

CXCR2 As a Novel Target for Overcoming Resistance to Tyrosine Kinase Inhibitors in Chronic Myelogenous Leukemia Cells

Ji-Hea Kim

Korea University College of Medicine and School of Medicine

Seung-Jin Lee

Korea University College of Medicine and School of Medicine

Ka-Won Kang

Korea University Anam Hospital

Byung-Hyun Lee

Korea University Anam Hospital

Yong Park

Korea University Anam Hospital

Byung Soo Kim (✉ kbs0309@korea.ac.kr)

Korea University Anam Hospital

Research

Keywords: CXCR2, IL-8, mTOR, c-Myc, tyrosine kinase inhibitors, drug resistance

Posted Date: January 25th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-152459/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Chronic myeloid leukemia (CML) is a reciprocal translocation disorder driven by a breakpoint cluster region (*BCR*)-Abelson leukemia virus (*ABL*) fusion gene that stimulates abnormal tyrosine kinase activity. Tyrosine kinase inhibitors (TKIs) are effective in the treatment of Philadelphia chromosome (Ph)⁺ CML patients. However, the appearance of TKI-resistant CML cells is a hurdle in CML treatment. Therefore, it is necessary to identify novel treatments that could target a different mechanism than that of tyrosine kinases.

Methods: The study was designed to verify whether C-X-C chemokine receptor 2 (CXCR2) could be a novel target for TKI-resistant CML treatment. We examined CXCR2 ligands from CML patient samples and TKI-resistant CML cell lines. Then, we inhibited CXCR2 and examined the effects on cell proliferation and apoptosis using immunoblotting and flow cytometry. The CXCR2 inhibition effect was also confirmed using a mouse xenograft model with TKI-sensitive and -resistant CML cells.

Results: Interleukin 8 (IL-8), a CXCR2 ligand, was significantly increased in the bone marrow serum of initially diagnosed CML patients. CML cell lines expressed CXCR2, regardless of their sensitivity to TKIs. IL-8 stimulated CXCR2, mTOR, and c-Myc mRNA expression in CML cell lines. CXCR2 antagonists suppressed the proliferation of CML cells via cell cycle arrest in the G2/M phase. In addition, CXCR2 inhibition attenuated mTOR, c-Myc, and BCR-ABL expression, leading to CML cell apoptosis, irrespective of TKI responsiveness. Moreover, SB225002, a CXCR2 antagonist, caused higher cell death in CML cells than TKIs. Using a mouse xenograft model, we confirmed that SB225002 suppresses CML cells, with a prominent effect on TKI-resistant CML cells.

Conclusions: Taken together, our findings demonstrate that IL-8 is a prognostic factor to the progress of CML. Inhibiting the CXCR2-mTOR-c-Myc cascade is a promising therapeutic strategy to overcome TKI-sensitive and -insensitive CML. Thus, CXCR2 blockade is a novel therapeutic strategy to treat CML, and SB225002, a commercially available CXCR2 antagonist, might be a drug candidate to treat TKI-resistant CML.

Background

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by the Philadelphia chromosome (Ph), which involves a translocation between the Abelson leukemia virus (*ABL*) and breakpoint cluster region (*BCR*) genes (t9;22) (q34;q11) (1–3). This *BCR-ABL* fusion gene stimulates abnormal tyrosine kinase activity and mediates multiple signaling cascades, including the JAK-STAT (4, 5), MAPK-ERK (6, 7), and PI3K pathways (8, 9). These signaling pathways lead to leukemogenesis by stimulating proliferation and arresting differentiation, thereby leading to the accumulation of immature hematopoietic stem cells (HSCs) (10). The prognosis of patients with CML dramatically improved after the development of the first-generation tyrosine kinase inhibitor (TKI) imatinib mesylate. However, approximately 15% of CML patients treated with TKIs report primary cytogenetic resistance or loss of the

achieved complete cytogenetic response due to the acquisition of drug resistance (11). Second- and third-generation TKIs such as nilotinib, dasatinib, and ponatinib have been developed to overcome this problem (12, 13). Despite the development of additional TKI-based treatments, treatment failure still occurs due to leukemic stem cell (LSC) and CML insensitivity to TKIs (14). CML cell survival is sustained by resistance mechanisms that enable the evasion to TKIs (15). Therefore, it is necessary to research new therapeutic targets to overcome TKI resistance in patients with CML.

C-X-C chemokine receptor 2 (CXCR2) is G-protein-coupled receptor that is overexpressed in various solid cancer types, including breast (16), gastric (17), colon (18), melanoma (19), ovarian (20), pancreatic (21), and prostate (22) cancers. Interleukin 8 (IL-8), a CXCR2 ligand, is highly associated with cellular stemness and drug resistance by inhibition of drug-induced apoptosis in resistant cells (23–25). Inhibiting the IL-8/CXCR2 axis reduces myelodysplastic syndrome and acute myeloid leukemia progression (26, 27). However, the relationship between CXCR2 and CML progression has not been fully elucidated. We hypothesized that CXCR2 inhibitors might be novel candidates for CML treatment because our previous report showed that CXCR2 is vital for the survival and self-renewal of normal human HSC/hematopoietic progenitor cells and cancer cells (28–31). Furthermore, we identified the CXCR2-mTOR-c-Myc cascade as a novel and crucial signaling pathway for human pluripotent stem cell (hPSC) and human HSC (hHSC) proliferation (29, 30, 32). In the present study, we investigated the role of CXCR2 in CML cells and assessed whether commercially available CXCR2 antagonists effectively suppress CML progression.

Methods

Patient samples

This study was approved by the internal review board of the Korea University Anam Hospital (IRB No. 2015AN0267). Bone marrow (BM) blood from CML patients ($n = 38$) and healthy donors ($n = 10$) was collected after obtaining written informed consent, in accordance with the Declaration of Helsinki. BM mononuclear cells (BMMNCs) were separated, as described previously (30). CD34⁺ BMMNCs were sorted using a Super MACS instrument (Miltenyi Biotech Inc., Auburn, CA, USA). CML CD34⁺ cells were cultured in serum-free expansion medium (Sigma-Aldrich, St. Luis, MO, USA) containing a recombinant human cytokine cocktail (StemCell Technologies, Vancouver, Canada). The remaining CD34⁻ cells were used to produce CML-derived BM stromal cells (BMSCs), which were cultured in mesenchymal stem cell growth medium (Lonza, Walkersville, MD, USA).

Reagents and cell lines

The CXCR2 antagonists, SB225002 and SB265610, were obtained from Calbiochem (San Diego, CA, USA) and Tocris Bioscience (Bristol, United Kingdom), respectively. Imatinib and nilotinib were purchased from Selleck Chemicals (Munich, Germany). Drug compounds were dissolved in Dimethyl Sulfoxide (DMSO) at a concentration of 10 mM. K562, KU812, and CD34⁺ cells were purchased from the American Type Culture Collection (Manassas, VA, USA). CML cells were maintained in RPMI 1640 medium (Corning, NY,

USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco Life Technologies, Grand Island, NY, USA) at 37 °C in a humidified atmosphere containing 5% CO₂.

Development of TKI-resistant cell lines

To establish imatinib- and nilotinib-resistant CML cell lines, K562 and KU812 cell lines were gradually exposed to imatinib (from 0.01 µmol/L to 10 µmol/L) or to nilotinib (from 0.1 nmol/L to 100 nmol/L). The resulting imatinib- and nilotinib-resistant cells were maintained with 1 µmol/L imatinib and 20 nmol/L nilotinib, respectively. *BCR-ABL* gene mutations generated during the acquisition of TKI resistance were identified using next-generation sequencing. Library preparation and data analysis are described in Additional File 1, Table S1.

Cytokine array analysis

A cytokine array analysis was performed on K562, K562 imatinib-resistant cells (K562/IR), and K562 nilotinib-resistant cells (K562/NR) cultured in cell culture-conditioned medium for 48 h and BM blood serum samples from CML patients. Serum samples were prepared by centrifugation for 10 min at 1000 × *g*. The human cytokine antibody array (Ray Biotech, Norcross, GA, USA) was performed according to the manufacturer's instructions (see in Additional File 1: Table S2), and dot blot quantitation was performed using Image Lab 5.0 software (Bio-Rad, Hercules, CA, USA).

ELISA

Human Interleukin-8 (IL-8) and Growth-Regulated Oncogene (GRO) alpha concentrations in culture supernatants and BM blood plasma were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The levels of ligands were measured using a standard curve provided with the kit. All samples were measured in duplicate.

Colony formation assay

CML cells were cultured for 72 h after treatment with SB225002 or vehicle. Treated cells were harvested, resuspended in Iscove's Modified Dulbecco's Medium (IMDM; Corning, NY, USA), and mixed with semisolid culture medium (methylcellulose H4034; Stem Cell Technologies, Grenoble, France). Cells were seeded onto 35 mm dishes (5 × 10³ cells/dish). Each assay was performed in duplicate, and the cells were counted after 12–14 culture days.

RT-qPCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. RT-qPCR was performed as previously described (30). SYBR Green PCR Master Mix was used with a CFX96 RT-PCR machine (Bio-Rad, Hercules, CA, USA) as follows: 95 °C for 60 s, followed by 95 °C for 10 s and 60 °C for 60 s for 45 cycles. The primer sequences are listed in Table S3,

Additional File 1. To evaluate relative mRNA expression, the comparative $\Delta\Delta\text{Ct}$ method was used, with *GAPDH* as a reference gene.

Cell proliferation assay

CML cells were cultured in 96-well plates at 5×10^3 cells/well. After 24 h, cells were treated with SB225002 at different concentrations for 48 h. Cell proliferation was evaluated by adding 10 μL Cell Counting Kit-8 (CCK-8) reagent (Dojindo Laboratories; Japan) to each well and incubating it for 2 h. Absorbance at 450 nm was measured using a microplate spectrophotometer. The half maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism 6.0.

Cell cycle analysis

For DNA content analysis, cells were harvested and fixed with 80% ethanol overnight at -20°C . The cells were washed twice with PBS and incubated overnight at 37°C in 0.5 mL PBS containing 100 $\mu\text{g}/\text{mL}$ RNase A. Fixed cells were stained with 50 $\mu\text{g}/\text{mL}$ propidium iodide (PI) solution and analyzed using FACS Canto \boxtimes (BD, Franklin Lakes, NJ, USA). Cell cycle analysis was performed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Western blotting

Proteins were extracted using a protease inhibitor kit (Intron Biotechnology.; Seongnam, Korea). Immunoblotting was performed as previously described (29). Antibodies against the following proteins were used: CXCR2 and c-Myc (Abcam, Cambridge, UK); β -actin, cyclin B1, and p-CDC2 (Santa Cruz, CA, USA); and p-c-ABL, c-ABL, p-mTOR, mTOR, p-Akt, Akt, caspase-3, and cleaved PARP (Cell Signaling, Danvers, MA, USA).

Apoptosis assay

CML cells were seeded in six-well plates and treated with 1 μM SB225002 for 48 h. Cells were washed with PBS and resuspended in 0.5 mL binding buffer containing 1 $\mu\text{g}/\text{mL}$ PI and 1 $\mu\text{g}/\text{mL}$ FITC-labeled annexin V (Koma Biotechnology, Seoul, Korea). After incubating cells for 15 min at room temperature, apoptotic cells were quantified by measuring the percentage of annexin V⁺ cells using a BD FACS Canto II flow cytometer (BD, Franklin Lakes, NJ, USA). The data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Transduction of lentivirus shRNA for CXCR2 silencing and activation

The pLKO.1-shRNA control and shRNA CXCR2 transduction particles were purchased from Sigma-Aldrich (St. Louis, MO, USA). For CXCR2 upregulation, control and CXCR2 lentiviral activation particles were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). K562 cells were resuspended in virus-containing media and mixed with 8 $\mu\text{g}/\text{mL}$ polybrene. The cells were centrifuged at $800 \times g$ for 2 h at 32°C . Virus-containing medium was removed, and the cell pellet was resuspended in culture media. To

select stably infected cells, the medium was replaced with fresh medium containing puromycin after 72 h.

Animal experiments

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea University College of Medicine (IRB NO. KOREA-2018-0058) and performed according to institutional and national guidelines. Female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (6–8-weeks-old) were purchased from Koatech (Pyeongtaek, South Korea). To establish the xenograft model, 5×10^6 K562, K562/imatinib resistance (IR), or K562/nilotinib resistance (NR) cells were suspended in culture media with Matrigel (356234; BD Bioscience, San Jose, CA, USA), and 100 μ L suspended cells were implanted subcutaneously into the right flank. One week after inoculation, the mice were randomly divided into control and drug treatment groups. The control group was injected with DMSO, while the SB225002 (20,33), imatinib (34,35), and nilotinib (36) groups were intraperitoneally injected with 10 mg/kg of the respective drugs three times per week. The injections were continued for 14 days until tumors reached 20 mm, which is the maximum allowable size following animal ethics guidelines. Tumor size was ascertained three times a week, and the volume was calculated using the following formula: volume (mm^3) = (length \times height²)/2.

Immunohistochemistry

Formalin-fixed tissue was embedded in paraffin, and 3 μ m-thick sections were cut. Slides were cleared in xylene and rehydrated with gradient ethanol to deparaffinize them. Samples were treated in citrate buffer for antigen retrieval. Then, samples were incubated overnight with CXCR2, c-Myc, and c-ABL (Abcam, Cambridge, UK), and mTOR (Invitrogen, Waltham, MA, USA) primary antibodies, followed by incubation with a biotinylated secondary anti-mouse antibody. Secondary antibody staining was visualized with DAB solution, and the samples were counterstained with hematoxylin. Images were analyzed using an Olympus BX 53 microscope (Tokyo, Japan).

Whole-genome sequencing

DNA was extracted from TKI-sensitive and -resistant cells (K562, K562/IR, K562/NR, KU812, KU812/IR, and KU812/NR). Libraries were prepared using TruSeq Nano DNA Sample Prep kits (Illumina, Inc., San Diego, CA, USA). DNA sequencing was performed using a NovaSeq 6000 system (Illumina, Inc.). The sequences were aligned to the reference genome (hg19) using the Isaac aligner v.iSAAC-03.16.12.05 (37). The Isaac aligner tool was used to remove adapter sequences and low-quality reads. Aligned reads were sorted according to location information. Single nucleotide variants and small indel mutations were confirmed to extract the variants calls using the Genome Variant Call Format File (gVCF) tool. The influence of mutations on the amino acid sequence was predicted using SnpEff v.4.2 (38) and SnpSift v.4.2 (39). Mutation annotations were added from the known variant databases dbSNP (40), COSMIC (41), ClinVar (42), and ExAC (43). Consensus sequences were obtained based on accumulated information in a specific region using Mpileup in Samtools v. 1.3.1 (44).

Statistical analysis

All results were obtained from at least three independent experiments. The data are represented as the mean \pm standard deviation (SD). Statistical differences were calculated using Student's *t*-test to compare two groups and one-way analysis of variance (ANOVA) with Tukey's *post hoc* test for comparison of more than two groups. $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism V6.0 (GraphPad Software, San Diego, CA, USA).

Results

IL-8 is secreted by CML patient-derived BM cells and stimulates CXCR2 to activate BCR-ABL

We used a semi-quantitative cytokine array to detect 80 human cytokines in BM serum and conditioned medium from BM-derived stromal cells (BMSC-CM). The serum and BMSCs were collected from six CML patients without concomitant diseases at initial diagnosis to compare their cytokine levels with those of two healthy donors. BM sera from CML patients had higher CXCR2 ligand levels, such as GRO $\alpha/\beta/\gamma$ (CXCL1/2/3) and IL-8 (CXCL8), than those from healthy donors (Fig. 1a and b). However, only IL-8 expression was significantly higher in BMSC-CM from two CML patients than in those from healthy donors (Fig. 1c and d). To find whether the level of cytokine differences after acquired resistance to TKIs, we established imatinib-resistance (IR) and nilotinib-resistance (NR) CML cell lines (see Additional File 2: Fig. S1A-D). Cytokine levels were similarly measured in the conditioned medium from TKI-sensitive (K562), imatinib-resistant (K562/IR), and nilotinib-resistant (K562/NR) cell lines, with high IL-8 expression observed in all of them. However, GRO $\alpha/\beta/\gamma$ was expressed only in TKI-sensitive cell lines (Fig. 1e and f). We hypothesized that IL-8 may be more important for the development of CML than GRO $\alpha/\beta/\gamma$ because the IL-8 secreted by CD34⁺ cells and CML patient-derived BMSCs was higher than in healthy donors. To investigate this hypothesis, IL-8 and GRO- α levels were measured in the BM serum of 37 patients with Ph⁺ CML (27 at initial diagnosis and ten undergoing TKI treatment) and compared to those of ten healthy donors. The mean age and white blood cell counts, regardless of TKI treatment history, were significantly higher in patients with Ph⁺ CML than in healthy donors, and many CML patients had a history of chronic disease. The CML patient and healthy donor characteristics are summarized in Table S4, Additional File 1. IL-8 levels in CML patients at initial diagnosis were significantly higher than in healthy donors and the CML patients undergoing TKI treatment (Fig. 1g), but GRO levels were not (Fig. 1h). These findings suggest that IL-8 is important for CML pathogenesis.

To investigate the activity of the receptors associated with IL-8, CXCR1 and CXCR2 protein expression was measured in CML cell lines. CXCR2 expression was approximately three-fold higher in K562 and KU812 cells than in normal CD34⁺ cells (Fig. 1i). CXCR2 expression in TKI-resistant cells was proportional to upregulated BCR-ABL expression, suggesting a relationship between CXCR2 and BCR-ABL (Fig. 1j). To investigate this relationship, CXCR2 was stimulated with IL-8, and changes in CXCR2, mTOR, c-Myc, and BCR-ABL mRNA expression were measured in CML cell lines (Fig. 1k). IL-8 stimulation significantly increased BCR-ABL, CXCR2, mTOR, and c-Myc mRNA expression in all CML cell lines, regardless of TKI

sensitivity. Therefore, IL-8 appears to play a role in CML pathogenesis by stimulating BCR-ABL. Further, CXCR2 may be a novel target for the treatment of CML that is resistant to well-known TKIs.

CXCR2 inhibition attenuates CML cell viability, regardless of TKI sensitivity

To confirm CXCR2 as a novel target for CML treatment, the effect of the commercially available CXCR2 antagonists, SB225002 and SB265610, on CML cell proliferation was determined. CML cell viability decreased in a dose-dependent manner upon treatment with both SB225002 and SB265610 (Fig. 2a). SB225002 was selected for further experiments, as it had a lower IC₅₀. To confirm the effect of SB225002 in the BM environment, which is comprised of CD34⁺ cells and BMSCs, we observed changes in the viability of BM CD34⁺ cells and BMSCs obtained from two CML patients and from healthy donors. CML CD34⁺ cell viability decreased with increasing SB225002 dose, but normal CD34⁺ cells maintained no effect their viability to 10 μM SB225002. In BMSCs, the CML-BMSC viability decreased to around 50%–60% at doses between 1 to 10 μM SB225002, but to a much lower degree than in CML CD34⁺ cells at 10 μM SB225002. The viability of normal BMSCs was maintained at 80% at 10 μM SB225002 (Fig. 2b). These findings suggest that SB225002 specifically suppresses CML CD34⁺ cell proliferation without having any significant influence on normal CD34⁺ cells and BMSCs. Different SB225002 concentrations reduced K562/IR, K562/NR, KU812/IR, and KU812/NR cell viability in a dose-dependent manner (Fig. 2c), and incubation with 1 μM SB225002 considerably decreased cell proliferation (Fig. 2d). To evaluate the hematopoietic effects of this antagonist, we performed a colony formation assay using methylcellulose and CML cell lines. We observed that SB225002 potently decreased the colony size and total colony number in all CML cell lines (Fig. 2e). These results suggest that CXCR2 inhibition attenuates CML cell lines, and CML CD34⁺ cell proliferation, regardless of TKI sensitivity.

CXCR2 inhibition induces CML cell apoptosis through G2/M cell cycle arrest

To examine whether CXCR2 inhibition affects cell cycle distribution, we investigated changes in the cell cycle phases in CML cell lines after SB22002 treatment. The proportion of G2/M phase cells significantly increased, concomitant with a decrease in the proportion of cells in the G0/G1 and S phases, in every CML cell line after treatment with either DMSO or SB225002 for 12 h (Fig. 3a). The expression of the G2/M phase regulators cyclin B1 and p-CDC2 decreased after SB225002 treatment (Fig. 3b), suggesting that SB225002 induces G2/M arrest by downregulating cyclin B1 and CDC2 proteins in CML cells. Next, pro-apoptotic proteins, such as cleaved PARP and cleaved caspase-3, were investigated to clarify the observed growth arrest and apoptosis effects. SB225002 treatment induced a significant increase in cleaved PARP and cleaved caspase-3 protein levels (Fig. 3c). The proportion of apoptotic cells was assessed using annexin V/PI staining. Apoptotic cell death was increased by approximately 15% following SB225002 treatment (Fig. 3d). These data demonstrate that SB225002 inhibits cell proliferation and induces apoptosis through G2/M cell cycle arrest in CML cells, irrespective of TKI sensitivity.

CXCR2 inhibition mediates Akt/mTOR and c-Myc signaling pathways

To investigate the effect of CXCR2 on BCR-ABL signaling, BCR-ABL activity was evaluated after treatment with SB225002. We observed reduced BCR-ABL phosphorylation that was directly proportional to CXCR2 expression in both TKI-sensitive and -resistant K562 cells (Fig. 4a). BCR-ABL signaling mediates cell growth through multiple signaling pathways, including PI3K/Akt/mTOR (45,46) and Ras/MAPK (47). Therefore, the inhibition of signaling pathways affecting BCR-ABL after CXCR2 knockdown was investigated. Silencing CXCR2 reduced BCR-ABL, Akt, mTOR, and c-Myc phosphorylation (Fig. 4b). Next, we confirmed the upregulation of those genes using CXCR2 lentiviral activation particles (Fig. 4c). This result suggests that regulating CXCR2 controls Akt/mTOR, c-Myc, and BCR-ABL levels. Loss of CXCR2 expression suppressed proliferation of and colony formation in K562 cells (Fig. 4d and e). Treatment with CXCR2 antagonists inhibited p-Akt, p-mTOR, and c-Myc in TKI-sensitive and -resistant cells, but did not inhibit p-Erk (Figure 4f; See Fig. S2, Additional File 2). These data indicate that inhibiting CXCR2 signaling reduces BCR-ABL activity, even with TKI resistance. Subsequently, the changes in the above-mentioned signaling pathways were explored in K562 cells after TKI treatment. Akt/mTOR expression decreased after TKI treatment, similar to the effects of SB225002 treatment. c-Myc expression was not suppressed in TKI-resistant CML cells, in contrast to its suppression in TKI-sensitive cells (Fig. 4g and h). Taken together, these results suggest that CXCR2 inhibition suppresses CML cell growth through the Akt/mTOR and c-Myc signaling pathways and that c-Myc may be associated with the acquisition of TKI resistance.

CXCR2 is a novel target for treating TKI-resistant CML

Based on our previous findings, we hypothesized that CXCR2 may be a novel target for suppressing TKI-resistant CML cell proliferation. We compared the effects of SB225002, imatinib, and nilotinib on TKI-sensitive and -resistant K562 cell lines. In TKI-sensitive K562 cells, reduced p-BCR-ABL, p-AKT, p-mTOR, and c-Myc expression was observed after treatment with any of the drugs. CXCR2 expression was only reduced by SB225002. Further, SB225002 suppressed p-BCR-ABL, p-AKT, p-mTOR, and c-Myc expression in both imatinib- and nilotinib-resistant K562 cells (Fig. 5a). We assessed whether downregulating BCR-ABL and c-Myc expression after SB225002 treatment induced cell death in TKI-resistant CML cells. The rate of apoptosis in SB225002-treated TKI-sensitive K562 cells was similar to that of TKI-treated cells. The proportion of annexin V⁺ cells significantly increased (20%) in TKI-resistant K562 cells treated with SB225002, but not in cells treated with TKIs. To confirm the applicability of these findings in clinical practice, the apoptotic effect of the drugs was tested in CD34⁺ cells from three CML patients, including a case with Ph⁻ atypical CML. The SB225002 apoptotic effect was comparable to that of TKIs on cells from a patient initially diagnosed with Ph⁺ CML and was superior to that of TKIs on cells from a patient showing imatinib-resistant and another with Ph⁻ atypical CML (Fig. 5b). We measured the apoptotic effect of these drugs and ponatinib on TKI-resistant CML patient-derived cells and observed no significant difference in apoptosis between ponatinib and nilotinib treatment (See Fig. S3, Additional File 2). Cleaved caspase-3 and PARP levels were unaffected by the different drugs in TKI-sensitive CML cells, but they were higher in TKI-resistant CML cells treated with SB225002 than in those treated with TKIs (Fig. 5c). These data suggest that CXCR2 is a novel target that could overcome TKI resistance for the treatment of CML.

CXCR2 inhibition suppresses *in vivo* CML cell proliferation, particularly for TKI-resistant cells

To evaluate the *in vivo* effects of CXCR2 inhibition on CML cells, a xenograft model was constructed by injecting K562, K562/IR, and K562/NR cells into NOD/SCID mice. In mice receiving TKI-sensitive K562 cells, all of the treatment groups showed significant differences in tumor size. In the TKI-resistant K562 group, SB225002 treatment resulted in a decreased tumor volume and weight compared to the other treatment groups (Fig. 6a and b; See Fig. S4, Additional File 2). Immunohistochemical staining of the tumor masses revealed that TKI-sensitive K562 tumors treated with SB225002 had lower BCR-ABL inhibition than those in the TKI groups. The cell proliferation marker Ki67 was similarly suppressed in all treatment groups. However, the TKI-resistant K562 tumors treated with SB225002 had the highest BCR-ABL and Ki67 inhibition, and the Ki67 levels in the TKI groups were similar to those of the vehicle treatment in the nilotinib-resistant group. c-Myc in imatinib-resistant K562 tumors was not reduced by imatinib, nor was c-Myc reduced by imatinib or nilotinib in nilotinib-resistant K562 tumors (Fig. 6c). These findings suggest a possible role of c-Myc in the development of TKI resistance, highlighting the importance of elucidating the role of CXCR2 in TKI-resistant CML. Finally, we measured CD31 expression to rule out the effect of these drugs on angiogenesis and observed that their effect on angiogenesis was not significant (See Fig. S5, Additional File 2). Collectively, these results suggest that CXCR2 inhibition suppresses *in vivo* proliferation of CML cells, particularly TKI-resistant cells.

Discussion

Ph⁺ CML patients with multiple TKI treatment failures and without ABL kinase domain mutations represent a population with predominantly BCR-ABL-independent mechanisms of resistance and limited treatment options (48–51). Only 27% of resistant or intolerant patients achieved significant major molecular responses in the PACE trial (52). In this study, we aimed to examine the drivers of BCR-ABL-independent resistance and to identify clinically relevant compounds for the eradication of TKI-resistant cells. All established cell lines with IR or NR expressed some mutations in introns, but not in the transcript region (See Table S4, Additional File 1). Therefore, the TKI resistance mechanism in these cells might be associated with BCR-ABL-independent resistance. We observed that cytokine levels in the BM of CML patients showed high IL-8 expression. IL-8 levels in the BM of CML patients at initial diagnosis were significantly higher than in that of CML patients who underwent TKI treatment or of normal healthy donors. This observation suggests a role for IL-8 and related receptors in CML pathogenesis. Although IL-8 binds both CXCR1 and CXCR2, we observed that CXCR2 is predominantly expressed in CML, despite the higher affinity of IL-8 for CXCR1. In addition, CD34⁺ cells and BMSCs derived from CML patients with elevated IL-8 levels had reduced cell proliferation after treatment with a CXCR2 antagonist. The BM niche protects LSCs from pharmacological treatment by providing a sanctuary where the microenvironment enables essential homing and engraftment (34, 53, 54). Our results imply that the blockade of CXCR2 signaling affects the CML-BM niche, rather than healthy BM. These findings suggest that inhibiting CXCR2 signaling could affect both autocrine and paracrine IL-8, which also controls the CML-BM niche.

Our results showed the potential genes involved in TKI resistance and the therapeutic mechanism in CML. SB225002 inhibited proliferation and induced apoptosis through the BCR-ABL, AKT/mTOR, and c-Myc pathways in TKI-sensitive and -resistant cells. A recent study from our group verified that the CXCR2-mTOR-c-Myc cascade mediates hHSC proliferation (30) during reprogramming to induced pluripotent stem cells (29) and maintains hPSC characteristics (32). Several transcription factors, such as c-Myc, Nanog, Sox2, and OCT4, are regulated by IL-8 (55). Previous studies showed reduced IL-8 levels in K562 cells and CML patient serum samples treated with dasatinib and nilotinib (56). c-Myc is related to BCR-ABL transformation and plays a crucial role in CML progression from the chronic phase to blast crisis. Inhibiting c-Myc induces reduced BCR-ABL activity (47)-(57) because c-Myc binds to the *BCR* promoter and directly regulates *BCR* activity (58). These reports indicate that IL-8 secretion depends on BCR-ABL activity. Considering these results, our data suggest that after acquiring TKI resistance, TKIs are not sufficient to inhibit BCR-ABL. Thus, IL-8 secretion is maintained, and downstream signaling is sustained in TKI-resistant cells. These results imply that downregulating CXCR2 signaling is more effective because of the suppressed expression of both BCR-ABL and c-Myc in TKI-resistant CML. Our study corroborates that the regulation of CXCR2 affects the BCR-ABL, Akt/mTOR, and c-Myc pathways and contributes to eradicating CML *in vitro* and *in vivo*.

Conclusions

CXCR2 might be a novel target for the treatment of CML independent from tyrosine kinase inhibition. Indeed, CXCR2 inhibition might be especially valuable to treat TKI-resistant CML.

Abbreviations

ABL: Abelson leukemia virus

BCR: Breakpoint cluster region

BM: Bone marrow

BMMNC: Bone marrow mononuclear cell

BMSC: Bone marrow stromal cell

BMSC-CM: Conditioned medium from bone marrow-derived stromal cells

CCK-8: Cell Counting Kit-8

CML: Chronic myeloid leukemia

CXCR2: C-X-C chemokine receptor 2

CDC: Cell division control protein

GRO: Growth-regulated oncogene

HSC: Hematopoietic stem cells

hHSC: Human hematopoietic stem cells

hPSC: Human pluripotent stem cell

IL-8: Interleukin 8

IR: Imatinib resistance

LSC: Leukemic stem cells

NOD/SCID: Non-obese diabetic/severe combined immunodeficiency

NR: Nilotinib resistance

PACE Pacing, graded Activity, and Cognitive behaviour therapy

Ph: Philadelphia chromosome

PARP: Poly ADP-ribose polymerase

PI: Propidium iodide

RT-qPCR: Quantitative real-time polymerase chain reaction

shRNA: Short hairpin RNA

TKI: Tyrosine kinase inhibitor

Declarations

Ethics approval and consent to participate

This study was approved by the internal review board of the Korea University Anam Hospital (IRB No. 2015AN0267). Animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea University College of Medicine (IRB NO. KOREA-2018-0058) and performed according to institutional and national guidelines.

Consent for publication

We have obtained consent to publish from the participants to report individual patient data.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the Bio & Medical Technology Development Program of the National Research Foundation, which is funded by the Ministry of Science & ICT (2017M3A9C8060403).

Acknowledgments

Not applicable.

Authors' contributions

JHK and BSK conceived and designed the study and drafted the manuscript. JHK and SJL performed the experiments. BSK, GWK, BHL, and YP collected and analyzed the clinical data. All authors read and approved the final manuscript.

References

1. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science*. 1990 Feb;247(4944):824–30.
2. McWhirter JR, Wang JY. Activation of tyrosinase kinase and microfilament-binding functions of c-abl by bcr sequences in bcr/abl fusion proteins. *Mol Cell Biol*. 1991;11(3):1553–65.
3. Chen Y, Peng C, Li D, Li S. Molecular and cellular bases of chronic myeloid leukemia. *Protein Cell*. 2010;1(2):124–32.
4. Sonoyama J, Matsumura I, Ezoe S, Satoh Y, Zhang X, Kataoka Y, et al. Functional cooperation among Ras, STAT5, and phosphatidylinositol 3-kinase is required for full oncogenic activities of BCR/ABL in K562 cells. *J Biol Chem*. 2002;277(10):8076–82.
5. Traer E, MacKenzie R, Snead J, Agarwal A, Eiring AM, O'Hare T, et al. Blockade of JAK2-mediated extrinsic survival signals restores sensitivity of CML cells to ABL inhibitors. *Leukemia*. 2012;26(5):1140–3.
6. Kang CD, Yoo SD, Hwang BW, Kim KW, Kim DW, Kim CM, et al. The inhibition of ERK/MAPK not the activation of JNK/SAPK is primarily required to induce apoptosis in chronic myelogenous leukemic K562 cells. *Leuk Res*. 2000;24(6):527–34.
7. Chorzalska A, Ahsan N, Rao RSP, Roder K, Yu X, Morgan J, et al. Overexpression of Tpl2 is linked to imatinib resistance and activation of MEK-ERK and NF- κ B pathways in a model of chronic myeloid leukemia. *Mol Oncol*. 2018;12(5):630–47.

8. Bertacchini J, Heidari N, Mediani L, Capitani S, Shahjahani M, Ahmadzadeh A, et al. Targeting PI3K/AKT/mTOR network for treatment of leukemia. *Cell Mol Life Sci* [Internet]. 2015;72(12):2337–47. Available from: <http://dx.doi.org/10.1007/s00018-015-1867-5>
9. Zhang F, Li K, Yao X, Wang H, Li W, Wu J, et al. A miR-567-PIK3AP1-PI3K/AKT-c-Myc feedback loop regulates tumour growth and chemoresistance in gastric cancer. *EBioMedicine* [Internet]. 2019;44:311–21. Available from: <https://doi.org/10.1016/j.ebiom.2019.05.003>
10. Yang L, Shi P, Zhao G, Xu J, Peng W, Zhang J, et al. Targeting cancer stem cell pathways for cancer therapy [Internet]. Vol. 5, *Signal Transduction and Targeted Therapy*. Springer US; 2020. Available from: <http://dx.doi.org/10.1038/s41392-020-0110-5>
11. Bradeen HA, Eide CA, O'Hare T, Johnson KJ, Willis SG, Lee FY, et al. Comparison of imatinib mesylate, dasatinib (BMS-354825), and nilotinib (AMN107) in an N-ethyl-N-nitrosourea (ENU)-based mutagenesis screen: High efficacy of drug combinations. *Blood*. 2006;108(7):2332–8.
12. O'Hare T, Eide CA, Deininger MWN. Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. *Blood*. 2007;110(7):2242–9.
13. Hughes T, Saglio G, Branford S, Soverini S, Kim DW, Müller MC, et al. Impact of baseline BCR-ABL mutations on response to nilotinib in patients with chronic myeloid leukemia in chronic phase. *J Clin Oncol*. 2009;27(25):4204–10.
14. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, Druker BJ. Erratum: Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest*. 2011;121(3):1222.
15. Massimino M, Stella S, Tirrò E, Romano C, Pennisi MS, Puma A, et al. Non ABL-directed inhibitors as alternative treatment strategies for chronic myeloid leukemia. *Mol Cancer*. 2018;17(1):1–15.
16. Xu H, Lin F, Wang Z, Yang L, Meng J, Ou Z, et al. CXCR2 promotes breast cancer metastasis and chemoresistance via suppression of AKT1 and activation of COX2. *Cancer Lett*. 2018 Jan 1;412:69–80.
17. Wang Z, Liu H, Shen Z, Wang X, Zhang H, Qin J, et al. The prognostic value of CXC-chemokine receptor 2 (CXCR2) in gastric cancer patients. *BMC Cancer*. 2015;15(1):1–8.
18. Lee YS, Choi I, Ning Y, Kim NY, Khatchadourian V, Yang D, et al. Interleukin-8 and its receptor CXCR2 in the tumour microenvironment promote colon cancer growth, progression and metastasis. *Br J Cancer*. 2012;106(11):1833–41.
19. Sharma B, Singh S, Varney ML, Singh RK. Targeting CXCR1/CXCR2 receptor antagonism in malignant melanoma. *Expert Opin Ther Targets*. 2010;14(4):435–42.
20. Devapatla B, Sharma A, Woo S. CXCR2 inhibition combined with sorafenib improved antitumor and antiangiogenic response in preclinical models of ovarian cancer. *PLoS One*. 2015;10(9):1–16.
21. Steele CW, Karim SA, Leach JDG, Bailey P, Upstill-Goddard R, Rishi L, et al. CXCR2 Inhibition Profoundly Suppresses Metastases and Augments Immunotherapy in Pancreatic Ductal Adenocarcinoma. *Cancer Cell*. 2016 Jun 13;29(6):832–45.

22. Li Y, He Y, Butler W, Xu L, Chang Y, Lei K, et al. Targeting cellular heterogeneity with CXCR2 blockade for the treatment of therapy-resistant prostate cancer [Internet]. Vol. 11, *Sci. Transl. Med.* 2019. Available from: <http://stm.sciencemag.org/>
23. Abraham RT. Chemokine to the Rescue: Interleukin-8 Mediates Resistance to PI3K-Pathway-Targeted Therapy in Breast Cancer. *Cancer Cell* [Internet]. 2012;22(6):703–5. Available from: <http://dx.doi.org/10.1016/j.ccr.2012.11.012>
24. Wang Y, Qu Y, Niu XL, Sun WJ, Zhang XL, Li LZ. Autocrine production of interleukin-8 confers cisplatin and paclitaxel resistance in ovarian cancer cells. *Cytokine*. 2011;56(2):365–75.
25. Kumar S, O'Malley J, Chaudhary AK, Inigo JR, Yadav N, Kumar R, et al. Hsp60 and IL-8 axis promotes apoptosis resistance in cancer. *Br J Cancer* [Internet]. 2019;121(11):934–43. Available from: <http://dx.doi.org/10.1038/s41416-019-0617-0>
26. Kuett A, Rieger C, Perathoner D, Herold T, Wagner M, Sironi S, et al. IL-8 as mediator in the microenvironment-leukaemia network in acute myeloid leukaemia. *Sci Rep* [Internet]. 2015;5(July):1–11. Available from: <http://dx.doi.org/10.1038/srep18411>
27. Schinke C, Giricz O, Li W, Shastri A, Gordon S, Barreyro L, et al. IL8-CXCR2 pathway inhibition as a therapeutic strategy against MDS and AML stem cells. *Blood*. 2015;125(20):3144–52.
28. Kuntz EM, Baquero P, Michie AM, Dunn K, Tardito S, Holyoake TL, et al. Targeting mitochondrial oxidative phosphorylation eradicates therapy-resistant chronic myeloid leukemia stem cells. *Nat Med*. 2017;23(10):1234–40.
29. Lee S-J, Kang K-W, Kim J-H, Lee B-H, Jung J-H, Park Y, et al. CXCR2 Ligands and mTOR Activation Enhance Reprogramming of Human Somatic Cells to Pluripotent Stem Cells. *Stem Cells Dev*. 2020 Feb;29(3):119–32.
30. Kang KW, Lee SJ, Kim JH, Lee BH, Kim SJ, Park Y, et al. Etoposide-mediated interleukin-8 secretion from bone marrow stromal cells induces hematopoietic stem cell mobilization. *BMC Cancer*. 2020;20(1):1–15.
31. Sinclair A, Park L, Shah M, Drotar M, Calaminus S, Hopcroft LEM, et al. CXCR2 and CXCL4 regulate survival and self-renewal of hematopoietic stem/progenitor cells. *Blood*. 2016;128(3):371–83.
32. Jung JH, Kang KW, Kim J, Hong SC, Park Y, Kim BS. CXCR2 Inhibition in Human Pluripotent Stem Cells Induces Predominant Differentiation to Mesoderm and Endoderm Through Repression of mTOR, β -Catenin, and hTERT Activities. *Stem Cells Dev*. 2016;25(13):1006–19.
33. De Vasconcellos JF, Laranjeira ABA, Leal PC, Bhasin MK, Zenatti PP, Nunes RJ, et al. SB225002 induces cell death and cell cycle arrest in acute lymphoblastic leukemia cells through the activation of GLIPR1. *PLoS One*. 2015;10(8):1–19.
34. Beider K, Darash-Yahana M, Blaier O, Koren-Michowitz M, Abraham M, Wald H, et al. Combination of imatinib with CXCR4 Antagonist BKT140 overcomes the protective effect of stroma and targets CML in vitro and in vivo. *Mol Cancer Ther*. 2014;13(5):1155–69.
35. Wang S, Xie W, Wang D, Peng Z, Zheng Y, Liu N, et al. Discovery of a small molecule targeting SET-PP2A interaction to overcome BCR-ABL T315I mutation of chronic myeloid leukemia. *Oncotarget*.

- 2015;6(14):12128–40.
36. Yan F, Al-Kali A, Zhang Z, Liu J, Pang J, Zhao N, et al. A dynamic N 6-methyladenosine methylome regulates intrinsic and acquired resistance to tyrosine kinase inhibitors. *Cell Res* [Internet]. 2018;28(11):1062–76. Available from: <http://dx.doi.org/10.1038/s41422-018-0097-4>
 37. Racz C, Petrovski R, Saunders CT, Chorny I, Kruglyak S, Margulies EH, et al. Isaac: Ultra-fast whole-genome secondary analysis on Illumina sequencing platforms. *Bioinformatics*. 2013;29:2041-3.
 38. Agrovskii BS, Vorob'ev VV, Gurvich AS, Pokasov VV, Ushakov AN. Intensity fluctuations of pulsed laser radiation during thermal self-interaction in a turbulent medium. *Sov. J. Quantum Electron*. 1980;10:308.
 39. Ruden DM, Cingolani P, Patel VM, Coon M, Nguyen T, Land SJ, et al. Using *Drosophila melanogaster* as a model for genotoxic chemical mutational studies with a new program, SnpSift. *Front Genet*. 2012;3:1-9.
 40. Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, et al. dbSNP: The NCBI database of genetic variation. *Nucleic Acids Res*. 2001;29:308-11.
 41. Tate JG, Bamford S, Jubb HC, Sondka Z, Beare DM, Bindal N, et al. COSMIC: The catalogue of somatic mutations in cancer. *Nucleic Acids Res*. 2019;47:D941-7.
 42. Landrum MJ, Lee JM, Riley GR, Jang W, Rubinstein WS, Church DM, et al. ClinVar: Public archive of relationships among sequence variation and human phenotype. *Nucleic Acids Res*. 2014;42:D980-5.
 43. Karczewski KJ, Weisburd B, Thomas B, Solomonson M, Ruderfer DM, Kavanagh D, et al. The ExAC browser: Displaying reference data information from over 60 000 exomes. *Nucleic Acids Res*. 2017;45:D840-5.
 44. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. *Bioinformatics*. 2009;25:2078-9.
 45. Ding J, Romani J, Zaborski M, MacLeod RAF, Nagel S, Drexler HG, et al. Inhibition of PI3K/mTOR overcomes nilotinib resistance in BCR-ABL1 positive leukemia cells through translational down-regulation of MDM2. *PLoS One*. 2013;8(12):1–12.
 46. Nie ZY, Yang L, Liu XJ, Yang Z, Yang GS, Zhou J, et al. Morin inhibits proliferation and induces apoptosis by modulating the MIR-188-5p/PTEN/Akt regulatory pathway in CML cells. *Mol Cancer Ther*. 2019;18(12):2296–307.
 47. Sawyers CL, Callahan W, Witte ON. Dominant negative MYC blocks transformation by ABL oncogenes. *Cell*. 1992;70(6):901–10.
 48. Nestal de Moraes G, Souza PS, Costas FC de F, Vasconcelos FC, Reis FRS, Maia RC. The Interface between BCR-ABL-Dependent and -Independent Resistance Signaling Pathways in Chronic Myeloid Leukemia. *Leuk Res Treatment*. 2012;2012:1–19.
 49. Ma L, Shan Y, Bai R, Xue L, Eide CA, Ou J, et al. A therapeutically targetable mechanism of BCR-ABL -Independent imatinib resistance in chronic myeloid leukemia. *Sci Transl Med*. 2014;6(252).

50. Eiring AM, Kraft IL, Page BD, O'Hare T, Gunning PT, Deininger MW. STAT3 as a mediator of BCR-ABL1-independent resistance in chronic myeloid leukemia. *Leuk Suppl.* 2014;3(S1):S5–6.
51. Lu T, Wei D, Yu K, Ma D, Xiong J, Fang Q, et al. Betulinic acid restores imatinib sensitivity in BCR-ABL1 kinase-independent, imatinib-resistant chronic myeloid leukemia by increasing HDAC3 ubiquitination and degradation. *Ann N Y Acad Sci.* 2020;1467(1):77–93.
52. Cortes JE, Kim D-W, Pinilla-Ibarz J, le Coutre P, Paquette R, Chuah C, et al. A Phase 2 Trial of Ponatinib in Philadelphia Chromosome-Positive Leukemias. *N Engl J Med.* 2013;369(19):1783–96.
53. Mitchell R, Copland M. Defining niche interactions to target chronic myeloid leukemia stem cells. *Haematologica.* 2020;105(1):2–4.
54. Houshmand M, Simonetti G, Circosta P, Gaidano V, Cignetti A, Martinelli G, et al. Chronic myeloid leukemia stem cells. *Leukemia [Internet].* 2019;33(7):1543–56. Available from: <http://dx.doi.org/10.1038/s41375-019-0490-0>
55. Hasan T, Caragher SP, Shireman JM, Park CH, Atashi F, Baisiwala S, et al. Interleukin-8/CXCR2 signaling regulates therapy-induced plasticity and enhances tumorigenicity in glioblastoma. *Cell Death Dis [Internet].* 2019;10(4). Available from: <http://dx.doi.org/10.1038/s41419-019-1387-6>
56. Hantschel O, Gstoettenbauer A, Colinge J, Kaupe I, Bilban M, Burkard TR, et al. The chemokine interleukin-8 and the surface activation protein CD69 are markers for Bcr-Abl activity in chronic myeloid leukemia. *Mol Oncol.* 2008 Oct;2(3):272–81.
57. Oudat R, Khan Z, Glassman AB. Detection of trisomy 8 in Philadelphia chromosome-positive CML patients using conventional cytogenetic and interphase fluorescence in situ hybridization techniques and its relation to c-myc involvement. *Ann Clin Lab Sci.* 2001;31(1):68–74.
58. Sharma N, Magistrini V, Piazza R, Citterio S, Mezzatesta C, Khandelwal P, et al. BCR/ABL1 and BCR are under the transcriptional control of the MYC oncogene. *Mol Cancer [Internet].* 2015;14(1):1–11. Available from: <http://dx.doi.org/10.1186/s12943-015-0407-0>

Figures

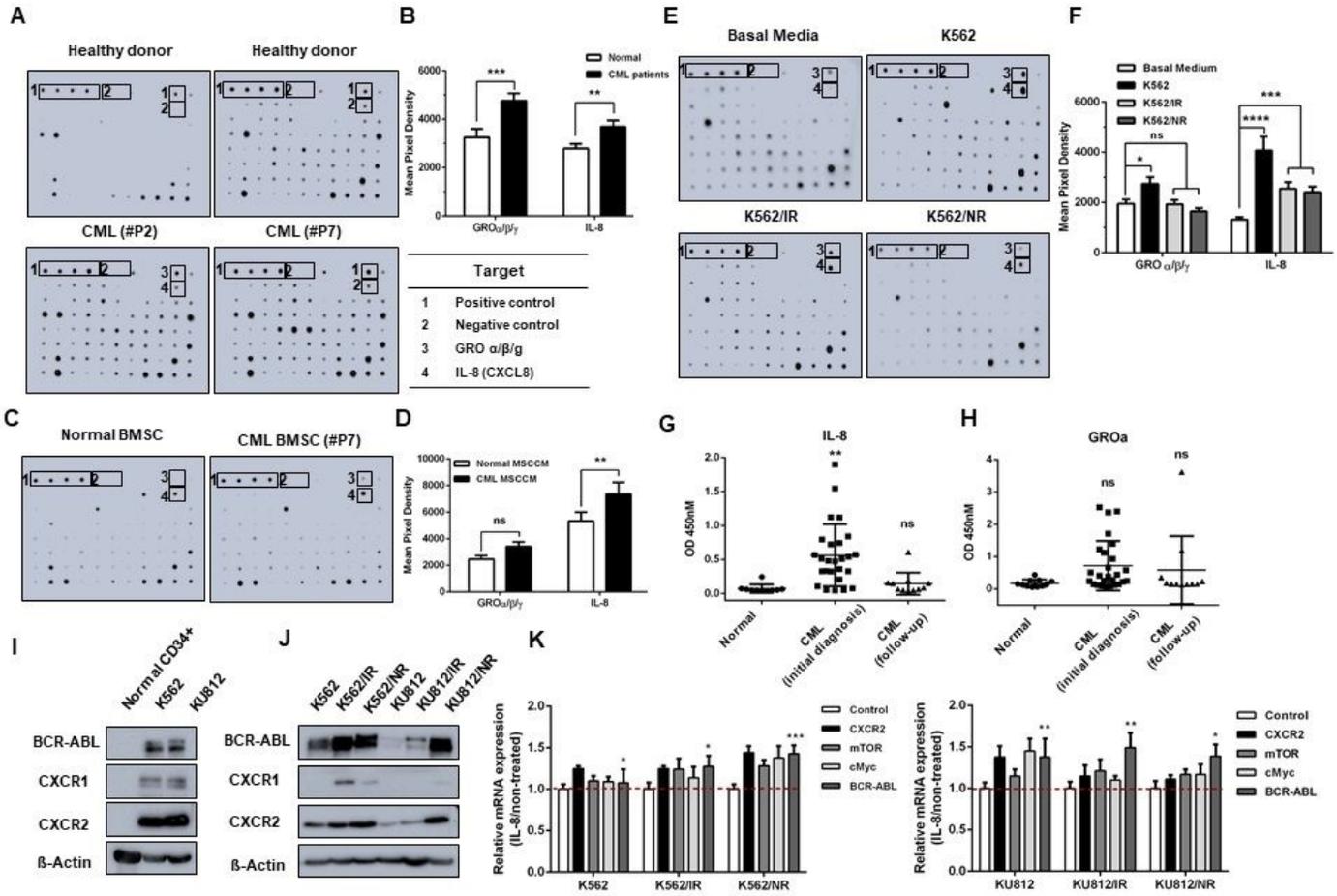


Figure 1

CML patient samples express IL-8, which activates CXCR2 and induces BCR-ABL activation. a A human cytokine array measuring various cytokines and chemokines in bone marrow (BM) serum of CML patients. b Bar graphs indicate the median value of the expression level of each cytokine. $**P < 0.01$; $***P < 0.001$. c Cytokines were detected using a human cytokine array in cell culture medium from normal human bone marrow-derived mesenchymal stromal cells (BMSCs) and CML-BMSCs. d Bar graphs indicate the median value of the expression level of each cytokine. $**P < 0.01$. e Simultaneous detection of multiple cytokines in culture-conditioned medium from K562, imatinib-resistant K562/IR, and nilotinib-resistant K562/NR cells at 48 h. f Bar graphs indicate the median value of the expression level of each cytokine. $*P < 0.05$; $***P < 0.001$; $****P < 0.0001$. g-h IL-8 (g) and GRO- α (h) protein levels in CML-BM serum samples (at first diagnosis, $n = 26$; TKI-treated patients, $n = 11$) compared to normal CD34+ cells ($n = 10$) determined using ELISA. Values are presented as the mean absolute amount of each protein (pg/mL). $**P < 0.01$. i CXCR2 expression in K562, KU812, and normal CD34+ cells detected using western blot. j Quantification of protein levels in TKI-sensitive (K562.KU812), imatinib-resistant (K562/IR and KU812/IR), and nilotinib-resistant (K562/NR and KU812/NR) cells detected using western blotting with

the indicated antibodies. k RT-qPCR of CXCR2, mTOR, c-Myc, and BCR-ABL mRNA levels in K562, K562/IR, K562/NR, KU812, KU812/IR, and KU812/NR cells after treatment with 50 ng/mL IL-8 for 5 min. Data are presented as the mean \pm SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

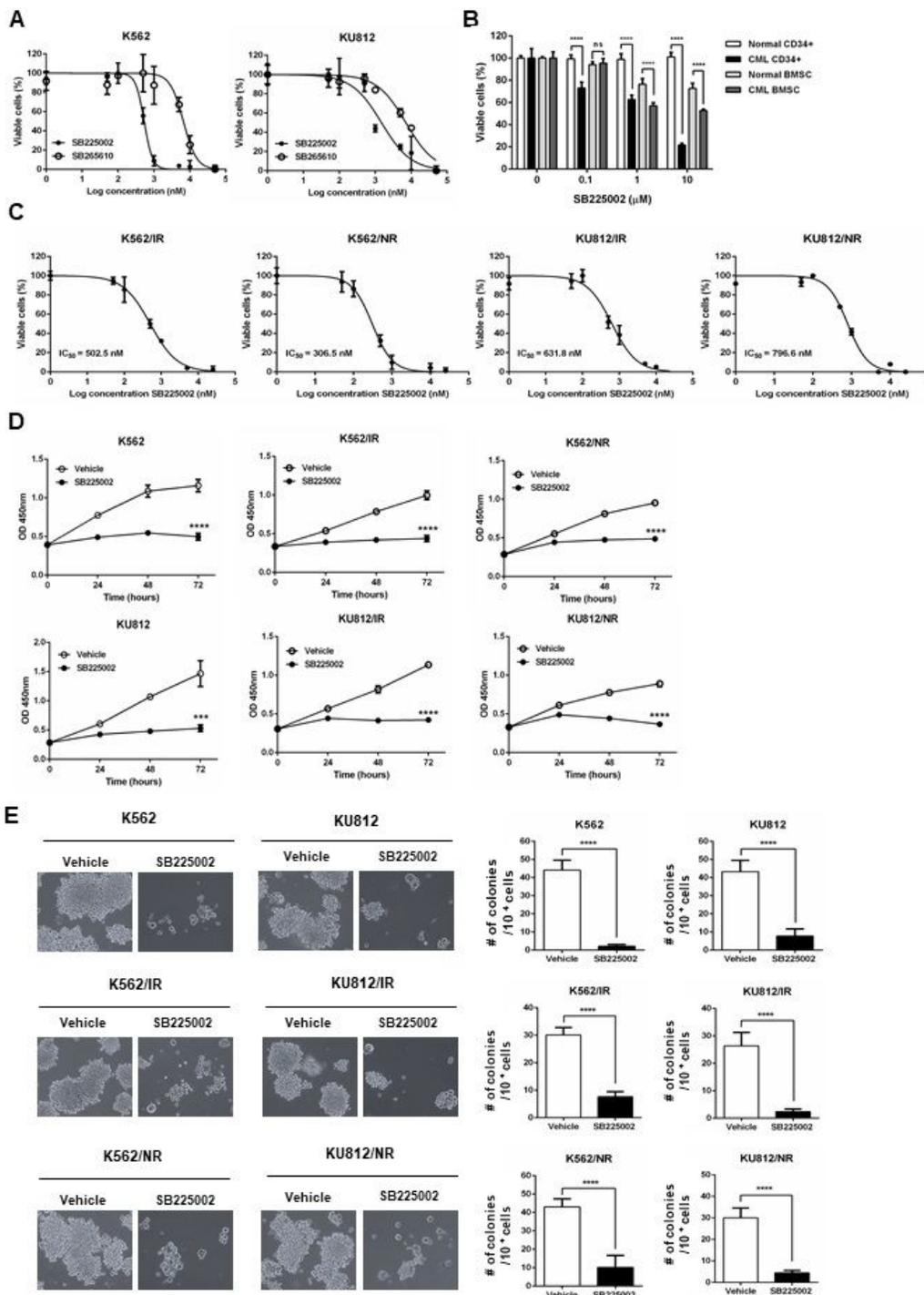


Figure 2

CXCR2 inhibition attenuates CML cell viability, regardless of TKI sensitivity. a Cell viability was measured using nonlinear regression analyses in K562 and KU812 cells with or without treatment with increasing

SB225002 or SB265610 doses for 48 h. Cell proliferation was detected using the CCK-8 assay. b Normal CD34+, CML CD34+, normal BMSC, and CML-BMSC cells were incubated with the indicated SB225002 dose for 48 h, followed by CCK-8 cell proliferation assays. Data are shown as mean \pm SD of three independent experiments. ****P < 0.0001. c Imatinib-resistant (K562/IR and KU812/IR) and nilotinib-resistant (K562/NR and KU812/NR) cells were treated with increasing SB225002 concentrations for 48 h. IC50 values were determined using Prism software. d Effect of SB225002 on cell proliferation in TKI-sensitive and -resistant CML cells. Parental, imatinib-, or nilotinib-resistant cells were cultured with 1 μ M SB225002 for 72 h. Representative data are shown as the mean \pm SD of three independent experiments. ***P < 0.001; ****P < 0.0001 versus control. e Colony-forming cell (CFC) assays were performed after treatment with DMSO or SB225002 for 48 h in TKI-sensitive and -resistant cells. Representative images show the colony sizes after vehicle or SB225002 treatment. Magnification, 100 \times . The number of colonies was calculated counting the total cells with Trypan blue staining on day 12. Each measurement was performed in duplicate. The data are presented as the mean \pm SD of three independent experiments. ****P < 0.0001.

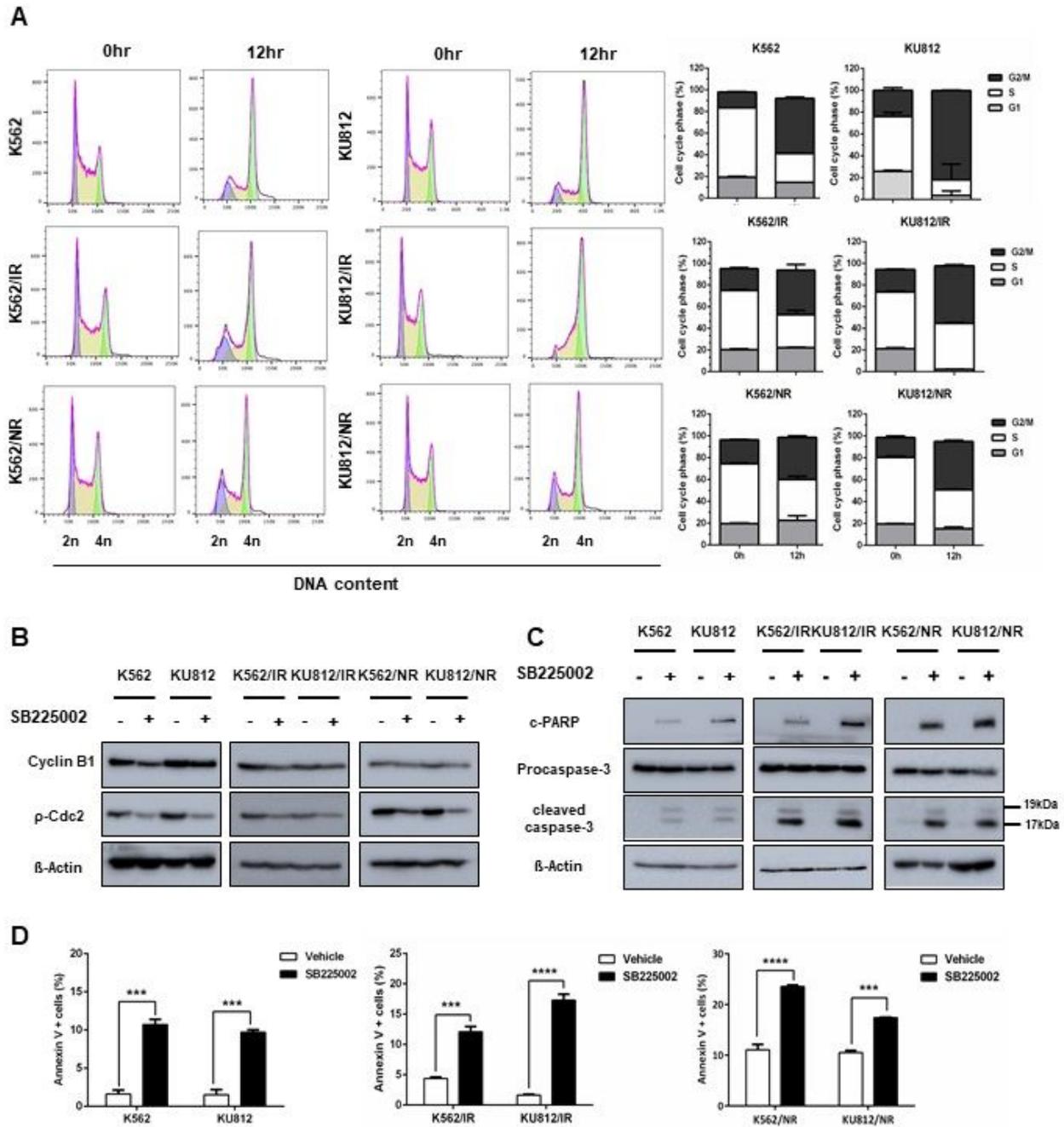


Figure 3

CXCR2 inhibition induces CML cell apoptosis through G2/M cell cycle arrest. a TKI-sensitive and -resistant cells were treated with SB225002 for 12 h. CML cells were stained with propidium iodide (PI), and cell cycle distribution was analyzed using flow cytometry. The proportion of each cell cycle phase is indicated. b Cell cycle regulatory protein levels were analyzed using western blotting with antibodies against the indicated proteins. c K562, K562/IR, and K562/NR cells were treated for 48 h with vehicle or

SB225002. Immunoblotting was used for the detection of apoptotic proteins, including cleaved caspase-3 and cleaved PARP. d Apoptosis was detected using annexin V-FITC and PI staining after culturing cells with or without 1 μ M SB225002 for 48 h. The proportion of apoptotic cells is indicated. Data represent the mean \pm SD of three independent experiments. ***P < 0.001; ****P < 0.0001.

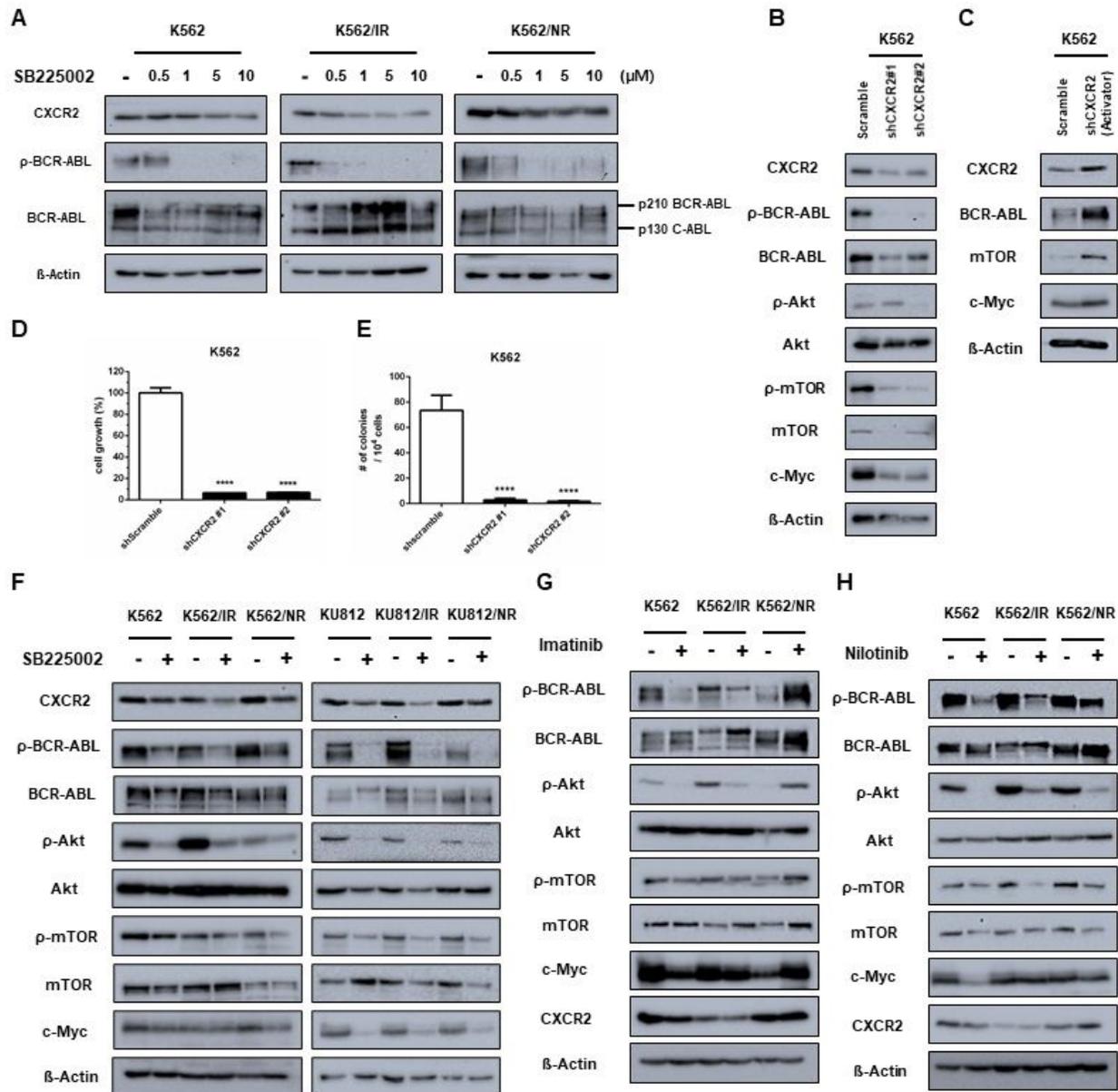


Figure 4

CXCR2 inhibition affects the Akt/mTOR and c-Myc signaling pathways. a K562, K562/IR, and K562/NR cells were treated with varying concentrations of SB225002 for 24 h. Protein levels of phosphorylated-BCR-ABL, BCR-ABL, and CXCR2 were detected using western blotting. b Western blotting to detect the expression of BCR-ABL, Akt, mTOR, and c-Myc in K562 cells infected with scramble or CXCR2 shRNA

lentiviruses. c BCR-ABL, mTOR, and c-Myc expression in K562 cells after transduction with control or CXCR2 lentiviral activation particles. d K562 cells were infected with the indicated lentiviruses, and cell proliferation was evaluated using CCK-8 assays after incubation for 96 h. ****P < 0.0001 versus lentivirus scramble. e Colony-forming assays were performed with lentivirus-infected K562 cells. The total number of cells was counted on day 14. ****P < 0.0001 versus lentivirus scramble. f Western blotting was performed to detect differences in BCR-ABL, Akt/mTOR, and c-Myc signaling between TKI-sensitive and -resistant CML cells after treatment with SB225002 for 24 h. g Western blot analysis was performed using antibodies against the indicated proteins on K562, K562/IR, and K562/NR cells treated with 1 μ M imatinib or h 20 nM nilotinib for 24 h.

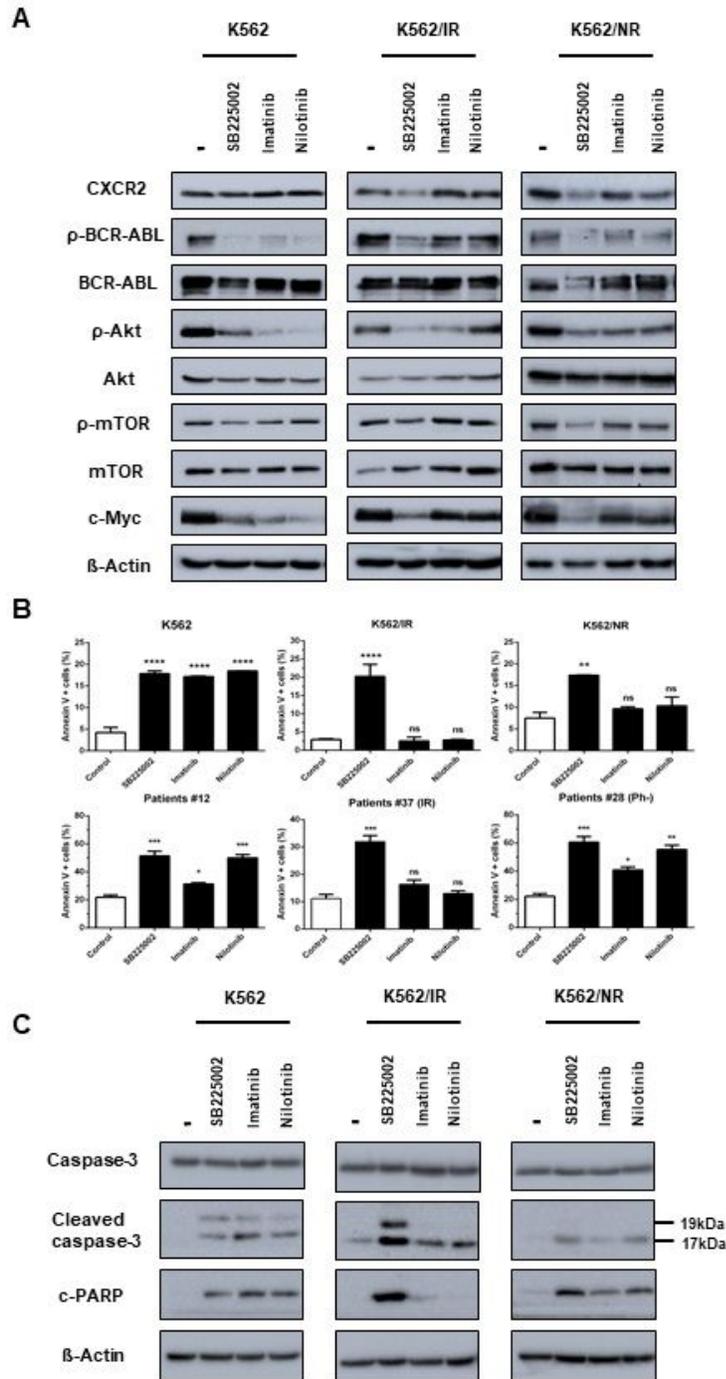


Figure 5

CXCR2 is a novel target to overcome TKI resistance in the treatment of CML. a Total proteins were extracted from K562, K562/IR, and K562/NR cells with or without 24 h SB225002, imatinib, or nilotinib treatment. Western blot analysis was performed using antibodies against the indicated proteins. b Apoptotic CML cells and patient-derived CD34+ cells were detected by staining cells with annexin V-FITC/PI and flow cytometry analysis after exposure to each drug for 48 h. Data represent the mean ± SD.

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 versus control. c After 48-h drug exposure, cleaved PARP and caspase-3 expression was measured in TKI-sensitive and -resistant cells.

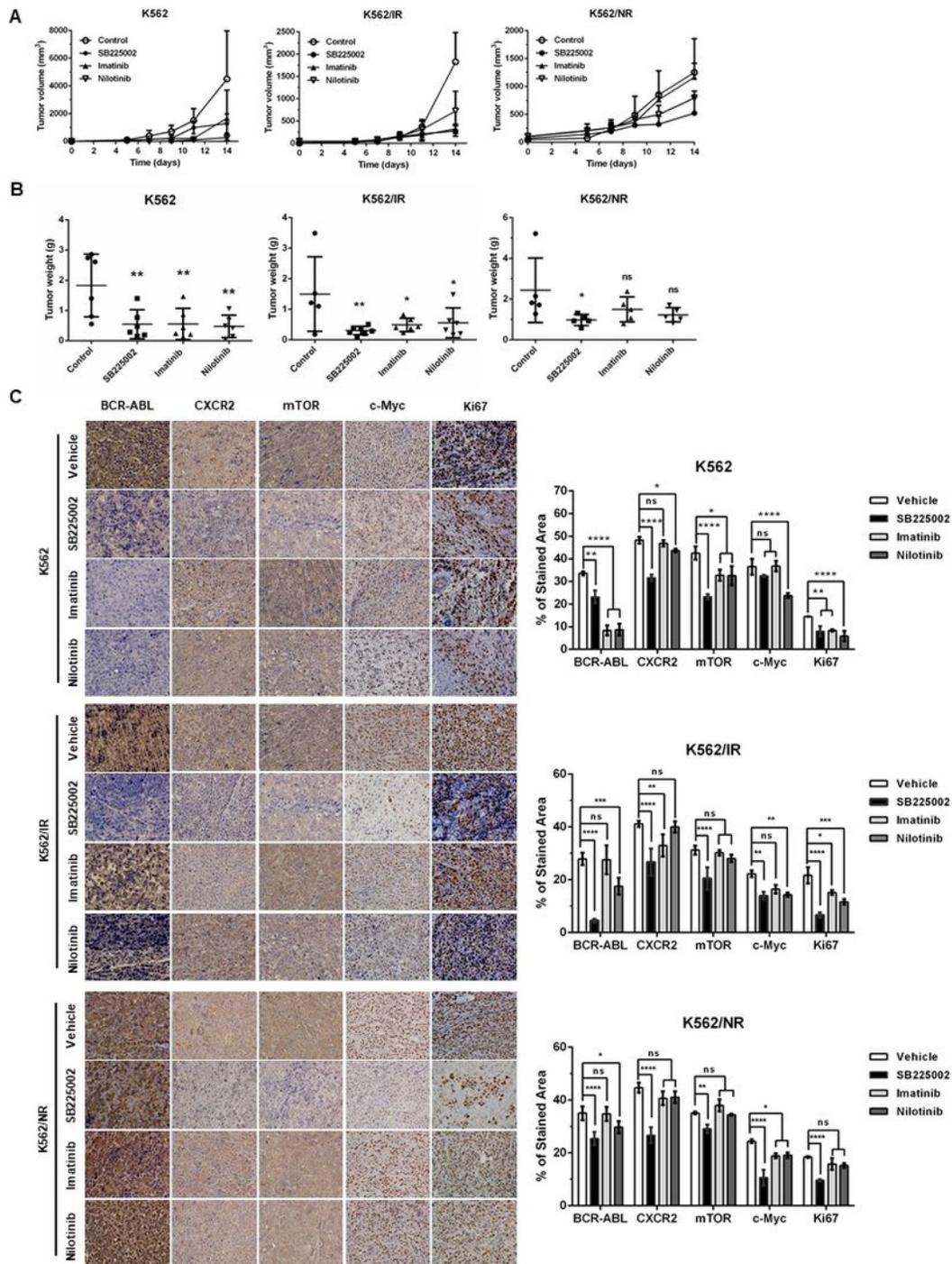


Figure 6

CXCR2 inhibition suppresses in vivo CML cell proliferation, particularly TKI-resistant cells. a K562, K562/IR, and K562/NR cells were injected subcutaneously into the right flank of NOD/SCID mice. Mice were intraperitoneally treated with 10 mg/kg SB225002, imatinib, or nilotinib three times per week. Tumor

volumes were measured with calipers and calculated using the following formula: $(\text{length} \times \text{width}^2)/2$. b The average tumor weight in each group (n = 6/group). c Representative images were detected indicated proteins using immunohistochemistry after a single dose of SB225002, imatinib, or nilotinib. BCR-ABL, CXCR2, mTOR, c-Myc, and Ki67 staining was detected in K562, K562/IR, and K562/NR cells in an in vivo xenograft model. Magnification, 40 \times . Bar graphs depict the quantification of the stained area. Data represent the mean \pm SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 versus vehicle.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.SupplementaryTable..docx](#)
- [Additionalfile2.Supplementaryfigure..docx](#)