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CXCR2, a novel target to overcome tyrosine kinase inhibitor resistance in chronic myelogenous leukemia cells

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ABSTRACT

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 treating 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 alternative treatments targeting tyrosine kinases. This study was designed to determine whether C-X-C chemokine receptor 2 (CXCR2) could be a novel target for TKI-resistant CML treatment. Interleukin 8 (IL-8), a CXCR2 ligand, was significantly increased in the bone marrow serum of initially diagnosed CML patients and TKI-resistant CML cell conditioned media. CXCR2 antagonists suppressed the proliferation of CML cells via cell cycle arrest in the G2/M phase. CXCR2 inhibition also 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 TKI-resistant CML cells than TKIs. Using a mouse xenograft model, we confirmed that SB225002 suppresses tumor growth, with a prominent effect on TKI-resistant CML cells. Our findings demonstrate that IL-8 is a prognostic factor for the progression of CML. Inhibiting the CXCR2-mTOR-c-Myc cascade is a promising therapeutic strategy to overcome TKI-sensitive and TKI-insensitive CML. Thus, CXCR2 blockade is a novel therapeutic strategy to treat CML, and SB225002, a commercially available CXCR2 antagonist, might be a candidate drug that could be used to treat TKI-resistant CML.

1. Introduction

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, BCR-ABL kinase domain (KD) point mutations have been identified in patients with resistance to imatinib. These mutations comprise amino acid substitutions, including M244V, G250E, T315, M351T, and F359V, [11] that lead to resistance by imatinib binding [12,13]. Further, approximately 15% of CML patients

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 behavior therapy; Ph, Philadelphia chromosome; PARP, Poly ADP-ribose polymerase; PI, Propidium iodide; shRNA, Short hairpin RNA; TKI, Tyrosine kinase inhibitor.

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treated with TKIs report primary cytogenetic resistance or loss of the achieved complete cytogenetic response due to drug resistance acquisition [14]. Second- and third-generation TKIs such as nilotinib, dasatinib, and ponatinib have been developed to overcome this problem [15,16]. Despite the development of additional TKI-based treatments, treatment failure still occurs due to leukemic stem cell (LSC) and CML insensitivity to TKIs [17]. CML cell survival is sustained by resistance mechanisms that enable the evasion to TKIs [18]. 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 [19], gastric [20], colon [21], melanoma [22], ovarian [22], pancreatic [23], and prostate [24] cancers. Interleukin 8 (IL-8), a CXCR2 ligand, is highly associated with cellular stemness and drug resistance by inhibiting drug-induced apoptosis in resistant cells [24–26]. Inhibiting the IL-8/CXCR2 axis reduces myelodysplastic syndrome and acute myeloid leukemia progression [27,28] 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 [29,30]. 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 [29,31,32]. In the present study, we investigated the role of CXCR2 in CML cells and assessed whether commercially available CXCR2 antagonists effectively suppress CML progression.

2. Materials and methods

2.1. 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 [29]. $CD34^+$ BMMNCs were sorted using a Super MACS instrument (Miltenyi Biotech Inc., Auburn, CA, USA). CML $CD34^+$ cells were cultured in a 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), cultured in a mesenchymal stem cell growth medium (Lonza, Walkersville, MD, USA).

2.2. 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; Sigma-Aldrich, St. Louis, MO, USA) 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% CO_2.

2.3. 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 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. *BCR-ABL* gene mutations generated during the acquisition of TKI resistance were identified using next-generation sequencing. Data analysis was described in Table 1.

Table 1
BCR-ABL kinase mutations in TKI-resistant cells.

Cell line	Chromosome base pair position	Reference	Alternative	Effect of variant	Mutation No./No. of transcripts
K562IR	133591459	CT	CTT, C	Intron variant	15/15
	133598821	A	G	Intron variant	1/24
KU812/IR	133598821	A	G	Intron variant	22/22
K562/NR	133653194	A	G	Intron variant	16/42
	13363080	TAG	T	Intron variant	1/34
KU812/NR	133683055	TTA	T	Intron variant	19/64
	133683088	T	TG	Intron variant	5/40

2.4. 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, and dot blot quantitation was performed using Image Lab 5.0 software (Bio-Rad, Hercules, CA, USA).

2.5. 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.

2.6. 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 semi-solid culture medium (methylcellulose H4034; Stem Cell Technologies, Grenoble, France). Cells were seeded onto 35 mm dishes (5×10^3 cells/dish). Each assay was performed in duplicate, and the cells were counted after 12–14 culture days.

2.7. 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 incubating cells with 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.

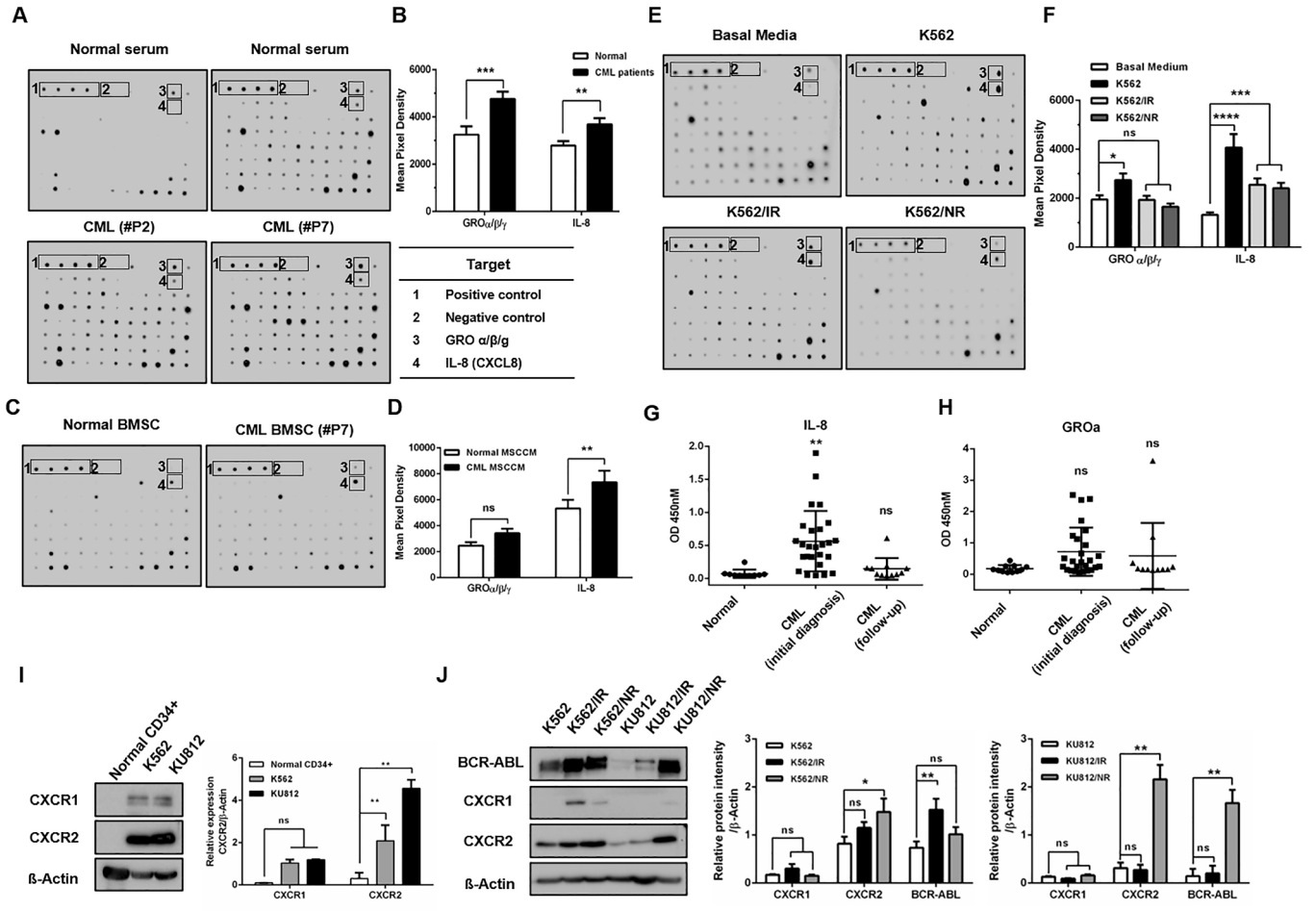

Fig. 1. CML patient samples express IL-8, which activates CXCR2 and induces BCR-ABL activation. (A) A human cytokine array measuring various cytokines and chemokines in bone marrow (BM) serum of CML patients. (B) Bar graphs indicate the median value of the expression level of each cytokine. $^{**}P < 0.01$; $^{***}P < 0.001$. (C) Cytokines were detected using a human cytokine array in a 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, $n = 29$; TKI-treated patients, $n = 10$) compared to normal $CD34^+$ cells ($n = 10$) determined using ELISA. Values are presented as the average optical density at 450 nm. $^{**}P < 0.01$. (I) CXCR2 expression in K562, KU812, and normal $CD34^+$ cells detected using western blot. (J) Quantification of protein levels in TKI-sensitive (K562 and 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. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

2.8. 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 μg/mL RNase A. Fixed cells were stained with 50 μg/mL propidium iodide (PI) solution and analyzed using FACS Canto II (BD, Franklin Lakes, NJ, USA). Cell cycle analysis was performed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

2.9. Western blotting

Proteins were extracted using a protease inhibitor kit (Intron Biotechnology.; Seongnam, Korea). Immunoblotting was performed as previously described [31]. Primary antibodies against the following proteins were used: CXCR2 (Cat# 65968) and c-Myc (Cat# 32072) were obtained from Abcam (Cambridge, UK); β-actin (Cat# sc-47778), cyclin B1 (Cat# sc-245), and p-CDC2 (Cat# sc-13601) from Santa Cruz Biotechnology (Dallas, TX, USA); p-c-ABL (Cat# 1861), c-ABL (Cat# 1861), p-mTOR (Cat# 5536), mTOR (Cat# 2972), p-Akt (Cat# 4060), Akt (Cat# 9272), caspase-3 (Cat# 9662), and cleaved PARP (Cat# 5625) were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.10. 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 μg/mL PI and 1 μg/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).

2.11. 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 μg/mL

Table 2
Characteristics of CML patients and healthy donors.

Category	Case No.	Age	Sex	Associated disease	Bone marrow serum (Fig. 1 G and H) IL-8	GRO-α	Remarks
CML Initial diagnosis	1	34	F	NA*	0.734	0.508	Fig. 1 A and B
	2	28	M	NA*	0.323	0.137	Fig. 1 A and B
	2	72	M	DM† & HTN‡	1.118	1.119	NA*
	3	75	M	CHD§ & DM†	0.723	0.382	NA*
	4	28	F	NA*	1.123	2.368	Fig. 1 A-D, Fig. 2 B
	5	75	F	DM† & HTN‡	0.61	3,616	NA*
	6	85	M	HTN‡	0.107	0.061	NA*
	7	42	F	NA*	0.508	0.276	Fig. 1 A-D, Fig. 2 B
	8	67	M	CRI|| & HTN‡	1.545	0.438	NA*
	9	37	M	NA*	0.842	0.661	Fig. 1 A and B
	10	30	M	NA*	0.539	1.23	Fig. 1 A and B
	11	61	F	HTN‡	0.205	1.706	NA*
	12	75	F	NA*	0.569	2.398	Fig. 5 B
	13	46	M	NA*	0.796	0.932	NA*
	14	55	F	DM†	0.36	1.397	Fig. 5 B
	15	63	M	COPD¶	0.512	0.214	NA*
	16	40	F	NA*	0.482	0.578	NA*
	17	48	F	NA*	0.566	0.409	NA*
	18	78	M	CVA# & HTN‡	0.332	0.086	NA*
	19	25	F	NA*	0.329	0.11	NA*
	20	60	M	HTN‡	0.473	0.253	NA*
	21	30	F	NA*	1.897	2.524	NA*
	22	31	F	NA*	0.045	0.145	NA*
	23	68	M	LC**	0.05	0.244	NA*
	24	67	F	DM† & HTN‡	0.071	0.318	NA*
	25	37	M	NA*	0.074	0.241	NA*
	26	63	M	CHD§	0.051	0.091	NA*
	27	67	F	NA*	0.329	0.115	NA*
	28	74	F	NA*	0.148††	0.154††	Ph- atypical CMLFig. 5 B
CML Follow-up	29	22	M	NA*	0.045	0.151	Imatinib Tx for 3 years
	30	65	M	Arrhythmia	0.085	0.359	Nilotinib Tx for 5 years
	31	46	F	DM†	0.046	0.152	Imatinib Tx for 10 years
	32	76	F	CHD§ & HTN‡	0.062	0.232	Imatinib Tx for 15 years
	33	35	M	NA*	0.063	0.139	Dasatinib Tx 3 years
	34	53	F	NA*	0.046	0.176	Nilotinib for 5 years
	35	62	M	HTN‡	0.157	0.149	Imatinib Tx for 3 years & Dasatinib Tx for 3 years
	36	26	F	NA*	0.051	0.082	Dasatinib Tx for 5 years
	37	50	M	NA*	0.189	0.126	Imatinib Tx for 3 years & Dasatinib Tx for 5 years
	38	71	F	DM† & HTN‡	0.151	1.188	Dasatinib Tx for 5 years & Nilotinib Tx for 5 years
Healthy donors	39	36	M	NA*	0.048	0.259	Bone marrow donor for allogeneic hematopoietic stem cell transplantation
	40	58	M	NA*	0.245	0.434	
	41	42	M	NA*	0.039	0.06	
	42	35	M	NA*	0.07	0.054	
	43	38	M	NA*	0.041	0.087	
	44	41	F	NA*	0.061	0,27	
	45	56	F	NA*	0.045	0.229	
	46	42	F	NA*	0.062	0.085	
	47	57	M	NA*	0.047	0.125	
	48	20	M	NA*	0.05	0.151	

*NA, not applicable.
†DM, diabetes mellitus; ‡HTN, hypertension; §CHD, chronic heart disease; ||CRI, chronic renal insufficiency; ¶COPD, chronic obstructive pulmonary disease; #CVA, cerebrovascular accident; **LC, liver cirrhosis.

polybrene. The cells were centrifuged at 800 × *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.

2.12. 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 μL suspended cells were implanted subcutaneously into the right flank. One week after inoculation, the mice were randomly divided into control and drug treatment groups (n = 11 mice per group). The control group was injected with DMSO, while the SB225002 [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 administered for 14 days. Tumor size was ascertained three times a week, and the volume was calculated using the following formula: volume (mm^3) = (length × height^2)/2.

2.13. Immunohistochemistry

Formalin-fixed tissue was embedded in paraffin, and 3 μm-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

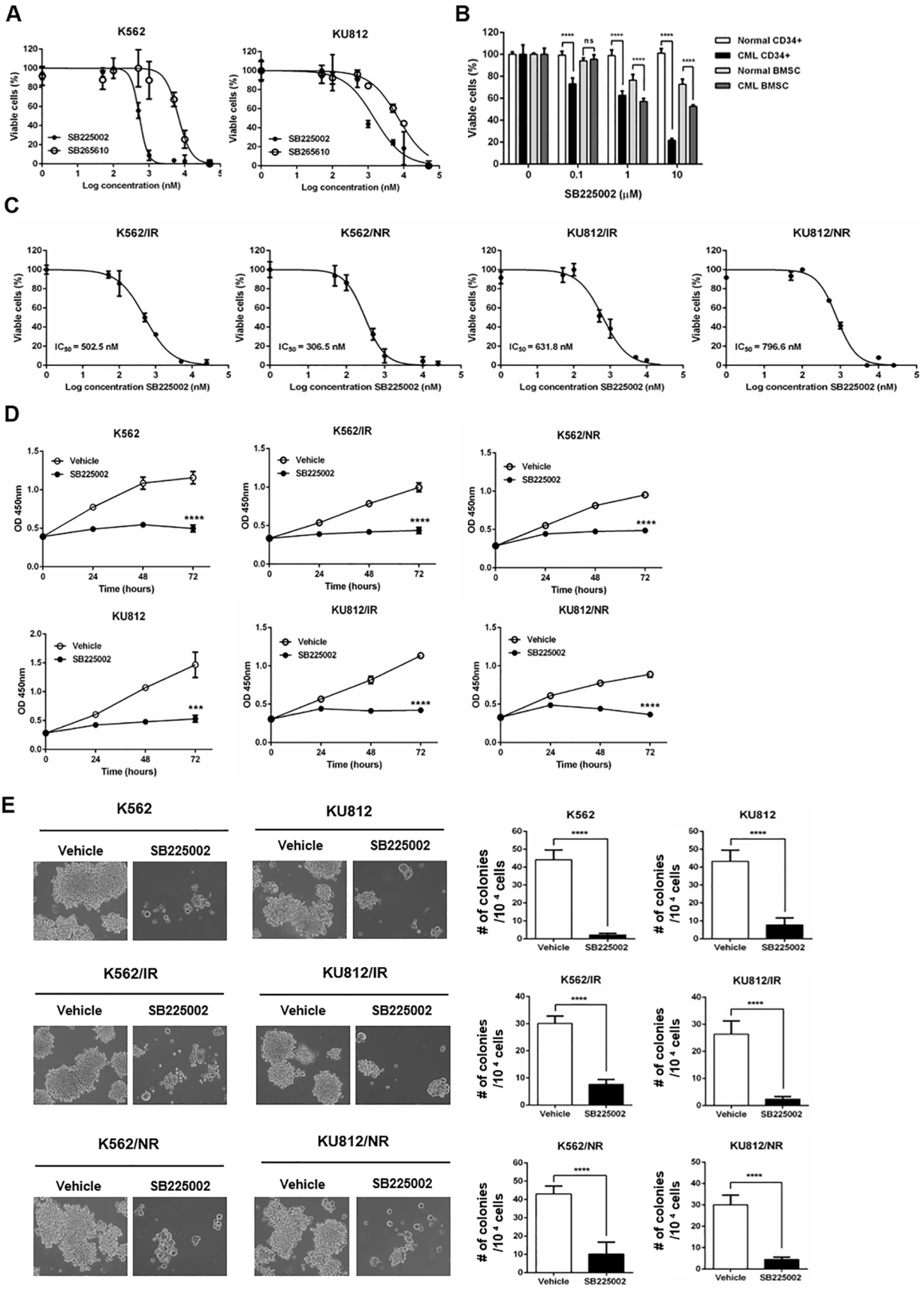

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Fig. 2. CXCR2 inhibition attenuates CML cell viability, regardless of TKI sensitivity. (A) Cell viability was measured using nonlinear regression analyses in K562 and KU812 cells with or without treatment with increasing SB225002 or SB265610 doses 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 ± 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. IC_{50} 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 μM SB225002 for 72 h. Representative data are shown as the mean ± 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×. The number of colonies was calculated by counting the total cells with Trypan blue staining on day 12. Each measurement was performed in duplicate. The data are presented as the mean ± SD of three independent experiments. $^{****}P < 0.0001$.

with primary antibodies against CXCR2 (Cat# 65968), c-Myc (Cat# 32072), and c-ABL (Cat# 16161) from Abcam (Cambridge, UK), and mTOR (Cat# AHO1232) from Thermo Fisher Scientific (San Jose, CA, USA), 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).

2.14. 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 [40], and ExAC [41]. Consensus sequences were obtained based on accumulated information in a specific region using Mpileup in SAMtools v. 1.3.1 [42].

2.15. Statistical analysis

All results were obtained from at least three independent experiments. The data are represented as the mean ± 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).

3. Results

3.1. 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 α/β/γ (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 healthy donors (Fig. 1C and D). To find whether the cytokine levels are altered after acquired resistance to TKIs, we established imatinib-resistance (IR) and nilotinib-resistance (NR) CML cell lines. 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 α/β/γ was expressed only in TKI-sensitive cell lines (Fig. 1E and F). We hypothesized that IL-8 might be more critical for CML development than GRO α/β/γ because the IL-8 level secreted by $CD34^+$ cells and CML patient-derived BMSCs was higher than that secreted by these cell types from 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) which were then compared to those of 10 healthy donors. The mean age and white blood cell count, 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 2. 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 receptors associated with IL-8, CXCR1 and CXCR2 protein expression were 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). Therefore, CXCR2 may be a novel target for the treatment of CML resistant to well-known TKIs.

3.2. 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 SB26561 (Fig. 2A). SB225002 was selected for further experiments, as it had a lower IC_{50}. To confirm the effect of SB225002 in the BM environment, which comprises $CD34^+$ cells and BMSCs, we observed changes in the viability of BM $CD34^+$ cells and BMSCs obtained from two CML patients and healthy donors. CML $CD34^+$ cell viability decreased with increasing SB225002 dose, but SB225002 had no effect on the viability of normal $CD34^+$ cells. In BMSCs, the CML-BMSC viability decreased to approximately 50%–60% at doses between 1 and 10 μM SB225002 but was affected by a much lower degree than that 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 significantly influencing 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

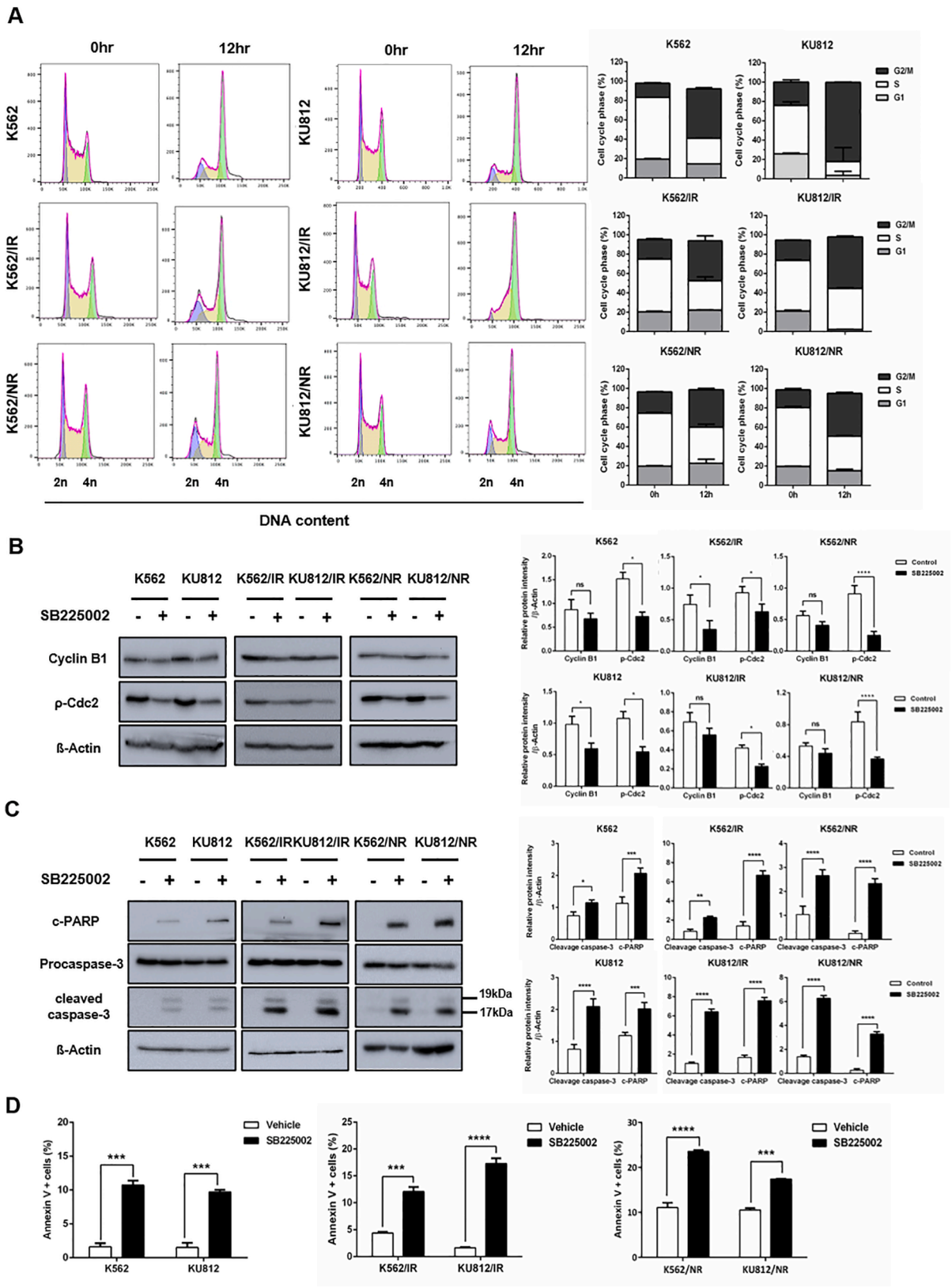

Fig. 3. CXCR2 inhibition induces CML cell apoptosis through G2/M cell cycle arrest. (A) TKI-sensitive and -resistant cells were treated with SB225002 for 12 h. CML cells were stained with propidium iodide (PI), and cell cycle distribution was analyzed using flow cytometry. The proportion of each cell 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 μM SB225002 for 48 h. The proportion of apoptotic cells is indicated. Data represent the mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

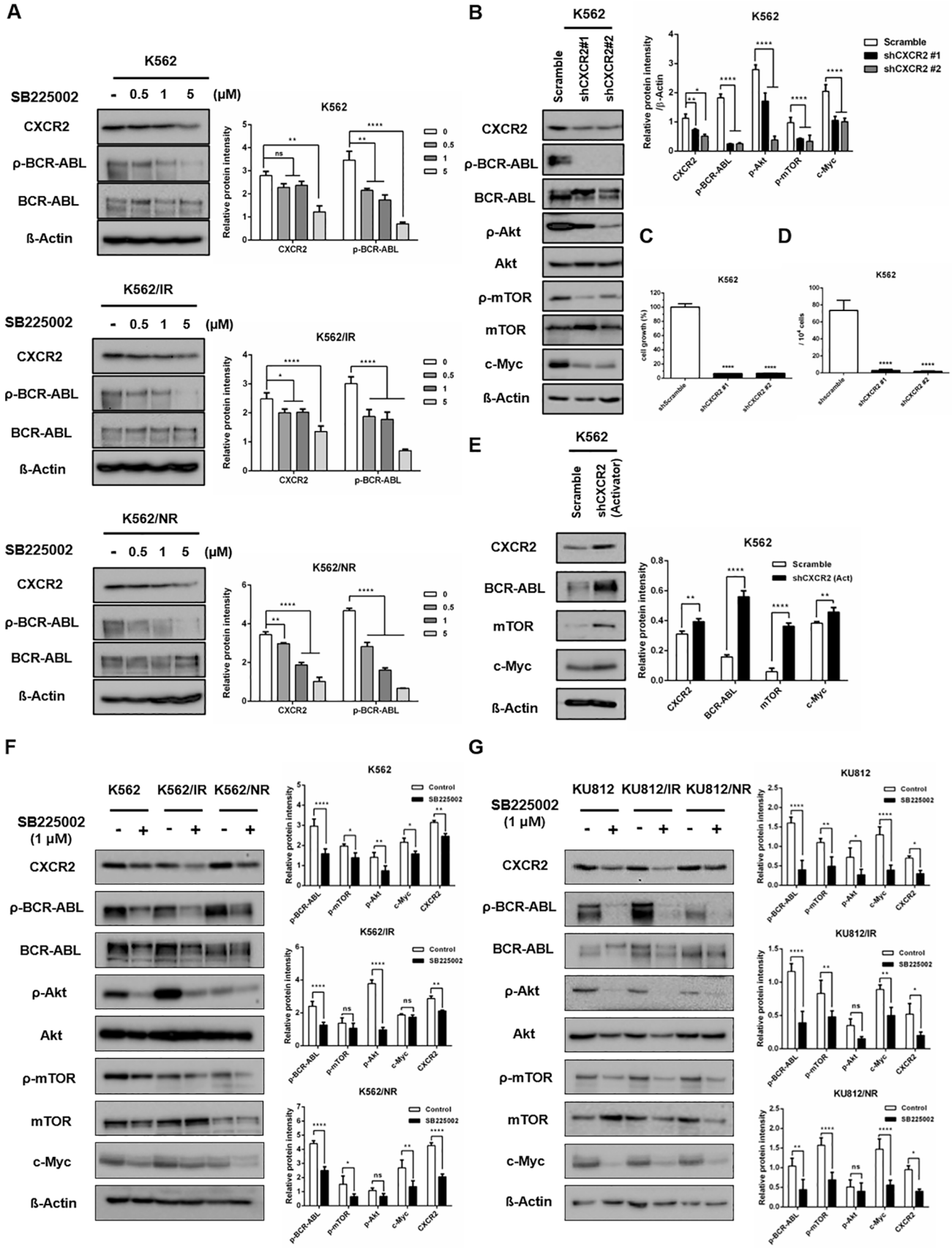

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Fig. 4. CXCR2 inhibition affects the Akt/mTOR and c-Myc signaling pathways. (A) K562, K562/IR, and K562/NR cells were treated with varying concentrations of SB225002 for 24 h. Protein levels of phosphorylated-BCR-ABL, BCR-ABL, and CXCR2 were detected 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) 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. (D) 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. (E) BCR-ABL, mTOR, and c-Myc expression in K562 cells after transduction with control or CXCR2 lentiviral activation particles. (F) Western blotting was performed to detect differences in BCR-ABL, Akt/mTOR, and c-Myc signaling between TKI-sensitive and -resistant K562 cells and (G) KU812 cells after treatment with 1 μM SB225002 for 24 h. Data represent the mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

cell lines (Fig. 2E). These results suggest that CXCR2 inhibition attenuates CML cell lines and CML CD34^{+} cell proliferation, regardless of TKI sensitivity.

3.3. 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 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 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.

3.4. 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 a reduction in BCR-ABL phosphorylation, which 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 [43,44] and Myc [45]. Therefore, the inhibition of signaling pathways affecting BCR-ABL after CXCR2 knockdown was investigated. Silencing CXCR2 reduced BCR-ABL, Akt, mTOR phosphorylation, and c-Myc (Fig. 4B). Loss of CXCR2 expression suppressed proliferation of and colony formation in K562 cells (Fig. 4C and D). Next, we confirmed the upregulation of those genes using CXCR2 lentiviral activation particles (Fig. 4E). This result suggests that regulating CXCR2 controls Akt/mTOR, c-Myc, and BCR-ABL levels. Treatment with CXCR2 antagonists inhibited p-Akt, p-mTOR, and c-Myc in TKI-sensitive and -resistant cells. (Fig. 4F and G). These data indicate that inhibiting CXCR2 signaling reduces BCR-ABL activity, even with TKI resistance. 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.

3.5. 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, reductions in p-BCR-ABL, p-AKT, p-mTOR, and c-Myc expression were observed after treatment with all the drugs. 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 in SB225002-treated TKI-resistant K562 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 clinical applicability of these findings, the drugs' apoptotic effect was tested in CD34^{+} cells from three CML patients, including a case with Ph^{-} atypical CML. The SB225002 apoptotic effect was comparable to TKIs on cells from a patient initially diagnosed with Ph^{+} CML and was superior to TKIs on cells from a patient showing imatinib-resistance and another with Ph^{-} atypical CML (Fig. 5B). 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 CML treatment.

3.6. 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. Two mice in the control group of K562 xenografts were excluded from the analysis because the diameters of their tumor masses exceeded the standard based on animal ethics guidelines, specifically a permissible diameter of masses of 20 mm in 14 days. The total number of mice analyzed in the control group of K562 xenografts was nine, and that in treatment groups was 11. The volumes of tumor masses were decreased in all treatment groups of K562 xenografts. Moreover, SB225002 had a better diminishing effect on tumor volume compared to that with K562/IR and K562/NR xenograft models after treatment with imatinib and nilotinib (Fig. 6A). In the K562 xenograft model, all treatments significantly reduced tumor weight compared to that in the vehicle group. The K562/IR xenograft model showed a significant reduction in mouse tumor weight upon treatment with SB225002 and nilotinib but not in imatinib. The K562/NR xenograft model presented with a meaningful reduction in tumor weight when treated with SB225002 only (Fig. 6B). Based on these results, we could verify that K562/IR cells acquired resistance to imatinib and that K562/NR cells are resistant to imatinib and nilotinib. As a result, CXCR2 inhibition effectively suppressed the proliferation of CML cells, and in particular, TKI-resistant cells, *in vivo*.

Immunohistochemical staining showed that tumor masses in the K562 group treated with SB225002 showed less BCR-ABL inhibition than those treated by TKIs, and the cell proliferation marker Ki67 was similarly suppressed in all treatment groups. However, the tumor masses of K562/IR and K562/NR mice treated with SB225002 presented with the highest inhibition of BCR-ABL and Ki67 expression among the groups, and the Ki67 levels in K562/IR and NR mice treated with imatinib and nilotinib were similar to those in animals treated with the vehicle. c-Myc in tumor masses of K562/IR mice was reduced by nilotinib and SB225002, whereas that in K562/NR mice was reduced only by SB225002 (Fig. 6C). These findings suggest a possible role of c-Myc in

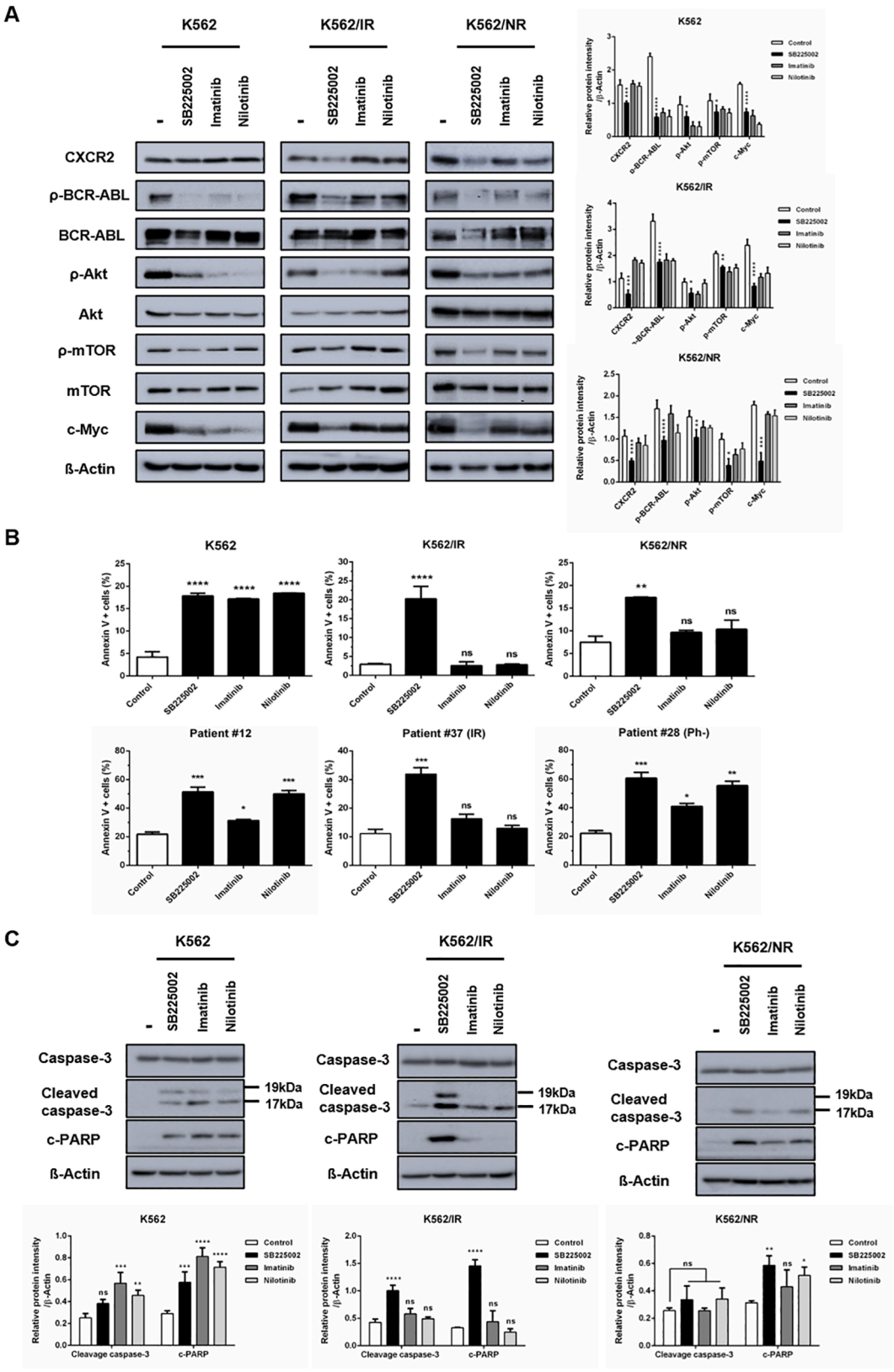

Fig. 5. CXCR2 is a novel target to overcome TKI resistance in the treatment of CML. (A) Total proteins were extracted from K562, K562/IR, and K562/NR cells with or without 24 h SB225002, imatinib, or nilotinib treatment. Western blot analysis was performed using antibodies against the indicated proteins. (B) Apoptotic CML cells (upper panel) and patient-derived $CD34^{+}$ cells (n = 3, lower panel) were detected by staining cells with annexin V-FITC/PI and flow cytometry analysis after exposure to each drug for 48 h. (C) After 48-h drug exposure, cleaved PARP and caspase-3 expression was measured in TKI-sensitive and -resistant cells. Data represent the mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ versus control.

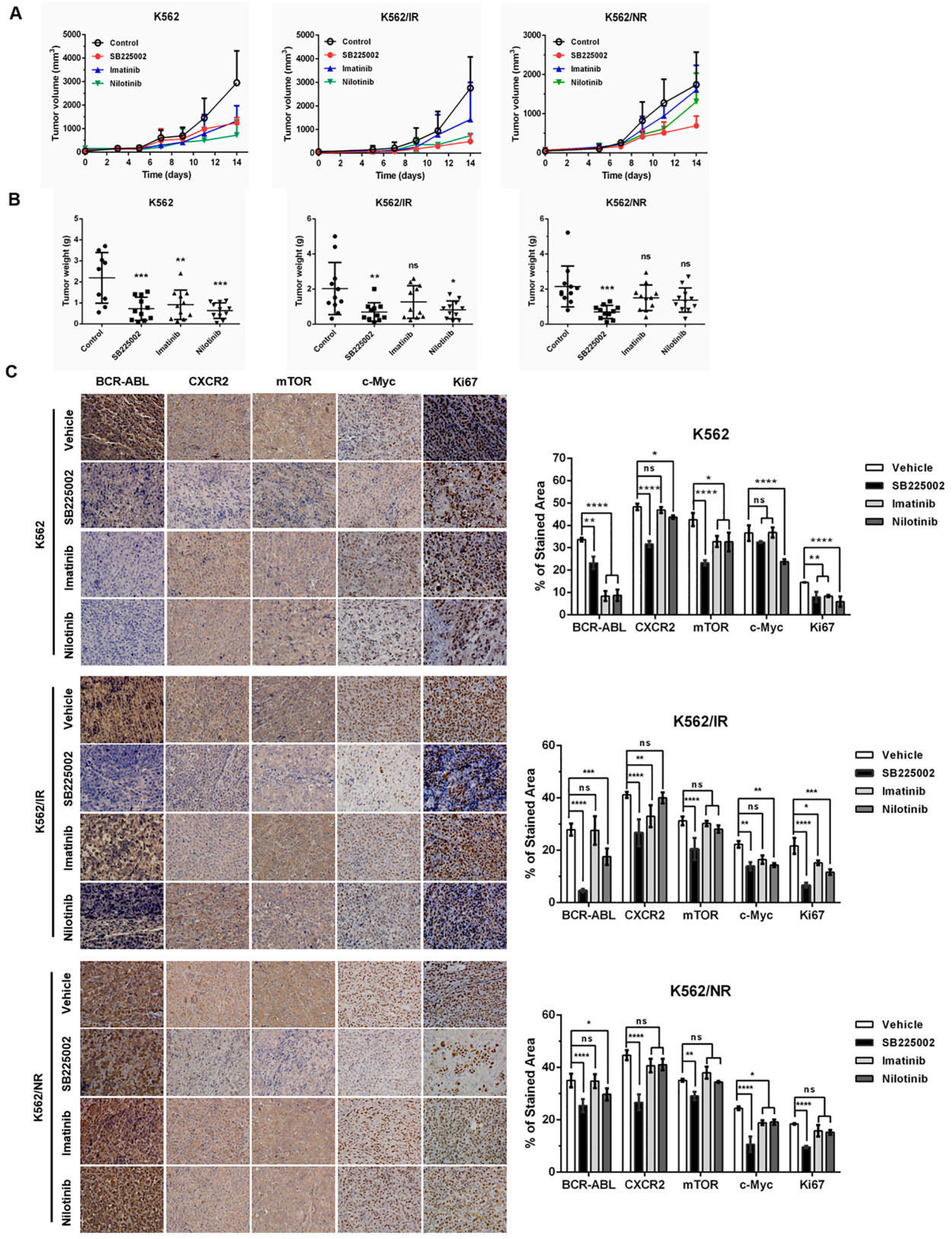

Fig. 6. CXCR2 inhibition suppresses *in vivo* CML cell proliferation, particularly TKI-resistant cells. (A) K562, K562/IR, and K562/NR cells were injected subcutaneously into the right flank of NOD/SCID mice. Mice were intraperitoneally treated with 10 mg/kg SB225002, imatinib, or nilotinib three times per week. Tumor volumes were measured with calipers and calculated using the following formula: (length × width^2)/2. (B) Tumors were excised from each group of mice on days 21 after K562, K562/IR, and K562/NR cell transplantation. Average tumor weights in each group are shown ($n = 11$/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 × . Bar graphs depict the quantification of the stained area. Data represent the mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ versus vehicle.

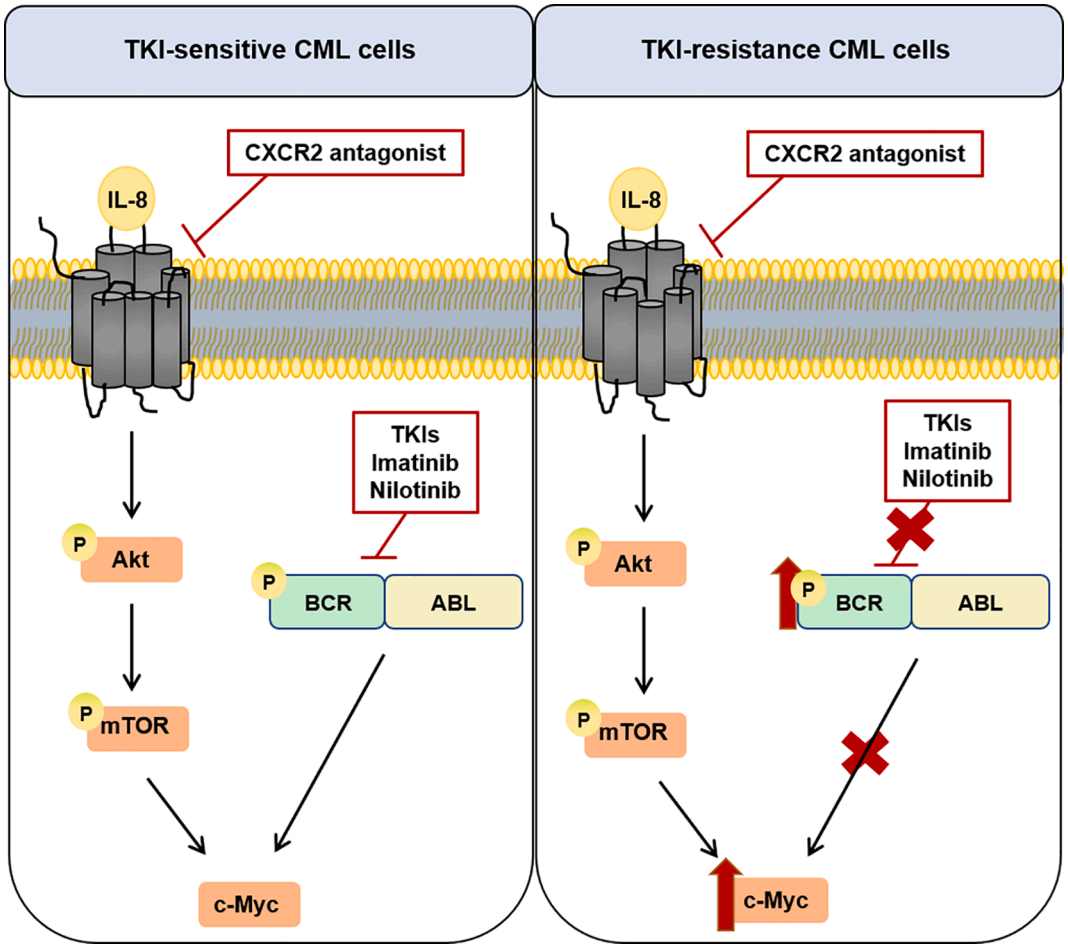

Fig. 7. Illustration of the CXCR2 pathway, regulated by SB225002, in TKI-sensitive and TKI-resistant CML cells. Highly expressed IL-8 promotes CXCR2 activation in CML. CXCR2 signaling blockade inhibits the Akt/mTOR, c-Myc pathway cascade. Imatinib- or nilotinib-treated TKI resistant cells showed both elevated BCR-ABL and c-Myc signaling activities in CML. Thus, downstream signaling is maintained in TKI-resistant cells. These results implicate that CXCR2 signaling is a promising target because of the reduced BCR-ABL and c-Myc expression in TKI-resistant CML.

the acquisition of TKI resistance and that targeting CXCR2 could overcome this resistance via c-Myc inhibition.

4. 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 [46–48]. Only 27% of resistant or intolerant patients achieved significant major molecular responses in the PACE trial [49]. This study aimed to examine the drivers of BCR-ABL-independent resistance and identify clinically relevant compounds for eradicating TKI-resistant cells. All established cell lines with IR or NR expressed some mutations in introns, but not in the transcript region (Table 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. Besides, $CD34^+$ 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 [50,51]. 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 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 the proliferation of human hematopoietic stem cells [29], enhances reprogramming to induced pluripotent stem cells [31], and maintains the characteristics of human pluripotent stem cells [32]. Several transcription factors, such as c-Myc, Nanog, Sox2, and OCT4, are regulated by IL-8 [10]. Previous studies showed the reduction of IL-8 levels in K562 cells and CML patient serum samples treated with dasatinib and nilotinib [52]. 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 [53,54] because c-Myc binds to the *BCR* promoter and directly regulates *BCR* activity [45]. These reports indicate that IL-8 secretion is associated with 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 (Fig. 7). Our study verifies that the regulation of CXCR2 affects the BCR-ABL, Akt/mTOR, and c-Myc pathways and contributes to eradicating CML cells regardless of acquired resistance to TKIs, *in vitro* and *in vivo*.

In conclusion, CXCR2 might be a novel target for treating CML independent from tyrosine kinase inhibition. Indeed, CXCR2 inhibition might be especially valuable to treat TKI-resistant CML.

CRediT authorship contribution statement

Ji-Hea Kim: Methodology, Formal analysis, Writing - original draft. **Seung-Jin Lee:** Investigation. **Ka-Won Kang:** Resources. **Byung-Hyun Lee:** Resources. **Yong Park:** Resources. **Byung-Soo Kim:** Resources, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] G.Q. Daley, R.A. Van Etten, D. Baltimore, Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome, Science 247 (1990) 824–830, https://doi.org/10.1126/science.2406902.
[2] Y. Chen, C. Peng, D. Li, S. Li, Molecular and cellular bases of chronic myeloid leukemia, Protein Cell 1 (2010) 124–132, https://doi.org/10.1007/s13238-010-0016-z.
[3] J.R. McWhirter, J.Y. Wang, Activation of tyrosinase kinase and microfilament-binding functions of c-abl by bcr sequences in bcr/abl fusion proteins, Mol. Cell. Biol. 11 (1991) 1553–1565, https://doi.org/10.1128/mcb.11.3.1553.
[4] E. Traer, R. MacKenzie, J. Snead, A. Agarwal, A.M. Eiring, T. O'Hare, B.J. Druker, M.W. Deininger, Blockade of JAK2-mediated extrinsic survival signals restores sensitivity of CML cells to ABL inhibitors, Leukemia 26 (2012) 1140–1143, https://doi.org/10.1038/leu.2011.325.
[5] J. Sonoyama, I. Matsumura, S. Ezoe, Y. Satoh, X. Zhang, Y. Kataoka, E. Takai, M. Mizuki, T. Machii, H. Wakao, Y. Kanakura, Functional cooperation among Ras, STAT5, and phosphatidylinositol 3-kinase is required for full oncogenic activities of BCR/ABL in K562 cells, J. Biol. Chem. 277 (2002) 8076–8082, https://doi.org/10.1074/jbc.M111501200.
[6] C.D. Kang, S.D. Yoo, B.W. Hwang, K.W. Kim, D.W. Kim, C.M. Kim, S.H. Kim, B. S. Chung, 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. 24 (2000) 527–534, https://doi.org/10.1016/S0145-2126(00)00010-2.
[7] A. Chorzalska, N. Ahsan, R.S.P. Rao, K. Roder, X. Yu, J. Morgan, A. Tepper, S. Hines, P. Zhang, D.O. Treaba, T.C. Zhao, A.J. Olszewski, J.L. Reagan, O. Liang, P. A. Gruppuso, P.M. Dubielecka, Overexpression of Tpl2 is linked to imatinib resistance and activation of MEK-ERK and NF-κB pathways in a model of chronic myeloid leukemia, Mol. Oncol. 12 (2018) 630–647, https://doi.org/10.1002/1878-0261.12186.
[8] J. Bertacchini, N. Heidari, L. Mediani, S. Capitani, M. Shahjahani, A. Ahmadzadeh, N. Saki, Targeting PI3K/AKT/mTOR network for treatment of leukemia, Cell. Mol. Life Sci. 72 (2015) 2337–2347, https://doi.org/10.1007/s00018-015-1867-5.
[9] F. Zhang, K. Li, X. Yao, H. Wang, W. Li, J. Wu, M. Li, R. Zhou, L. Xu, L. Zhao, A miR-567-PIK3AP1-PI3K/AKT-c-Myc feedback loop regulates tumour growth and chemoresistance in gastric cancer, EBioMedicine. 44 (2019) 311–321, https://doi.org/10.1016/j.ebiom.2019.05.003.
[10] L. Yang, P. Shi, G. Zhao, J. Xu, W. Peng, J. Zhang, G. Zhang, X. Wang, Z. Dong, F. Chen, H. Cui, Targeting cancer stem cell pathways for cancer therapy, Springer US, 2020. doi: 10.1038/s41392-020-0110-5.
[11] S. Soverini, S. Colarossi, A. Gnani, G. Rosti, F. Castagnetti, A. Poerio, I. Iacobucci, M. Amabile, E. Abruzzese, E. Orlandi, F. Radaelli, F. Ciccone, M. Tiribelli, R. Di Lorenzo, C. Caracciolo, B. Izzo, F. Pane, G. Saglio, M. Baccarani, G. Martinelli, Contribution of ABL kinase domain mutations to imatinib resistance in different subsets of Philadelphia-positive patients: by the GIMEMA working party on chronic myeloid leukemia, Clin. Cancer Res. 12 (2006) 7374–7379, https://doi.org/10.1158/1078-0432.CCR-06-1516.
[12] F.D. Böhmer, L. Karagyozov, A. Uecker, H. Serve, A. Botzki, S. Mahboobi, S. Dove, A single amino acid exchange inverts susceptibility of related receptor tyrosine kinases for the ATP site inhibitor STI-571, J. Biol. Chem. 278 (2003) 5148–5155, https://doi.org/10.1074/jbc.M209861200.
[13] A.S. Corbin, E. Buchdunger, F. Pascal, B.J. Druker, Analysis of the structural basis of specificity of inhibition of the Abl kinase by STI571, J. Biol. Chem. 277 (2002) 32214–32219, https://doi.org/10.1074/jbc.M111525200.
[14] H.A. Bradeen, C.A. Eide, T. O'Hare, K.J. Johnson, S.G. Willis, F.Y. Lee, B.J. Druker, M.W. Deininger, 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 108 (2006) 2332–2338, https://doi.org/10.1182/blood-2006-02-004580.
[15] T. O'Hare, C.A. Eide, M.W.N. Deininger, Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia, Blood 110 (2007) 2242–2249, https://doi.org/10.1182/blood-2007-03-066936.
[16] T. Hughes, G. Saglio, S. Branford, S. Soverini, D.W. Kim, M.C. Müller, G. Martinelli, J. Cortes, L. Beppu, E. Gottardi, D. Kim, P. Erben, Y. Shou, A. Haque, N. Gallagher, J. Radich, A. Hochhaus, Impact of baseline BCR-ABL mutations on response to nilotinib in patients with chronic myeloid leukemia in chronic phase, J. Clin. Oncol. 27 (2009) 4204–4210, https://doi.org/10.1200/JCO.2009.21.8230.
[17] A.S. Corbin, A. Agarwal, M. Loriaux, J. Cortes, M.W. Deininger, B.J. Druker, Erratum: Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity (Journal of Clinical Investigation (2011) 121, 1, (396-409) DOI: 10.1172/JCI35721), J. Clin. Invest. 121 (2011) 1222. doi: 10.1172/JCI46407.
[18] M. Massimino, S. Stella, E. Tirrò, C. Romano, M.S. Pennisi, A. Puma, L. Manzella, A. Zanghì, F. Stagno, F. Di Raimondo, P. Vigneri, Non ABL-directed inhibitors as alternative treatment strategies for chronic myeloid leukemia, Mol. Cancer. 17 (2018) 1–15, https://doi.org/10.1186/s12943-018-0805-1.
[19] H. Xu, F. Lin, Z. Wang, L. Yang, J. Meng, Z. Ou, Z. Shao, G. Di, G. Yang, CXCR2 promotes breast cancer metastasis and chemoresistance via suppression of AKT1 and activation of COX2, Cancer Lett. 412 (2018) 69–80, https://doi.org/10.1016/j.canlet.2017.09.030.
[20] Z. Wang, H. Liu, Z. Shen, X. Wang, H. Zhang, J. Qin, J. Xu, Y. Sun, X. Qin, The prognostic value of CXC-chemokine receptor 2 (CXCR2) in gastric cancer patients, BMC Cancer 15 (2015) 1–8, https://doi.org/10.1186/s12885-015-1793-9.
[21] Y.S. Lee, I. Choi, Y. Ning, N.Y. Kim, V. Khatchadourian, D. Yang, H.K. Chung, D. Choi, M.J. Labonte, R.D. Ladner, K.C. Nagulapalli Venkata, D.O. Rosenberg, N. A. Petasis, H.J. Lenz, Y.K. Hong, Interleukin-8 and its receptor CXCR2 in the tumour microenvironment promote colon cancer growth, progression and metastasis, Br. J. Cancer 106 (2012) 1833–1841, https://doi.org/10.1038/bjc.2012.177.
[22] B. Sharma, S. Singh, M.L. Varney, R.K. Singh, Targeting CXCR1/CXCR2 receptor antagonism in malignant melanoma, Expert Opin. Ther. Targets. 14 (2010) 435–442, https://doi.org/10.1517/14728221003652471.
[23] C.W. Steele, S.A. Karim, J.D.G. Leach, P. Bailey, R. Upstill-Goddard, L. Rishi, M. Foth, S. Bryson, K. McDaid, Z. Wilson, C. Eberlein, J.B. Candido, M. Clarke, C. Nixon, J. Connelly, N. Jamieson, C.R. Carter, F. Balkwill, D.K. Chang, T.R. J. Evans, D. Strathdee, A.V. Biankin, R.J.B. Nibbs, S.T. Barry, O.J. Sansom, J. P. Morton, CXCR2 inhibition profoundly suppresses metastases and augments immunotherapy in pancreatic ductal adenocarcinoma, Cancer Cell 29 (2016) 832–845, https://doi.org/10.1016/j.ccell.2016.04.014.
[24] Y. Li, Y. He, W. Butler, L. Xu, Y. Chang, K. Lei, H. Zhang, Y. Zhou, A.C. Gao, Q. Zhang, D.G. Taylor, D. Cheng, S. Farber-Katz, R. Karam, T. Landrith, B. Li, S. Wu, V. Hsuan, Q. Yang, H. Hu, X. Chen, M. Flowers, S.J. Mccall, J.K. Lee, B.A. Smith, J.W. Park, A.S. Goldstein, O.N. Witte, Q. Wang, M.B. Rettig, A.J. Armstrong, Q. Cheng, J. Huang, Targeting cellular heterogeneity with CXCR2 blockade for the treatment of therapy-resistant prostate cancer, 2019. http://stm.sciencemag.org/.
[25] R.T. Abraham, Chemokine to the Rescue: Interleukin-8 Mediates Resistance to PI3K-Pathway-Targeted Therapy in Breast Cancer, Cancer Cell 22 (2012) 703–705, https://doi.org/10.1016/j.ccr.2012.11.012.
[26] Y. Wang, Y. Qu, X.L. Niu, W.J. Sun, X.L. Zhang, L.Z. Li, Autocrine production of interleukin-8 confers cisplatin and paclitaxel resistance in ovarian cancer cells, Cytokine 56 (2011) 365–375, https://doi.org/10.1016/j.cyto.2011.06.005.
[27] A. Kuett, C. Rieger, D. Perathoner, T. Herold, M. Wagner, S. Sironi, K. Sotlar, H. P. Horny, C. Deniffel, H. Drolle, M. Fiegl, IL-8 as mediator in the microenvironment-leukaemia network in acute myeloid leukaemia, Sci. Rep. 5 (2015) 1–11, https://doi.org/10.1038/srep18411.
[28] C. Schinke, O. Giricz, W. Li, A. Shastri, S. Gordon, L. Barreyro, T. Bhagat, S. Bhattacharyya, N. Ramachandra, M. Bartenstein, A. Pellagatti, J. Boultwood, A. Wickrema, Y. Yu, B. Will, S. Wei, U. Steidl, A. Verma, IL8-CXCR2 pathway inhibition as a therapeutic strategy against MDS and AML stem cells, Blood 125 (2015) 3144–3152, https://doi.org/10.1182/blood-2015-01-621631.
[29] K.W. Kang, S.J. Lee, J.H. Kim, B.H. Lee, S.J. Kim, Y. Park, B.S. Kim, Etoposide-mediated interleukin-8 secretion from bone marrow stromal cells induces hematopoietic stem cell mobilization, BMC Cancer 20 (2020) 1–15, https://doi.org/10.1186/s12885-020-07102-x.
[30] A. Sinclair, L. Park, M. Shah, M. Drotar, S. Calaminus, L.E.M. Hopcroft, R. Kinstrie, A.V. Guitart, K. Dunn, S.A. Abraham, O. Sansom, A.M. Michie, L. Machesky, K. R. Kranc, G.J. Graham, F. Pellicano, T.L. Holyoake, CXCR2 and CXCL4 regulate survival and self-renewal of hematopoietic stem/progenitor cells, Blood 128 (2016) 371–383, https://doi.org/10.1182/blood-2015-08-661785.
[31] S.-J. Lee, K.-W. Kang, J.-H. Kim, B.-H. Lee, J.-H. Jung, Y. Park, S.-C. Hong, B.-S. Kim, CXCR2 ligands and mTOR activation enhance reprogramming of human somatic cells to pluripotent stem cells, Stem Cells Dev. 29 (2020) 119–132, https://doi.org/10.1089/scd.2019.0188.
[32] J.H. Jung, K.W. Kang, J. Kim, S.C. Hong, Y. Park, B.S. Kim, 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. 25 (2016) 1006–1019, https://doi.org/10.1089/scd.2015.0395.
[33] J.F. De Vasconcellos, A.B.A. Laranjeira, P.C. Leal, M.K. Bhasin, P.P. Zenatti, R. J. Nunes, R.A. Yunes, A.E. Nowill, T.A. Libermann, L.F. Zerbini, J.A. Yunes, SB225002 induces cell death and cell cycle arrest in acute lymphoblastic leukemia cells through the activation of GLIPR1, PLoS ONE 10 (2015) 1–19, https://doi.org/10.1371/journal.pone.0134783.
[34] K. Beider, M. Darash-Yahana, O. Blaier, M. Koren-Michowitz, M. Abraham, H. Wald, O. Wald, E. Galun, O. Eizenberg, A. Peled, A. Nagler, Combination of imatinib with CXCR4 Antagonist BKT140 overcomes the protective effect of stroma and targets CML in vitro and in vivo, Mol. Cancer Ther. 13 (2014) 1155–1169, https://doi.org/10.1158/1535-7163.MCT-13-0410.
[35] S. Wang, W. Xie, D. Wang, Z. Peng, Y. Zheng, N. Liu, W. Dai, Y. Wang, Z. Wang, Y. Yang, Y. Chen, Discovery of a small molecule targeting SET-PP2A interaction to overcome BCR-ABLT315I mutation of chronic myeloid leukemia, Oncotarget 6 (2015) 12128–12140, https://doi.org/10.18632/oncotarget.3665.
[36] F. Yan, A. Al-Kali, Z. Zhang, J. Liu, J. Pang, N. Zhao, C. He, M.R. Litzow, S. Liu, A dynamic N 6-methyladenosine methylome regulates intrinsic and acquired resistance to tyrosine kinase inhibitors, Cell Res. 28 (2018) 1062–1076, https://doi.org/10.1038/s41422-018-0097-4.
[37] C. Raczy, R. Petrovski, C.T. Saunders, I. Chorny, S. Kruglyak, E.H. Margulies, H. Y. Chuang, M. Källberg, S.A. Kumar, A. Liao, K.M. Little, M.P. Strömberg, S. W. Tanner, Isaac: ultra-fast whole-genome secondary analysis on Illumina sequencing platforms, Bioinformatics 29 (2013) 2041–2043, https://doi.org/10.1093/bioinformatics/btt314.
[38] B.S. Agrovskii, V.V. Vorob'ev, A.S. Gurvich, V.V. Pokasov, A.N. Ushakov, Intensity Fluctuations of Pulsed Laser Radiation During Thermal Self-Interaction in a Turbulent Medium., Sov. J. Quantum Electron. 10 (1980) 308–312. doi: 10.1070/qe1980v010n03abeh009978.
[39] P. Cingolani, V.M. Patel, M. Coon, T. Nguyen, S.J. Land, D.M. Ruden, X. Lu, Using Drosophila melanogaster as a model for genotoxic chemical mutational studies with a new program, SnpSift, Front. Genet. 3 (2012) 1–9, https://doi.org/10.3389/fgene.2012.00035.
[40] M.J. Landrum, J.M. Lee, G.R. Riley, W. Jang, W.S. Rubinstein, D.M. Church, D. R. Maglott, ClinVar: public archive of relationships among sequence variation and human phenotype, Nucleic Acids Res. 42 (2014) 980–985, https://doi.org/10.1093/nar/gkt1113.
[41] K.J. Karczewski, B. Weisburd, B. Thomas, M. Solomonson, D.M. Ruderfer, D. Kavanagh, T. Hamamsy, M. Lek, K.E. Samocha, B.B. Cummings, D. Birnbaum, M.J. Daly, D.G. MacArthur, The ExAC browser: displaying reference data information from over 60 000 exomes, Nucleic Acids Res. 45 (2017) D840–D845, https://doi.org/10.1093/nar/gkw971.
[42] H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, The sequence alignment/map format and SAMtools, Bioinformatics 25 (2009) 2078–2079, https://doi.org/10.1093/bioinformatics/btp352.
[43] J. Ding, J. Romani, M. Zaborski, R.A.F. MacLeod, S. Nagel, H.G. Drexler, H. Quentmeier, Inhibition of PI3K/mTOR overcomes nilotinib resistance in BCR-ABL1 positive leukemia cells through translational down-regulation of MDM2, PLoS ONE 8 (2013) 1–12, https://doi.org/10.1371/journal.pone.0083510.
[44] Z.Y. Nie, L. Yang, X.J. Liu, Z. Yang, G.S. Yang, J. Zhou, Y. Qin, J. Yu, L.L. Jiang, J. K. Wen, J.M. Luo, Morin inhibits proliferation and induces apoptosis by modulating the MIR-188-5p/PTEN/Akt regulatory pathway in CML cells, Mol. Cancer Ther. 18 (2019) 2296–2307, https://doi.org/10.1158/1535-7163.MCT-19-0051.
[45] C.L. Sawyers, W. Callahan, O.N. Witte, Dominant negative MYC blocks transformation by ABL oncogenes, Cell 70 (1992) 901–910, https://doi.org/10.1016/0092-8674(92)90241-4.
[46] G. Nestal de Moraes, P.S. Souza, F.C. de F. Costas, F.C. Vasconcelos, F.R.S. Reis, R. C. Maia, The Interface between BCR-ABL-Dependent and -Independent Resistance Signaling Pathways in Chronic Myeloid Leukemia, Leuk. Res. Treatment. 2012 (2012) 1–19. doi: 10.1155/2012/671702.
[47] L. Ma, Y. Shan, R. Bai, L. Xue, C.A. Eide, J. Ou, L.J. Zhu, L. Hutchinson, J. Cerny, H. J. Khoury, Z. Sheng, B.J. Druker, S. Li, M.R. Green, A therapeutically targetable mechanism of BCR-ABL – independent imatinib resistance in chronic myeloid leukemia, Sci. Transl. Med. 6 (2014), https://doi.org/10.1126/scitranslmed.3009073.
[48] A.M. Eiring, I.L. Kraft, B.D. Page, T. O'Hare, P.T. Gunning, M.W. Deininger, STAT3 as a mediator of BCR-ABL1-independent resistance in chronic myeloid leukemia, Leuk. Suppl. 3 (2014) S5–S6, https://doi.org/10.1038/leusup.2014.3.
[49] J.E. Cortes, D.-W. Kim, J. Pinilla-Ibarz, P. le Coutre, R. Paquette, C. Chuah, F. E. Nicolini, J.F. Apperley, H.J. Khoury, M. Talpaz, J. DiPersio, D.J. DeAngelo, E. Abruzzese, D. Rea, M. Baccarani, M.C. Müller, C. Gambacorti-Passerini, S. Wong, S. Lustgarten, V.M. Rivera, T. Clackson, C.D. Turner, F.G. Haluska, F. Guilhot, M. W. Deininger, A. Hochhaus, T. Hughes, J.M. Goldman, N.P. Shah, H. Kantarjian, A phase 2 trial of ponatinib in philadelphia chromosome-positive leukemias, N. Engl. J. Med. 369 (2013) 1783–1796, https://doi.org/10.1056/nejmoa1306494.
[50] R. Mitchell, M. Copland, Defining niche interactions to target chronic myeloid leukemia stem cells, Haematologica 105 (2020) 2–4, https://doi.org/10.3324/haematol.2019.234898.
[51] M. Houshmand, G. Simonetti, P. Circosta, V. Gaidano, A. Cignetti, G. Martinelli, G. Saglio, R.P. Gale, Chronic myeloid leukemia stem cells, Leukemia 33 (2019) 1543–1556, https://doi.org/10.1038/s41375-019-0490-0.
[52] O. Hantschel, A. Gstoettenbauer, J. Colinge, I. Kaupe, M. Bilban, T.R. Burkard, P. Valent, G. Superti-Furga, The chemokine interleukin-8 and the surface activation protein CD69 are markers for Bcr-Abl activity in chronic myeloid leukemia, Mol. Oncol. 2 (2008) 272–281, https://doi.org/10.1016/j.molonc.2008.07.003.
[53] S.A. Abraham, L.E.M. Hopcroft, E. Carrick, M.E. Drotar, K. Dunn, A.J. K. Williamson, K. Korfi, P. Baquero, L.E. Park, M.T. Scott, F. Pellicano, A. Pierce, M. Copland, C. Nourse, S.M. Grimmond, D. Vetrie, A.D. Whetton, T.L. Holyoake, Dual targeting of p53 and c-MYC selectively eliminates leukaemic stem cells, Nature 534 (2016) 341–346, https://doi.org/10.1038/nature18288.
[54] M. Albajar, M.T. Gómez-Casares, J. Llorca, I. Mauleon, J.P. Vaqué, J.C. Acosta, A. Bermúdez, N. Donato, M.D. Delgado, J. León, MYC in chronic myeloid leukemia: Induction of aberrant DNA synthesis and association with poor response to imatinib, Mol. Cancer Res. 9 (2011) 564–576, https://doi.org/10.1158/1541-7786.MCR-10-0356.