAT5 by flow cytometry**

Cells were serum-starved overnight, then incubated either with or without CXCL12 (200 ng/ml) for 5 and 30 minutes, washed with PBSx1, fixed with 4% PFA, permeabilized with ice-cold methanol (90% final), washed and immunostained with PE-conjugated anti-phospho-STAT5 antibody or isotype control (eBioscience).

**In vivo xenograft model of human CML in NOD/SCID mice**

Immunodeficient NOD/SCID mice (NOD/LtSz PrKdc scid/PrKdc scid) (27) were maintained under defined flora conditions at the Hebrew University Animal Pathogen Free Facility. The Animal Care Committee of the Hebrew University approved all experiments. Twenty four hours prior to intravenous (i.v.) injection of cells, mice were irradiated with 375 RAD. K562LG cells (5x10⁶/mouse) were injected i.v. and
monitored for tumor cell distribution 24 and 48 hours later. In vivo imaging was performed as previously described (28).

For local tumor generation, K562L cells \((5 \times 10^6/mouse)\) were injected subcutaneously (s.c.) into the left flank. Tumor growth was followed using caliper measurement. BKT140 was administered s.c. 400 \(\mu\)g per mouse per injection, in a site different to tumor injection. Imatinib was injected i.p., 10 mg/kg. Control animals were treated with saline.

**Statistical analyses**

Data are expressed as the mean ± standard deviation (SD), or standard error (SE). Statistical comparisons of means were performed by a two-tailed unpaired Student's \(t\) test.
RESULTS

High CXCR4 expression promotes in vitro survival and proliferation, but not migration of the K562 cells in response to CXCL12

We and others have demonstrated the role of CXCR4 in aberrant migration and adhesion of primary CML cells (19, 29). However, BCR-ABL-positive CML cell lines express low cell-surface and mRNA levels of CXCR4. In order to study the in vitro and in vivo roles of CXCL12/CXCR4 in CML, we generated a K562 cell line that expresses high levels of CXCR4, similar to the levels observed in primary CML CD34+ cells. For in vivo monitoring of tumor growth and migration, K562 cells were first stably transduced with a retrovirus carrying the luciferase (luc) gene (K562L). FACS analysis demonstrated that more than 80% of K562LG-CXCR4 cells and more than 72% of the control YTS-CXCR4 cells expressed high levels of CXCR4 on their cell surface (Supp. Fig. 1A, B).

To investigate the possible role of CXCR4 in mediating CML migration in response to CXCL12, K562 or K562LG-CXCR4 cells, were tested for their migration potential. Both cell lines migrated poorly in response to CXCL12. In contrast, Ph- cells overexpressing CXCR4 (YTS-CXCR4) increased their migration in response to CXCL12 by 8-folds compared to YTS cells not expressing the receptor CXCR4. These results indicate functional activity of the transduced CXCR4 receptor in YTS cells, while its signaling for chemotaxis is impaired in K562LG-CXCR4 cells (Supp. Fig. 1C).

Next, we generated single-cell clones with high and stable level of CXCR4 expression, comparable to CXCR4 expression by primary CML CD34+ cells (Supp. Fig. 1D, E). Neither K562 nor K562LG-CXCR4.86 cells expressed CXCR7 (Supp. Fig. 1F). K562 cells with high cell-surface CXCR4 (K562LG-CXCR4.86)
demonstrated dose-dependent in vitro proliferation response to CXCL12 stimulation that was higher compared to native K562 cells. Proliferative rate of K562LG-CXCR4.86 in response to CXCL12 was comparable with that of primary CD34+ CML cells (Supp. Fig. 1G).

**CXCR4 signaling in CML cells**

To delineate the signaling pathways activated by CXCR4 in CML cells, K562 and K562LG-CXCR4.86 cells were stimulated with CXCL12 and protein phosphorylation was analyzed by the Phospho-kinase Proteome Profiler Array. In high CXCR4-expressing cells CXCL12 treatment resulted in profound activation of survival pathways, including increase in phosphorylated Erk1/2, AKT, STAT5, STAT3, S6K, RSK1 and WNK1. In contrast, low CXCR4-expressing K562 cells poorly responded to CXCL12 stimulation, mainly by a delayed increase in pAKT (following 30 minutes of treatment) (Fig. 1A).

To verify the results and determine the level of signaling mediators’ activation in response to CXCR4 stimulation, FACS and immunoblot analysis were performed. CXCL12 treatment increased the levels of pSTAT5 in CXCR4-expressing K562 cells, as confirmed by phsopho-flow analysis (Fig. 1B). Furthermore, rapid phosphorylation of Erk1/2 and AKT occurred as early as 5 min in K562LG-CXCR4.86 cells, and started diminishing 30 minutes post-treatment. Conversely, in K562 cells, CXCL12 resulted in very weak Erk1/2 and delayed (post 30 min) AKT activation (Fig. 1C). Interestingly, increased phosphorylation of the mTOR target S6 were detected in CXCR4-expressing K562 cells. Together, these findings indicate that CXCR4 activates multiple pro-survival signaling pathways in CML cells.

**CXCR4-specific high affinity antagonist BKT140 inhibits proliferation of K562 cells**
The enhanced proliferative response of K562LG-CXCR4.86 cells to CXCL12 stimulation prompted us to study the effect of the CXCR4 antagonist BKT140 (Supp. Fig. 2) on CML cell survival and proliferation. K562 cells with low and high levels of CXCR4 were treated with increased concentrations of BKT140 for 48 hours. BKT140 treatment reduced viability and correspondingly increased cell death, in a dose-dependent manner, in both low- and high-CXCR4-expressing K562 cells. Notably, K562LG-CXCR4.86 cells were significantly more sensitive (p<0.01) to low concentrations of BKT140 (4 and 8 µM) than native K562 cells (Fig. 2A).

Since CXCL12 stimulated the growth of CXCR4-expressing K562 cells and has been shown to mediate chemoresistance of leukemic cells in BM milieu, we examined whether BKT140 can overcome the protective effect of exogenous CXCL12. Importantly, CXCL12 treatment did not inhibit BKT140-induced cytotoxicity against K562LG-CXCR4.86 cells (Fig. 2B).

These results illustrate the anti-proliferative potential of BKT140 in CML cells and indicate a role of CXCR4 in BKT140-induced anti-CML effect.

**BKT140 co-operates with imatinib in vitro and effectively inhibits CML growth**

To further elucidate the role of CXCR4 and its inhibitor BKT140 on CML growth in vitro, BKT140 was combined with the TKI imatinib and their effect on CML viability was evaluated. CML cells K562 and LAMA-84 were treated with low concentrations of imatinib (0.1 µM or 0.5 µM) either alone or in combination with BKT140 (8 µM) for 48 hours. Treatment of K562 cells with single agent imatinib (0.1 µM) or BKT140 (8 µM) did not significantly alter cell viability, while the combination of both agents promoted significant cell death. LAMA-84 cells demonstrated a higher sensitivity to imatinib and BKT140 single therapy, but similarly to K562, the combination of both agents significantly increased cell death in culture (Fig. 2C). This cytotoxic effect of
combinatory treatment with imatinib and BKT140 against CML cells was also demonstrated in microscopic evaluation of the cells in culture (Fig. 2D). These results suggest that BKT140 enhances the anti-CML effect of imatinib.

**BKT140 treatment induces mitochondrial-dependent apoptosis in CML cells**

To analyze the mechanism of BKT140-induced cytotoxicity in CML cells, we next examined phosphatidylserine exposure, a hallmark of apoptosis, using Annexin V combined PI staining method. An accumulation of Annexin-V positive cells was observed following imatinib treatment confirming early stage apoptosis induction, while BKT140 increased the number of both early apoptotic (Annexin V+/PI-) and late apoptotic/dead (Annexin V+/PI+) cells (Figure 2E). Moreover, combination of imatinib with BKT140 significantly increased the number of Annexin V/PI double positive cells, indicating subsequent cell death (Fig. 2E, F).

Next, we questioned whether the combined pro-apoptotic effect of imatinib and BKT140 is mediated through the mitochondria using DiOC6 staining. Mitochondrial membrane permeabilization is an important marker of intrinsic and extrinsic pathways of apoptosis induction (30). We measured the effects of BKT140 and imatinib on the mitochondrial membrane potential ($\Delta \Psi_m$) in K562 cells. Each agent alone promoted modest mitochondrial depolarization. Remarkably, the combination of BKT140 with imatinib significantly reduced the $\Delta \Psi_m$ and increased the percent of apoptotic cells to 68% (Fig. 2G, H). These results correspond with the results obtained from viability assays and detect apoptotic pathway with mitochondrial involvement.

**BKT140 reduces clonogenic potential of K562 cells and targets primary CML CD34+ cells in vitro**

Next, we assessed the effect of BKT140 on the clonogenic potential and colony formation ability of K562 cells. Figure 3A demonstrates that BKT140 alone markedly
inhibited soft agar colony growth, reducing the number and size of colonies comparing to untreated cells (p<0.01). Combination of BKT140 with imatinib further suppressed colony formation, achieving 97% suppression comparing to control (p<0.01) (Fig. 3B). We also determined the effect of BKT140 treatment on primary CML CD34+ cells procured from newly diagnosed CML patient. BKT140 alone decreased the percent of viable CML CD34+ cells in culture in a dose-dependent manner. However, co-treatment with imatinib further promoted cell viability loss (Fig. 3C, D). These findings demonstrate the ability of BKT140 to inhibit the clonogenic potential of CML cells and possibly to target CML stem cells.

**Effect of BKT140 treatment on apoptotic signaling in CML cells**

To investigate the involvement of apoptotic signaling molecules following BKT140 treatment, expression level of apoptosis related proteins were analyzed using antibody array. For this, K562 and LAMA-84 cells were treated with BKT140 (20 µM) for 24 hours, cell lysates were prepared and apoptosis antibody array was performed. Our results show that a number of apoptotic signaling proteins were modulated following treatment with BKT140 (Fig. 4A). Among the pro-apoptotic proteins, increase in catalase, TRAIL R2/DR5 and SMAC/Diablo was detected in both cell lines. Increase in pro-apoptotic Bad and Bax was determined in LAMA-84 cells. On the other hand, several anti-apoptotic proteins were down-regulated by BKT140, including BCL-2, BCL-XL and XIAP. Interestingly, significant reduction in HIF1a and HSP70 level was detected in both cell lines treated with BKT140. In order to confirm these results, effect of BKT140 treatment on chaperon protein levels in CML cells was evaluated by immunoblotting. As demonstrated in Figure 4B, BKT140 reduced the levels of HSP70 and HSP90 in both K562 and LAMA-84 cells in a time- and dose-dependent
manner. Moreover, reduction in BCL-2 and BCL-XL levels was observed in K562 cells treated with high concentrations of BKT140 (Fig. 4B).

In addition, BKT140 dose-dependently induced cytochrome C release, a late apoptotic event associated with loss of mitochondrial integrity. Combination of low-dose BKT140 (8 µM) with imatinib significantly increased imatinib-induced release of cytochrome C (Fig. 4C). These data suggest that BKT140 triggers mitochondrial alterations, followed by cytochrome C release and apoptosis.

**Interaction of CML cells with bone marrow stromal cells (BMSCs)**

In order to study the cross-talk between CML cells and the non-malignant BM counterparts, an in vitro co-culture system was established. For this purpose K562 and LAMA-84 cells were seeded on a pre-established monolayer of primary BM stromal cells. Microscopic observation revealed that K562 cells remained mainly in the floating fraction, whereas LAMA-84 cells incorporated into the BMSCs monolayer and produced so called “cobblestone” structures (Fig. 5A). Generated BMSCs produced detectable levels of CXCL12, confirmed by ELISA (Fig. 5B).

Effect of stromal cells on CML proliferation in serum-reduced conditions was evaluated using BrdU incorporation method. Population of CFSE-labeled CML cells was distinguished from stromal cells (Figure 5C). BMSCs significantly (p<0.01) increased the percent of proliferating K562 and LAMA-84 cells (Fig. 5D). Imatinib increased the surface expression of CXCR4 in LAMA-84 cells (Fig. 5E), demonstrating the undesirable up-regulating effect of TKIs on CXCR4, that can potentially promote the CML-stroma protective interactions.

Next, we tested the effect of BMSCs on imatinib sensitivity. In accordance with previous reports, incubation with stromal cells significantly reduced the sensitivity of K562 cells to imatinib and completely blocked its inhibitory effect in LAMA-84 cells,
therefore demonstrating the stromal-provided protection. We then used BKT140 to study whether the sensitivity of CML cells to imatinib can be restored by inhibition of the CXCR4 axis. Cell viability was tested using the XTT method. Interestingly, low concentration (8 µM) of single agent BKT140 in the presence of BMSC had no significant effect on K562 viability, and even increased the proliferation of LAMA-84 cells. However, a combination of BKT140 with imatinib or usage of higher concentration of BKT140 (20 µM) reversed the protective effect of BMSCs and significantly impaired CML proliferation (Fig. 5F).

These results suggest the ability of CXCR4 antagonist BKT140 to disrupt the interaction between CML and BM stromal cells, restoring the sensitivity to imatinib.

**BKT140 releases BMSC-induced CML cell dormancy and effectively targets CML cells in the presence of supportive BMSCs**

To evaluate the mechanisms involved in the anti-CML effects induced by imatinib and BKT140, we tested the cell cycle of K562 cells treated with each agent alone or in combination, in the absence or presence of BMSCs. Figure 5G demonstrates representative histograms of DNA content in K562 cells cultured for 48 hours in serum-deprived conditions. In the absence of stroma, each agent alone reduced the percent of proliferating (G2/M+S phase) cells. Combination of imatinib with BKT140 further significantly decreased the percent of cycling cells (Fig. 5H, a). Co-culture with BMSCs slightly increased the percent of K562 cells in G2/M+S phases, (from 22% without BMSCs to 30% in the presence of BMSCs, respectively). Imatinib treatment did not significantly affect the percent of cycling cells in the presence of stroma, whereas BKT140 slightly increased the percent of cycling cells. Importantly, the combination of imatinib with BKT140 considerably decreased the percent of proliferating cells even in the presence of BMSCs (Fig. 5H, b). These results could
reflect the fact that blockade of CXCR4/CXCL12 axis can overcome stroma-mediated CML cell quiescence and make the cells more susceptible to the anti-proliferative effect of imatinib.

**BMSCs enhance the imatinib-induced increase in BCL6 in a CXCR4-dependent manner**

Recently, a new mechanism for imatinib resistance in CML cells was reported. Proto-oncogenic transcription factor BCL6 was found to be elevated upon imatinib treatment and was required for the survival of CML initiating cells (31). Since BM stroma protects CML cells from imatinib-induced apoptosis, we studied whether BCL6 is involved in the stroma-mediated protection. Corresponding with previously reported data, imatinib increased BCL6 expression in both CML cell lines. Moreover, BCL6 mRNA levels were even further increased by imatinib treatment in the presence of BMSCs, suggesting the involvement of BCL6 in stroma-mediated resistance to TKIs. To evaluate the possible regulatory role of CXCR4 in imatinib-mediated up-regulation of BCL6, we assessed the levels of BCL6 in CXCR4-transduced K562 cells. K562LG-CXCR4.86 cells expressed significantly higher levels of BCL6 in comparison to the native K562, and imatinib treatment in co-culture of K562LG-CXCR4.86 and BMSCs further elevated BCL6 levels in the CXCR4-expressing K562 cells. Notably, BKT140 treatment effectively abrogated the imatinib-induced increase in BCL6 in K562 as well as in K562LG-CXCR4.86 and LAMA-84 cells (Fig. 5I).

**BKT140 effectively targets K562 CML cells in vivo**

To assess the in vivo activity of BKT140, a CML xenograft model was established. Luciferase-expressing K562 cells were injected i.v. or s.c. into NOD/SCID mice and monitored using a CCCD camera. Intravenously-injected K562L cells did not produce systemic disease and no spread to the vital organs was detected. In contrast,
subcutaneously-injected K562L cells (5x10^6) produced fast-growing locally invasive tumors, without systemic spread to the spleen or bone marrow (data not shown).

Therefore, we used the local tumor model to evaluate the effect of BKT140, alone or in combination with imatinib. Three days following cell inoculation, animals were started on treatment with BKT140 (400 µg/mouse), imatinib (10 mg/kg), or a combination of both agents in accordance to the schedule presented in Figure 6A. Imatinib dose was chosen based on previous report showing that daily treatment with 10 mg/kg partially inhibited tumor growth of BCR-ABL-transformed 32D cells (32). In accordance, imatinib treatment partially inhibited the growth of K562 xenograft, achieving 52% suppression in tumor size. Importantly, BKT140 single-agent treatment was able to significantly reduce tumor burden by 46%. Significantly, imatinib and BKT140 combined treatment essentially abrogated tumor growth, resulting in 95% reduction in tumor size and weight (Fig. 6B, C). Histological evaluation of hematoxylin and eosin (H&E)-stained tumor sections demonstrated that combination of BKT140 with imatinib promoted extensive necrotic tissue damage in treated tumors (Fig. 6D).

These results demonstrate the potent in vivo effect of BKT140 in tumor burden reduction of CML, and reinforce the role of CXCR4/CXCL12 axis in CML growth and progression in vivo.
DISCUSSION

In this study we investigated the role of CXCR4 in CML progression. Using a lentiviral transduction method CXCR4 was exogenously elevated in low-expressing K562 cell line to levels comparable with primary CD34+ CML cells. Our data indicate impaired migration ability and support a pro-survival role of CXCR4 signaling in CML. CXCR4 is expressed by a broad range of normal and malignant hematopoietic cells and regulates chemotaxis, cell survival and proliferation in CXCL12-expressing niches (33-37). Consistent with previously reported data, our results demonstrate that CXCR4 is important for CML survival and growth.

A well-known aspect of BCR–ABL transformation is its ability to activate multiple signaling pathways that lead to proliferation, reduced growth factor-dependence and apoptosis, and abnormal interaction with extra-cellular matrix and stroma. However, these pathways can be also triggered by external microenvironmental signals, in BCR-ABL-independent manner. Here we show that CXCR4 stimulation in CML cells results in profound activation of numerous signaling pathways, resulting in phosphorylation of Erk1/2, AKT, p70S6K, STAT3 and STAT5. MAPK, PI3K and JAK-STAT signaling are known to be involved in BCR-ABL-independent TKI-resistance and represent targeting opportunities in CML (38). For example, STAT5 was shown to mediate imatinib resistance and was up-regulated in leukemic cells from imatinib-resistant patients (39). Similarly, CML cell lines cultured with HS5 stromal cells were protected from imatinib-induced apoptosis by STAT3 up-regulation (40). Here we provide evidence for a mechanistic role of CXCR4 in activation of pro-survival signaling pathways in CML that was not previously described.
A large body of evidence, including our previous work, indicates that the p210\(^{\text{BCR-ABL}}\) onco-protein impairs the function of CXCR4/CXCL12 pathway in CML cells (19). Using leukemia MO7e cells Geay et al. demonstrated that p210\(^{\text{BCR-ABL}}\) inhibits the chemotactic response to CXCL12 and down-regulates CXCR4 expression (21). Ptasznik and colleagues demonstrated that p210\(^{\text{BCR-ABL}}\) alters CXCL12-mediated adhesion through beta2 integrin (41). The mechanism underlying BCR-ABL-mediated inhibition of CXCR4 function in CML cells involves the Src-related kinase Lyn. It was found that BCR-ABL constitutively activates Lyn in CML cells, resulting in Lyn becoming unresponsive to CXCL12-mediated signaling and promoting the egress of immature cells from the BM (20). Importantly, it was demonstrated that TKI treatment restores CXCR4 expression and CXCR4-mediated migration toward the BM niche, therefore promoting stroma-mediated resistance of CML cells to TKI (22).

In agreement with previously reported data demonstrating an up-regulation of CXCR4 in K562 and KBM-5 cells upon imatinib treatment, our results show a similar increase in CXCR4 surface levels in LAMA-84 cells, therefore supporting a protective role for CXCR4-mediated stroma interaction. Indeed, we found that adhesion to BMSCs increased the proliferation of CML cells and protected them from imatinib-induced apoptosis. Furthermore, previous work has demonstrated that adherent sub-populations of CML cell lines retained increased malignant properties (16).

Based on this data, we hypothesized that in the presence of BMSCs, CXCR4 blockade with the specific antagonist BKT140 could restore CML cells’ sensitivity to imatinib. Indeed, CXCR4 inhibition is a recently developed strategy to enhance the anti-tumor activity of chemotherapy drugs via interference with stroma-mediated
drug resistance in different solid and hematological cancers, including CML. CXCR4 inhibition with the small molecule inhibitor AMD3100 (plerixafor) was found to be effective in combination with TKIs both in vitro and in vivo. Thus, pre-treatment with AMD3100 in vitro increased the imatinib-induced apoptosis of stroma-protected the BV173 lymphoid BCR-ABL-positive cells in culture, and significantly reduced the repopulating CML content in BM and spleen of SCID mice (42). Another work demonstrated that combination of AMD3100 with second-generation TKI nilotinib restored the sensitivity of stroma-protected human K562 and KU812F CML cells in vitro. In addition, AMD3100 enhanced nilotinib-induced reduction of 32D.p210 cell-produced tumor in vivo in a murine model of CML (43). However, AMD3100 did not demonstrate single-agent activity against CML cells and had no additive effect to TKI-induced apoptosis of CML cells cultured without BMSCs.

We previously demonstrated a role for the CXCR4 antagonist BKT140 in mobilization of hematopoietic stem cells from the BM (44). In addition, BKT140 was shown to mediate a potent cytotoxic activity against various hematological malignancies, including MM, APL and NHL cells and effectively cooperates with bortezomib and rituximab against MM and NHL cells, respectively (24, 26). In the current work, we were able to show that BKT140 treatment induced a modest inhibition of CML cell growth in vitro. Combining BKT140 with imatinib significantly increased the anti-CML effect, inducing cell apoptosis with mitochondrial involvement. Furthermore, BKT140 treatment ameliorated the BMSC-mediated resistance to imatinib and effectively targeted CML cells when combined with imatinib.

Importantly, this report provides the first evidence illuminating the molecular mechanism of BKT140-triggered apoptosis. BKT140 treatment increased the level of
the H$_2$O$_2$-specific scavenger catalase, while simultaneously down-regulated the chaperon proteins HSP70 and HSP90 in CML. These changes suggest the involvement of cellular chaperons in BKT140-mediated cell death. Chaperons were shown to be important regulators of apoptosis. HSPs protect cells from stress-induced apoptosis and have been shown to interact with different key apoptotic proteins and to block caspases activation (45). Furthermore, a pro-apoptotic role of catalase via inhibition of HSP70, promotion of cytochrome c release and caspase 3 activation was demonstrated in leukemic U937 cell line (46). Chaperons are also implicated in CML pathogenesis. Disruption of chaperone function by HDACis was shown to enhance TKI activity (47).

Our findings of mitochondrial destabilization with subsequent release of cytochrome C and SMAC/Diablo following BKT140 treatment may represent secondary apoptotic events. Nevertheless, deeper characterization of the mechanism underlying BKT140-induced apoptosis should be performed.

Interestingly, single-agent BKT140 treatment of CML cells in the presence of stroma increased the percent of cycling cells. Interaction with BMSCs is particularly important in CML. It was suggested that BMSCs can preserve the proliferative capacity and renewal ability of CML cells by inhibition of cell cycle progression and mediation of anti-apoptotic signals, thus protecting CML progenitors and leukemia stem cells from TKIs action and preventing full CML elimination (48-50). The increase in proportion of cycling cells upon BKT140 treatment in the presence of BMSCs may actually indicate that BKT140 could release the CML cells from BMSC-induced quiescent state. Thus, interference with BMSCs-associated CML cell quiescence using CXCR4 blockade may be one of the mechanisms by which BKT140 restores sensitivity to TKI treatment.
Our results identify BCL6 as another factor that may be involved in the stroma-provided protection from imatinib-induced apoptosis. Recent works have demonstrated that up-regulation of BCL6 is induced by TKIs, mediates resistance to TKIs and enables the survival of CML stem cells and Ph+ ALL cells (31, 51). In accordance with these reports, we show that imatinib increased expression of BCL6 in K562 and LAMA-84 cells. Furthermore, we found that imatinib treatment in the presence of BMSCs further elevated BCL6 mRNA levels, indicating a role for stroma in BCL6-mediated TKI resistance. Moreover, CXCR4-transduced K562 cells demonstrated a higher basal, as well as TKI-induced levels of BCL6, suggesting the possible role of CXCR4 in BCL6 regulation in CML cells. Importantly, BKT140 treatment effectively mitigated the imatinib-induced increase in BCL6 levels in the absence as well as the presence of BMCSs. Although the mechanisms by which BMSCs affect the imatinib-induced increase in CML cells in BCL6 levels requires further studies, our data indicates the importance of CXCR4/CXCL12 axis in this process and further substantiate that CXCR4 is rational target for combinational therapy in CML.

Collectively, our results demonstrate the important role of CXCR4 in activation of major down-stream regulators of proliferation and survival responses in CML cells, and suggest that CXCR4 is an important mediator in CML-stromal protective interactions. CXCR4 inhibition with BKT140 efficiently cooperated with imatinib, overcoming the protective effect of the BM stroma. BKT140 treatment reduced the stroma-mediated dormancy and restored elevated BCL6 levels to baseline levels, therefore re-sensitizing CML cells to imatinib and effectively promoting CML cell death. Moreover, BKT140 demonstrated single-agent anti-CML activity in vivo and significantly potentiated imatinib activity against K562-produced tumors. These
results provide the rational basis for CXCR4-targeted therapy in combination with TKI to override drug resistance and eliminate residual disease.

ACKNOWLEDGMENTS

This work was supported (in part) by scientific grant from Guy Weinshtock Foundation to AN, and Sarousy Foundation grant to AN. We also acknowledge the Israeli Jack Craps foundation for funding this research.

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43. Weisberg E, Azab AK, Manley PW, Kung AL, Christie AL, Bronson R, et al. Inhibition of CXCR4 in CML cells disrupts their interaction with the bone marrow microenvironment and sensitizes them to nilotinib. Leukemia 2012;26:985-90.
FIGURE LEGENDS

Figure 1. CXCR4 stimulation activates multiple signaling pathways in CML cells.

(A) Human phospho-kinase proteome profiler array was used to screen for activation of specific signaling pathways in CML cells following CXCR4 stimulation. K562 and K562LG-CXCR4.86 cells were serum-starved overnight and exposed to CXCL12 (200 ng/ml) for 5 and 30 minutes. Cells were lysed and relative level of phosphorylated kinases was analyzed. (B) Intracellular levels of phospho-STAT5 in K562LG-CXCR4.86 cells in response to CXCL12 activation was monitored by flow cytometry. Columns indicate the mean fluorescent intensity (MFI) of pSTAT5-PE staining in un-stimulated versus CXCL12-stimulated cells. Results represent the average of triplicates ±STDEV (**p<0.01). (C) K562 and K562LG-CXCR4.86 cells were serum-starved overnight and treated with CXCL12 (200 ng/ml) as in (A). Expression of the indicated proteins was analyzed by immunoblotting. Blots were stripped and re-probed with anti-β-actin antibody to ensure equal loading and transfer of protein (50 µg each lane).

Figure 2. BKT140 demonstrates potent cytotoxic activity against CML cells and co-operates with imatinib in vitro.

(A) K562 and K562LG-CXCR4.86 cells were cultured in serum-reduced medium (1%) with different concentrations of BKT140 (4, 8 and 20 µM) for 48 hours. Cell viability was determined using PI-exclusion and FACS count. Decrease in viability is presented as percent from untreated control. Percent of dead (PI-positive) cells were determined. (B) K562LG-CXCR4.86 cells were cultured with CXCL12 (500 ng/ml),
BKT140 (8 and 20 µM) or combination of both for 48 hours. Number of viable cells in culture was determined by FACS. Data represents the mean of triplicates ±STDEV (**p<0.01). (C-F) K562 and LAMA-84 cells were cultured in serum-reduced medium (1% FCS) in the absence or presence of BKT140 (8 µM), imatinib (0.1 µM and 0.2 µM), or combination of both agents for 48 hours. (C) Viability was determined using PI exclusion by FACS. Data represents the mean of triplicates ±STDEV (**p<0.01). (D) Representative images of untreated or treated CML cells following 48 hours of incubations depict the presence of apoptotic cells. (E, F) Apoptosis was detected using Annexin V-FITC/PI staining. Percent of early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptotic cells was determined. Data is presented as mean of triplicates ±STDEV (**p<0.01). (G, H) K562 cells were treated with imatinib, BKT140 or combination of both agents for 12 hours and mitochondrial membrane potential (ΔΨm) was determined using DiOC6 staining. Percent of apoptotic cells with reduced ΔΨm and subsequent staining intensity was detected. Data is presented as mean of triplicates ±STDEV (**p<0.01).

Figure 3. BKT140 suppresses colony formation and targets primary CML CD34+ cells in vitro.

(A, B) Following pre-treatment with BKT140 (20 µM), imatinib (0.5 µM) or combination of both agents for 24 hours, K562 cells were plated in triplicates in soft agar and colonies were photographed and counted after 14 days. Chart shows mean values ±STDEV and p-value of two independent experiments. (C, D) Mononuclear cells from BM sample of CML patient in chronic phase were incubated in the absence or presence of BKT140 (8 and 20 µM), imatinib (0.5 µM) or combination of both agents for 48 hours. Percent of viable CD34+ cells was determined by 7-AAD
counterstain and FACS analysis. 7-AAD-positive dead cells were gated out. Data is presented as mean of triplicates ±STDEV (**p<0.01).

**Figure 4. Effect of BKT140 treatment on apoptotic signaling pathways.**

(A) K562 and LAMA-84 were treated with 20 µM BKT140 for 24 hours. The cells were lysed, and the expression of several pro- and anti-apoptotic proteins was measured as described in the Materials and Methods section. The relative expression of these proteins compared with that of cells not treated with BKT140 is presented. The controls are considered as 1 (dotted line). (B) K562 and LAMA-84 cells were incubated in serum-reduced medium (1% FCS) in the absence or presence of various concentrations of BKT140, for 24 and 48 hours. Expression of the indicated proteins was monitored by immunoblotting. Blots were stripped and re-probed with anti-β-actin antibody to ensure equal loading and transfer of protein (50 µg each lane). (C) K562 and LAMA-84 cells were incubated in serum-reduced medium (1% FCS) in the absence or presence of BKT140 (8 µM), imatinib (0.25 µM) or combination of both agents for 48 hours. Release of mitochondrial cytochrome C to cytosol was measured by specific immunolabeling and flow cytometry analysis. Decrease in staining intensity represents the release of cytochrome C from mitochondria to cytosol. Percent of low fluorescent cell population comparing to control cells, was quantified. Data is presented as mean of triplicates ±STDEV (**p<0.01).

**Figure 5. BM stromal cells support the survival and proliferation of CML cells**

K562 and LAMA-84 cells were pre-stained with CFSE and co-cultured in serum-reduced conditions (0.1%) for 72 hours in the absence or presence of BMSCs. (A) Representative images of CML cells on the monolayer of BMSCs are presented (original magnification of x400). (B) CXCL12 levels in conditioned medium produced by BMSCs, measured by ELISA. (C) The analysis was gated on CFSE-
labeled cells. Representative plots of unlabeled BMSCs and CFSE-labeled K562 cells incubated in the absence or presence of BMSCs are depicted. (D) Proliferation of CFSE-stained K562 and LAMA-84 cells was determined using BrdU incorporation method. Data represents the mean of triplicates ±STDEV (**p<0.01). (E) CXCR4 expression by K562 and LAMA-84 cells cultured in the absence or presence of imatinib. (F) CML cells were treated with imatinib (0.2 µM for K562 or 0.1 µM for LAMA-84), BKT140 (8 µM) or combination of both agents in the absence or presence of BMSCs for 48 hours. Viability was determined using XTT method. Data represents the mean of triplicates ±STDEV (**p<0.01). (G, H) K562 cells were incubated with imatinib (0.2 µM), BKT140 (8 µM) or combination of both agents in the absence or presence of BMSCs for 48 hours and cell cycle analysis was performed using 7-AAD staining. Percent of cycling cells (in G2/M and S phases) is presented as mean of triplicates ±STDEV (**p<0.01). (I) K562, K562LG-CXCR4.86 and LAMA-84 cells were pre-treated with imatinib, BKT140 or combination of both agents for 24 hours in the absence or presence of BMSCs and subjected to qPCR analysis. Expression of BCL6 was evaluated. Data represents the mean of triplicates from two separate experiments ±STDEV, all normalized against β2-microglobulin expression (**p<0.01).

**Figure 6. BKT140 displays anti-CML activity and cooperates with imatinib in vivo in xenograft model**

K562L cells (5x10⁶ per mouse) were injected subcutaneously into the flank of NOD/SCID mice. Treatment was started 72 hours following cell inoculation. BKT140 (400 µg per injection, s.c.), imatinib (10 mg/kg, i.p.) or combination of both were administered according to the schedule, total of 11 injections. Control mice were injected with saline. (A) Treatment schedule. (B) Representative images of harvested
subcutaneous tumors. (C) Tumor size (length x width, cm$^2$) and tumor weight (gr), presented as mean ± STDEV of 4 mice (**p<0.01, Student's t test). (D) Representative images of hematoxylin and eosin stained section of K562L-produced xenograft tumors, magnification of x200 and x400.
Figure 1

A. K562

B. K562LG-CXCR4.86

C. K562  K562LG-CXCR4.86

CXCL12 (200ng/ml)  0  5'  30'  0  5'  30'

pErk1/2

pAKT (S473)

pS6

β-actin

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Figure 2

A. Viable cells (% of ctrl)

![Bar chart showing viable cells over K562 and K562LG-CXCR4.86 with varying BKT140 concentrations.](image1)

B. Dead cells (%)

![Bar chart showing dead cells over K562 and K562LG-CXCR4.86 with varying BKT140 concentrations.](image2)

C. Proliferating cells (% of ctrl)

![Bar chart showing proliferating cells over K562 and K562LG-CXCR4.86 with varying BKT140 concentrations.](image3)

D. CTRL Imatinib (0.1 µM)

![Images showing cell morphology](image4)

E. Early apoptosis

![Flow cytometry analysis showing early and late apoptosis with BKT140 and Imatinib](image5)

F. Late apoptosis

![Flow cytometry analysis showing early and late apoptosis with BKT140 and Imatinib](image6)

G. DiOC6

![Images showing cell morphology with DiOC6](image7)

H. DiOC6

![Images showing cell morphology with DiOC6](image8)
Figure 3

A.

CTRL

BKT140

Imatinib

Imatinib + BKT140

B.

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C.

Live

Dead

7-AAD

Isotype

D.

% CD34+ 7-AAD

CD34

Imatinib 0.5 µM

BKT 8 µM

BKT 20 µM

Imatinib + BKT 8 µM

Imatinib + BKT 20 µM
Figure 4

A. K562

Relative protein expression

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LAMA-84

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B. K562

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LAMA-84

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C. Isotype

control

BKT140

8 µM

Imatinib

0.25 µM

Imatinib + BKT140

Cyt C

% Cytochrome C release

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Figure 5

A. K562  LAMA-84

X40  X40

B. CXCL12 (pg/ml)

BMSCs-1  BMSCs-2

80  60  40  20  0

C. BMSC  K562  K562 + BMSC

CFSE

10  10^2  10^3  10^4

D. no BMSC  + BMSC

K562  LAMA-84

13%  35%

19%  42%

E. ctrl  Imatinib

K562  LAMA-84

F. Cell growth (% of control)

K562

LAMA-84

no BMSC  + BMSC

F. Cell growth (% of control)

G. CTRL  Imatinib  BKT140  BKT140+Imatinib

DNA content

H. no BMSC  + BMSC

G2/M+S phase (% of cells)

-  -  +  +

-  -  +

Imatinib 0.2 µM  -  -  +  +

BKT140 8 µM  -  -  +  +

I. BCL-6

K562

K562LG-CXCR4.86

CTRL  Imatinib  BKT  Imat+BKT

J. BCL-6

K562

LAMA-84

CTRL  Imatinib  BKT  Imat+BKT

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Figure 6

A.

s.c.
K562L
5x10^6

Terminate

Days
0  2  4  6  8  10  12  14  16  18

BKT140 s.c
Imatinib i.p.

B.

vehicle
BKT140
Imatinib
Imatinib+BKT140

C.

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D.

vehicle
x20
x40

Imatinib
x20
x40

BKT140
x20
x40

Imatinib+BKT140
x20
x40
Combination of Imatinib with CXCR4 antagonist BKT140 overcomes the protective effect of stroma and targets CML in vitro and in vivo

Katia Beider, Merav Darash-Yahana, Orly Blaier, et al.

Mol Cancer Ther Published OnlineFirst February 6, 2014.

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