CXCR2 inhibition overcomes ponatinib intolerance by eradicating chronic myeloid leukemic stem cells through PI3K/Akt/mTOR and dipeptidylpeptidase IV (CD26)

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A B S T R A C T

This study explores the therapeutic potential of targeting CXCR2 in patients afflicted with ponatinib-resistant chronic myeloid leukemia (CML). Ponatinib, a third-generation tyrosine kinase inhibitor (TKI), was initially designed for treating patients with CML harboring the T315I mutation. However, resistance or intolerance issues may lead to treatment discontinuation. Additionally, TKIs have exhibited limitations in eradicating quiescent CML stem cells. Our investigation reveals the activation of CXC chemokine receptor 2 (CXCR2) signaling in response to chemotherapeutic stress. Treatment with the CXCR2 antagonist, SB225002, effectively curtails cell proliferation and triggers apoptosis in ponatinib-resistant CML cells. SB225002 intervention also results in the accumulation of reactive oxygen species and disruption of mitochondrial function, phenomena associated with TKI chemoresistance and apoptosis. Furthermore, we demonstrate that activated CXCR2 expression induces the activity of dipeptidylpeptidase IV (DPP4/CD26), a CML leukemic stem cell marker, and concomitantly inhibits the PI3K/Akt/mTOR pathway cascades. These findings underscore the novel role of CXCR2 in the regulation of not only ponatinib-resistant CML cells, but also CML leukemic stem cells. Consequently, our study proposes that targeting CXCR2 holds promise as a viable therapeutic strategy for addressing patients with CML grappling with ponatinib resistance.

1. Introduction

Chronic myeloid leukemia (CML) is a hematological malignancy driven by an abnormal chromosome, the Philadelphia chromosome [1]. This genetic alteration results in the formation of the BCR-ABL1 fusion protein, known for its activation of tyrosine kinase activity and various signaling pathways, including JAK/STAT [2,3], PI3K/AKT [4], and MAPK/ERK [5]. While tyrosine kinase inhibitors (TKIs) such as imatinib have significantly improved survival in patients with CML, long-term therapy often leads to acquired resistance, particularly due to mutations like T315I in the *BCR-ABL1* gene [6–9]. Ponatinib, a third-generation TKI, emerged as a promising option targeting TKI-resistant mutations [10,11]. However, resistance and intolerance issues persist, prompting the need for novel therapeutic strategies, especially to address quiescent CML stem cells that evade TKI treatment [12–14].

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Chemokines and their receptors, such as CXC chemokine receptor 2 (CXCR2), are known to regulate diverse biological processes, including leukocyte migration, tumor growth, and metastasis through G protein-coupled receptors [15,16]. Dysregulation of cytokines and their receptors has implications in hematopoietic malignancies, impacting cell viability [17,18]. CXCR2 has shown a preference for inhibiting immature hematopoietic stem cells, suggesting its role in hematopoietic malignancies [19].

The CXCL8-CXCR1/2 axis is associated with tumorigenesis and metastasis, influencing cancer stem cell behavior and chemotherapy sensitivity [20–23]. Therapeutic stress can upregulate IL-8 gene expression and secretion in various cancers, contributing to cancer stem cell characteristics [23–25].

Previous research identified elevated levels of IL-8 and CXCR2 in CML, even in TKI-resistant cases [26]. Inhibiting CXCR2 signaling proved more effective than TKIs in such cases. However, the mechanism whereby CXCR2 induces resistance to third-generation TKIs and confers cancer stem cell properties in CML remains unclear.

This study aims to explore the therapeutic potential of CXCR2 as a target in ponatinib-resistant CML. Additionally, it investigates whether inhibiting CXCR2 signaling induces leukemic stem cell features and elucidates the underlying mechanisms. The findings of this study are intended to shed light on the pathogenesis of CML cells and identify potential therapeutic targets.

2. Materials and methods

2.1. Reagents

SB225002 (Calbiochem, San Diego, CA) was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) at a concentration of 1 mM, and ponatinib (Selleck Chemicals, Munich, Germany) was dissolved in DMSO at a concentration of 100 μM. All reagents were treated separately, and the final concentration remained below 0.5 % of DMSO for cell culture without cytotoxicity.

2.2. Cell culture

The human leukemia cell lines K562 and KU812 were procured from the American Type Culture Collection (Manassas, VA). They were cultured at 37 °C in 5 % CO2 in RPMI 1640 medium (Hyclone, Logan UT) with 10 % fetal bovine serum and 1 % penicillin-Streptomycin (Gibco Life Technologies, Grand Island, NY). Ponatinib-resistant cells (designated as K562/PR and KU812/PR) were generated by exposing the cells to gradually increasing ponatinib concentrations in a stepwise manner. To initiate the process, a basal concentration of 0.1 nmol/L of ponatinib was introduced into the cell cultures. Over 1–2 weeks, this initial concentration was sustained until apoptotic events were longer observed. Subsequently, the concentration of ponatinib was elevated by a factor of 2 with each increment. This progressive elevation continued until the concentration of 100 nmol/L of ponatinib was attained. The development of ponatinib resistance was confirmed, and the resistant cells were then maintained with ponatinib at 100 nmol/L.

2.3. Whole-genome sequencing

The sequencing libraries were prepared using the TruSeq PCR-free DNA High Throughput Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Briefly, 1 μg of genomic DNA was sheared using adaptive focused acoustic technology (Covaris LLC, Woburn, MA), and the fragmented DNA was end-repaired to create 5′-phosphorylated, blunt-ended dsDNA molecules. Following end-repair, DNA was size-selected using a bead-based method. These DNA fragments were then subjected to the addition of a single 'A' base and ligation of the TruSeq DNA UD indexing adapters. Following the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms), purified libraries were quantified using qPCR and validated using the high-sensitivity DNA chip (Agilent Technologies, Santa Clara, CA). NovaSeq (Illumina) was used for sequencing. Macrogen (Seoul, Korea) performed all procedures.

2.4. Cytokine array analysis

To acquire the conditioned medium, 3×10^5 cells were plated onto 60 mm culture dishes and subsequently cultured for 48 h. After incubating the conditioned medium with membranes (Ray Biotech, Norcross, GA), array images were obtained, according to the manufacturer's protocol. Briefly, the cell culture supernatants were incubated with membranes for 24 h at 4 °C, and subsequently subjected to thorough washing using wash buffers. An antibody cocktail, which had been biotinylated, was introduced and incubated with the membranes for another 24 h at 4 °C and the membranes were washed. HRP-streptavidin was added and incubated for 2 h at room temperature. Chemiluminescence was detected using a ChemiDOC imaging system (Bio-Rad, Hercules, CA). Image Lab 5.0 software was used to obtain densitometry data.

2.5. Cell viability assay

In 96 well plates, cells were seeded at a density of 1×10^4 cells per well and treated with drugs after 24 h. Cell viability was assessed by incubating cells with 10 μL of EZ-Cytox reagent (DoGEN, Korea) for 2 h, followed by the measurement of absorbance at 450 nm using a SpectraMax Plus 384 spectrophotometer (Molecular Devices Corporation, CA, USA). GraphPad Prism was used to calculate the half-maximal inhibitory concentration (IC_{50}).

2.6. Colony formation assay

Ponatinib-resistant cells were treated with SB225002 for 48 h before being seeded in Iscove's Modified Dulbecco's Medium (Thermo Fisher Scientific, Waltham, MA) and semisolid culture medium (Stem Cell Technologies, Grenoble, France) at a density of 5×10^3 cells/dish. After 14 d, the resulting colonies were harvested and dissociated with PBS, and the total number of cells was counted.

2.7. Cell cycle analysis

The collected CML cells were fixed in 80 % ethanol for 24 h at $-20\ ^\circ$C. After washing with PBS, the fixed cells were resuspended and incubated in PBS containing RNase A (100 µg/mL) at 37 $^\circ$C for 30 min, followed by staining with 50 µg/mL of propidium iodide (PI). A FACS Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to examine cell cycle stages.

2.8. Flow cytometry

A total of 1×10^6 of CML cells were subjected to treatment for 10 min at 4 $^\circ$C with specific antibodies or isotype control antibodies. Subsequently, the cells were washed with PBS. Flow cytometric analysis was performed using a BD FACS Canto II flow cytometer (BD, Franklin Lakes, NJ, USA), and data were analyzed using the FlowJo software (FlowJo LLC, Ashland, OR). The following antibodies against specific proteins were utilized for flow cytometric analysis and were procured from Miltenyi Biotec Inc. (Auburn, CA): CXCR2 (#130-104-886) and CD25 (#130-113-283), CD35 (#130-106-652), CD93 (#130-113-119), IL-1RAcP (#130-108-686), and CD26 (#130-126-362).

For the apoptosis assay, cells from SB225002-treated CML patients were collected, washed with PBS, and stained with 1 µg/mL propidium iodide (PI) and 1 µg/mL annexin V labeled with FITC, according to the manufacturer's instructions (Koma Biotechnology, Seoul, Korea). A BD FACS Canto II flow cytometer was used to assess the proportion of apoptotic cells, and the resulting data were analyzed using FlowJo software.

2.9. Reactive oxygen species measurements

Cells were incubated with 5 µM of MitoSOX reagent (Thermo Fisher Scientific, Waltham, MA) in Hank's balanced salt solution (HBSS) at 37 $^\circ$C for 10 min in the dark. The cells were then washed with warm HBSS, and flow cytometry was used to determine the level of MitoSOX fluorescence.

2.10. Mitochondrial membrane potential

Overall, one million cells were suspended in a cell growth medium and stained with 2 µM JC-1 (Thermo Fisher Scientific, Waltham, MA), both with and without treatment with 50 µM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), for 30 min at 37 $^\circ$C. The resulting stained cells were subsequently analyzed via flow cytometry, with the CCCP-treated samples used for standard compensation.

2.11. Immunoblotting

To ensure uniformity, the cells were lysed with a PRO-PREP™ solution (Intron Biotechnology, Korea), and the protein concentration was determined using the Bradford assay with a microplate reader. SDS-PAGE was used to separate total proteins, which were then transferred to PVDF membranes. The membranes were then treated with primary antibodies overnight at 4 $^\circ$C before being incubated with HRP-conjugated antibodies at a temperature of 25 $^\circ$C for 1 h. All images were captured using a ChemiDOC imaging system (Bio-Rad), and the signal was measured using Image Lab 5.0 software (Bio-Rad). Primary antibodies against the following

Table 1
List of primer sequences used for real-time PCR.

Gene	Direction	Primer sequence (5' → 3')
GAPDH	Forward	GAGTCCACTGGCGTCTTCAC
	Reverse	TACAAGCAGTACCCACACTT
CXCR2	Forward	CAATGAATGAATGAATGGCTAAG
	Reverse	TTGTGCCTGCTTGGAACTTTTGAAA
SOX2	Forward	GCCGAGTGGAAACTTTTGTCG
	Reverse	GGCAGCGTGTACTTATCCTTCT
OCT4	Forward	TCTCGCCCCCTCCAGGT
	Reverse	CTGCTTCGCCCTCAGGC
Nanog	Forward	AAAGAATCTTCACCTATGCC
	Reverse	GAAGGAAGAGGAGAGACAGT
KLF4	Forward	TCGCTTCCTCTTCCTCCGACACA
	Reverse	GCGAACTCACACAGGCGAGAAACC
c-Myc	Forward	GCTGCTTAGACGCTGGATTT
	Reverse	TAACGTTGAGGGGCATCG

proteins were used: CXCR2 (#65968) and c-Myc (#32072), SOX2 (#137385), and CD26 (#114033) were obtained from Abcam (Cambridge, UK); β-actin (#47778), cyclin B1 (#245), and p-CDC2 (#13601), Nanog (#293121) from Santa Cruz Biotechnology (Dallas, TX, USA); p-CrkL (#3181), p-mTOR (#5536), mTOR (#2972), p-Akt (#4060), Akt (#9272), caspase-3 (#9662), and cleaved PARP (#5625) were purchased from Cell Signaling Technology (Danvers, MA, USA). γH2AX (#07–164) was obtained from Millipore. The following secondary antibodies were used: Anti-rabbit IgG, HRP-linked antibody (#7074) obtained from Cell signaling technology and goat anti-mouse IgG-HRP conjugate (#1706516) was purchased from Bio-Rad.

2.12. Quantitative RT-PCR

Total RNA was extracted from cells using the TRIzol reagent, following the manufacturer's instructions. The extracted RNA was then reverse transcribed into cDNA using a commercially available kit (Intron). On a Bio-Rad instrument, the quantity of RNA extracted was determined using quantitative real-time PCR (qRT-PCR) with SYBR green PCR master mix. The qRT-PCR protocol included denaturation at 95 °C for 60 s, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 60 s. The comparative ΔΔCt method was used to assess the fold change in gene expression levels relative to the control. Table 1 lists the primer sequences used. In addition, GAPDH was used as an internal control to normalize the gene expression levels.
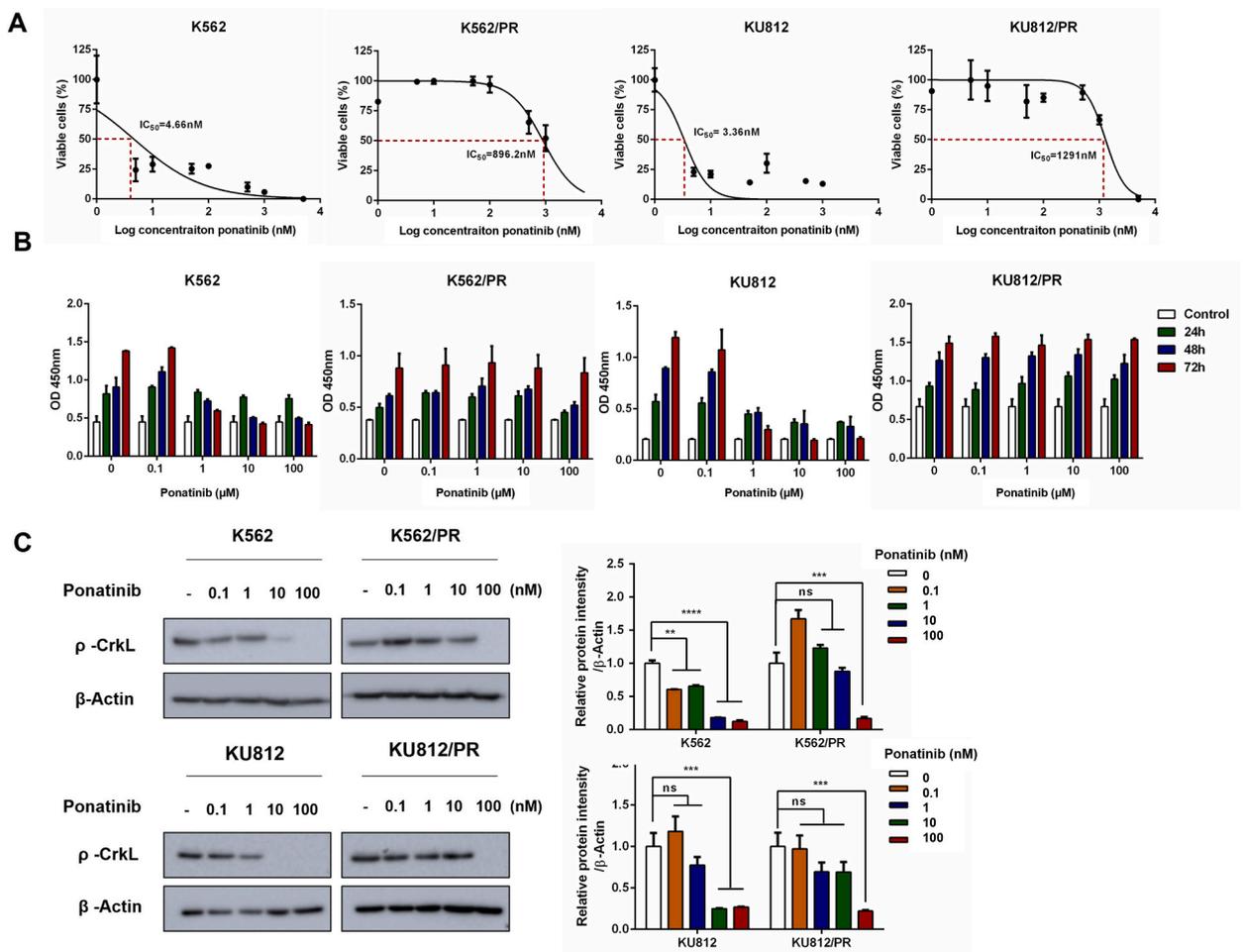
Fig. 1. Validation of ponatinib sensitivity following resistance in CML cell lines. (A) K562, KU812, and ponatinib-resistant (K562/PR and KU812/PR) cell lines were treated with increasing doses of ponatinib for 48 h. The viability of cells was confirmed using nonlinear regression analyses, and the IC$_{50}$ values were obtained using Prism software. (B) The time-dependent cell proliferation in various doses was tested for 72 h via the WST-1 assay. (C) The variations in phosphorylated CrkL levels were examined using immunoblotting analysis after 24 h of treatment with increasing concentrations of ponatinib. The results are expressed as the mean ± standard deviation (SD), and the significance levels were denoted as **P < 0.01, ***P < 0.001, ****P < 0.0001, and ns (no significance). The complete, unaltered images of the band are depicted in Supplementary Fig. 1.

2.13. Transduction of lentivirus shRNA for CXCR2 silencing and activation

A total of 5 x 10°4 CML cells were resuspended in the medium containing 5 μg/mL polybrene to facilitate the transduction process. CML cells were transduced with lentiviral particles designed to activate or silence CXCR2 expression (Santa Cruz Biotechnology, Dallas, TX, USA). After centrifuging the cells at $800 \times g$ for 2 h at 32 °C, the medium was replaced with fresh culture media. After 72 h of transfection, the medium was replaced with a fresh medium containing puromycin to select infected cells.

2.14. Statistical analysis

The results were obtained from at least three independent experiments and were expressed as mean ± standard deviation. Statistical analysis was conducted with GraphPad Prism V6.0 (GraphPad Software, Inc., La Jolla, CA) using either Student's *t*-tests or two-way analysis of variance (ANOVA). P-values <0.05 were considered statistically significant and marked as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns (not significant).

3. Results

3.1. BCR-ABL-independent proliferation in ponatinib-resistant CML cells

Previous studies have demonstrated the potential of CXCR2 inhibition as a strategy to overcome TKI resistance in imatinib- and
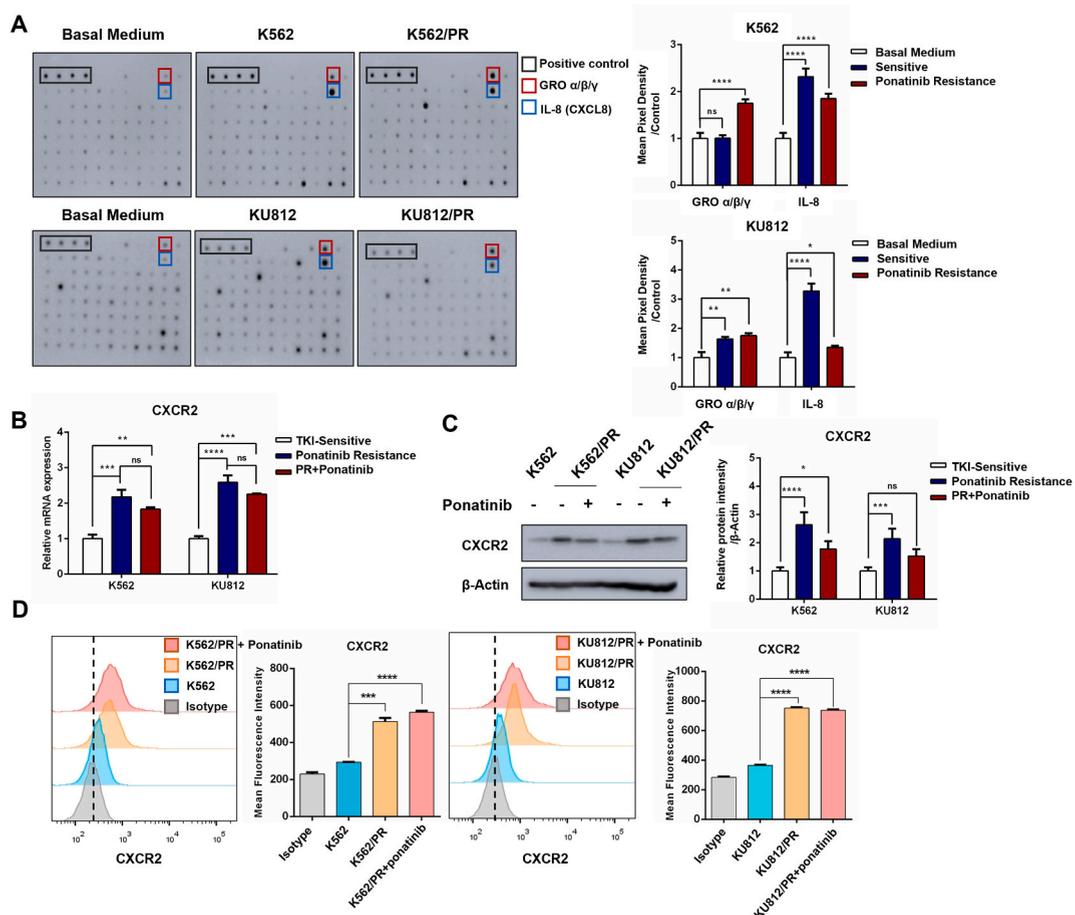
Fig. 2. Elevated expression of CXCR2 and its ligands in ponatinib-resistant cells. (A) A human cytokine array comparing various cytokines and chemokines in TKI-sensitive (K562 and KU812) and ponatinib-resistant (K562/PR and KU812/PR) cell culture media. (B) BCR-ABL and CXCR2 mRNA expression in CML cell lines treated with or without 100 nM ponatinib. (C) Western blotting was used to detect the basal expression levels of CXCR2 in ponatinib-resistant CML cells (K562/PR and KU812/PR) treated with or without 100 nM ponatinib. The complete, unaltered images of the band are depicted in Supplementary fig. 2 (D) Flow cytometry was used to analyze the expression level of CXCR2, and bar graphs were used to represent the mean fluorescence intensity (MFI) expression of CML cells. The error bars denote the mean ± SD, and significance levels are indicated as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and ns (no significance).
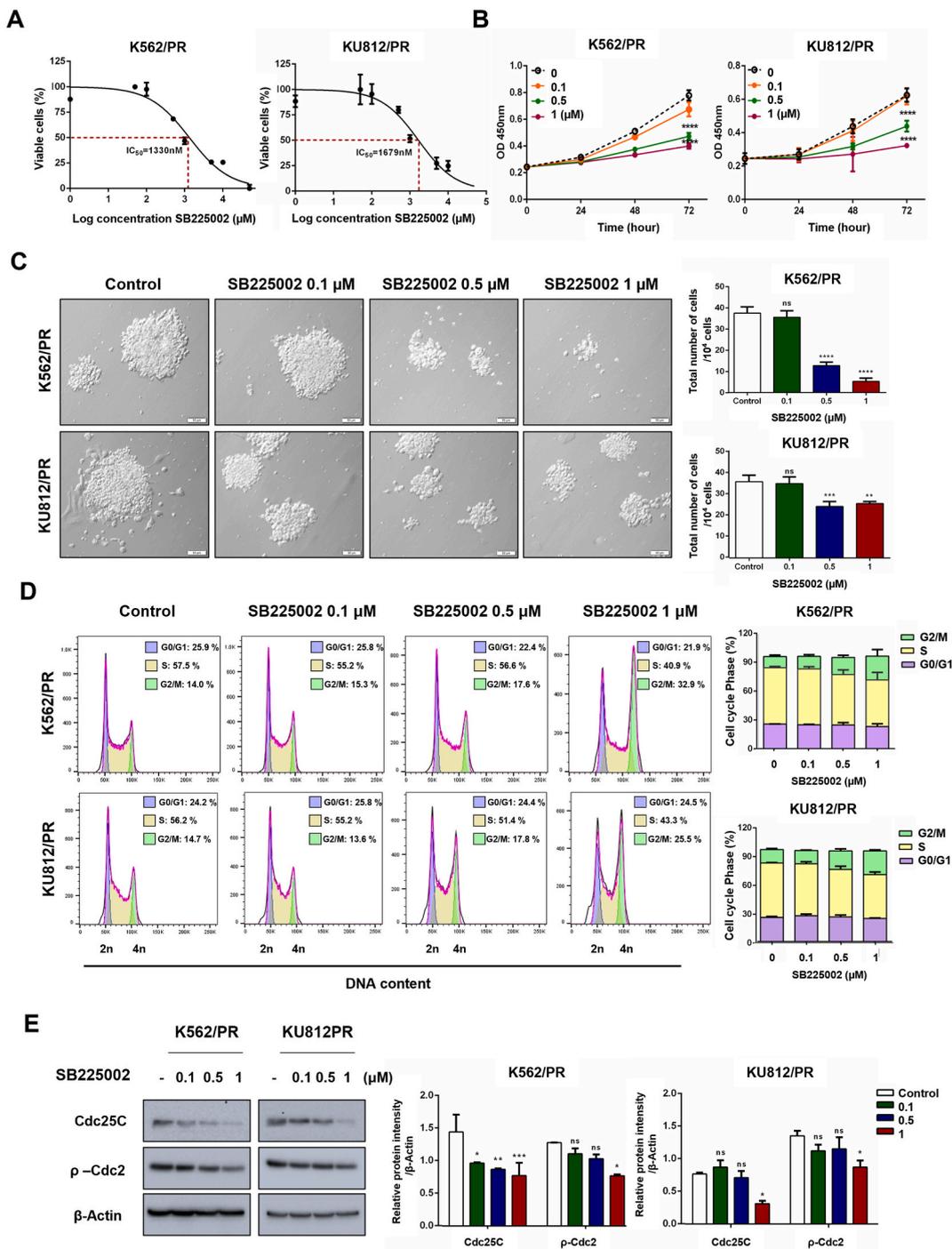
Fig. 3. SB225002, a CXCR2 antagonist, inhibited cell growth via G2/M cell cycle arrest in ponatinib-resistant CML cells. (A) The cell viability of K562/PR and KU812/PR cells was assessed for 48 h of treatment with different concentrations (0.05–50 μM) of SB225002. (B) Cell proliferation of K562/PR and KU812/PR cells was assessed after 72 h of treatment with SB225002 at various doses. (C) The colony-forming cell (CFC) assay was performed on ponatinib-resistant CML cell lines exposed to SB225002. Representative images of the colony sizes after vehicle or SB225002 treatment with a magnification of 100X were obtained, and the total number of cells was counted using Trypan blue staining on day 14. (D) The cell cycle distribution of CML cells was determined after treatment with SB225002 for 24 h, followed by staining with propidium iodide (PI). FlowJo software was used to calculate the proportion of cells in each cell cycle phase. (E) Western blotting was used to determine the protein levels of Cdc25 and phosphorylated Cdc2. Significance levels are indicated as **P < 0.01, ***P < 0.001, ****P < 0.0001, and ns (no significance). The complete, unaltered images of the band are depicted in Supplementary Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

nilotinib-resistant CML cells [26]. However, the effectiveness of this approach against various forms of acquired TKI resistance remains uncertain, particularly considering that ponatinib was specifically designed to target the T315I mutation, one of the most common mutations associated with TKI resistance.

To investigate this, we established ponatinib-resistant CML cells, denoted as K562/PR and KU812/PR, and determined their IC$_{50}$ values (Fig. 1A). Remarkably, even when exposed to 100 nM of ponatinib, these acquired ponatinib-resistant cells continued to proliferate (Fig. 1B). We proceeded to assess BCR-ABL activity in these cells after subjecting them to increasing concentrations of ponatinib, as indicated by CrkL phosphorylation. Intriguingly, ponatinib-resistant CML cells exhibited sustained activation of BCR-ABL following high-dose ponatinib treatment. In contrast, TKI-sensitive cells displayed a dose-dependent reduction in CrkL activity (Fig. 1C). These observations imply that ponatinib-resistant CML cells possess the capability to bypass BCR-ABL signaling, even in the presence of TKIs.

3.2. Expression of CXCR2 ligands in IL-8 and GRO-α and their receptor CXCR2 in ponatinib-resistant CML cells

To explore potential disparities in secreted cytokine levels between ponatinib-resistant and -sensitive cells, we conducted an analysis of 80 human cytokines in conditioned media samples. While both K562 and KU812 cells exhibited elevated IL-8 (CXCL8) levels when compared to basal medium, ponatinib-resistant cells displayed increased expression of GRO α/β/γ (CXCL1/2/3) and IL-8 (Fig. 2A). These observations contribute to the growing evidence emphasizing the crucial role of cytokines in cancer progression,
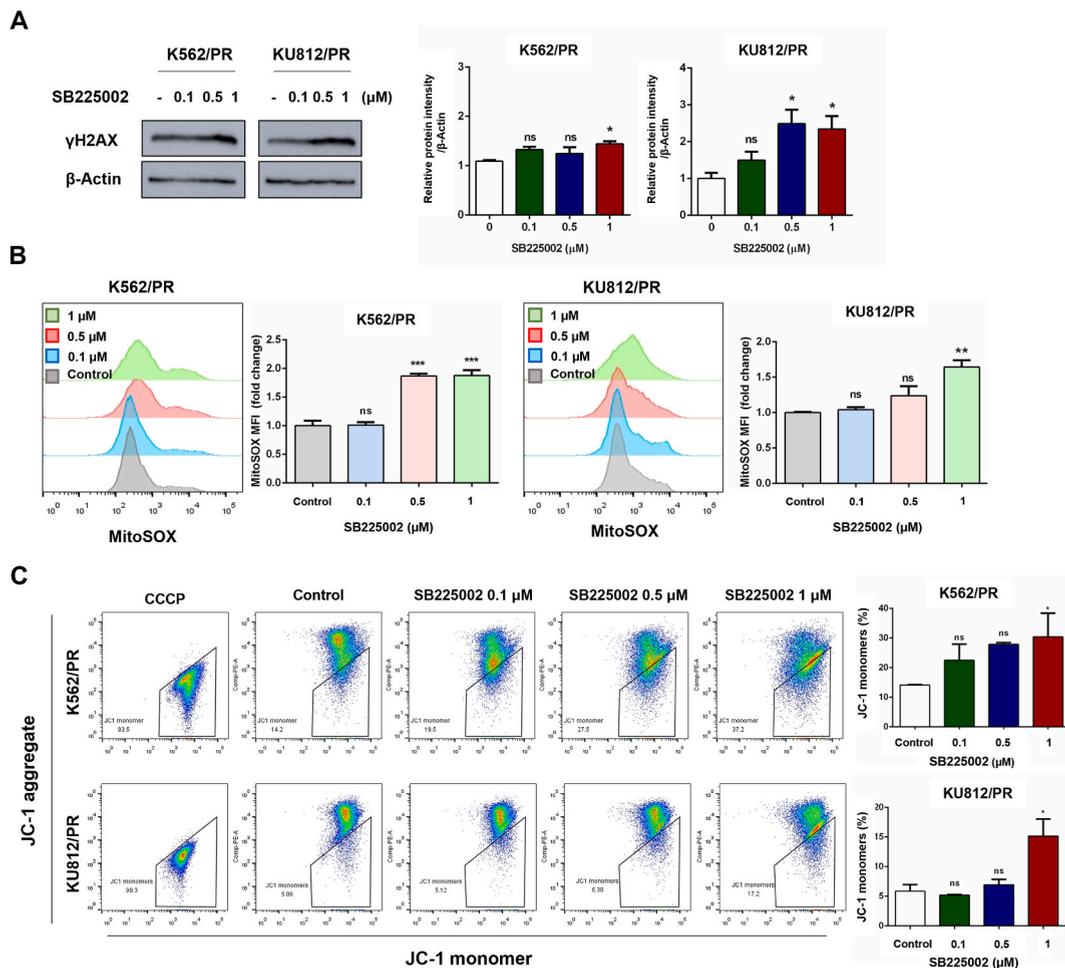
Fig. 4. CXCR2 inhibition induces DNA damage and mitochondrial dysfunction in ponatinib-resistant CML cells. (A) Western blot analysis indicates the expression levels of γH2AX after treatment with SB225002. The complete, unaltered images of the band are depicted in Supplementary fig. 4 (B) Ponatinib-resistant CML cell lines were treated with SB225002 at specific doses, and mitochondrial superoxide was detected using flow cytometry MitoSOX staining, as reflected by fold changes in mean fluorescence intensity (MFI) values. (C) Loss of Δψm was determined using flow cytometry in K562/PR and KU812/PR cells after incubation with SB225002 for 48 h. As a positive control, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used, and green JC-1 monomers were identified as low Δψm. The statistical significance levels of the results are indicated as **P < 0.01, ***P < 0.001, ****P < 0.0001, and ns (no significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

particularly in the context of drug resistance development.

As CXCR2 serves as the shared receptor for both IL-8 and GRO-α, we proceeded to analyze CXCR2 expression levels in both ponatinib-resistant and parental cell lines. Our findings revealed a significant upregulation of CXCR2 expression in K562/PR and KU812/PR cells when compared to the parental cell lines (Fig. 2B). Importantly, CXCR2 levels remained elevated even under ponatinib treatment conditions (Fig. 2C and D). These results strongly suggest a role of CXCR2 and cytokines in the development of ponatinib resistance.

3.3. SB225002-induced G2/M cell cycle arrest and inhibition of cell viability in ponatinib-resistant CML via CXCR2 inhibition

To investigate the functional relevance of CXCR2 in ponatinib-resistant CML cells, we examined the impact of the CXCR2 antagonist SB225002 on cell viability and proliferation. Our findings demonstrated that CXCR2 suppression resulted in a reduction in viable cells across all ponatinib-resistant cell lines (Fig. 3A). Notably, significant inhibition of proliferation was observed at SB225002 doses exceeding 0.5 μM (Fig. 3B). Furthermore, we substantiated the long-term effects of CXCR2 antagonist treatment using a colony-forming unit (CFU) assay, revealing a dose-dependent suppression of cell growth in ponatinib-resistant cell lines (Fig. 3C).

To gain deeper insights into the mechanisms underpinning the observed suppression of proliferation and induction of G2/M cell cycle arrest by SB225002 in ponatinib-resistant cells, we conducted an assessment of cell cycle distribution. Our results demonstrated a concentration-dependent increase in the proportions of G2/M arrested cells when compared to the control group (Fig. 3D). To corroborate the mechanism of induced cell cycle arrest, we analyzed the expression levels of key G2/M checkpoint regulatory proteins. Upon exposure to SB225002, K562/PR and KU812/PR cells exhibited reduced expression of Cdc25c and phosphorylated Cdc2 (Fig. 3E). These findings collectively suggest that targeting CXCR2 signaling effectively impedes cellular proliferation by inducing G2/M cell cycle arrest.

3.4. ROS induction and mitochondrial dysfunction induced by the CXCR2 inhibitor SB225002 in CML cells

Subsequent to chemotherapy, the activation of DNA repair mechanisms often triggers the DNA damage response (DDR), culminating in cell cycle arrest and apoptosis [27,28]. To elucidate whether the observed G2/M cell cycle arrest was attributed to DNA damage, we evaluated the expression of γH2AX, a pivotal protein within the DDR pathway. Notably, exposure to SB225002 resulted in
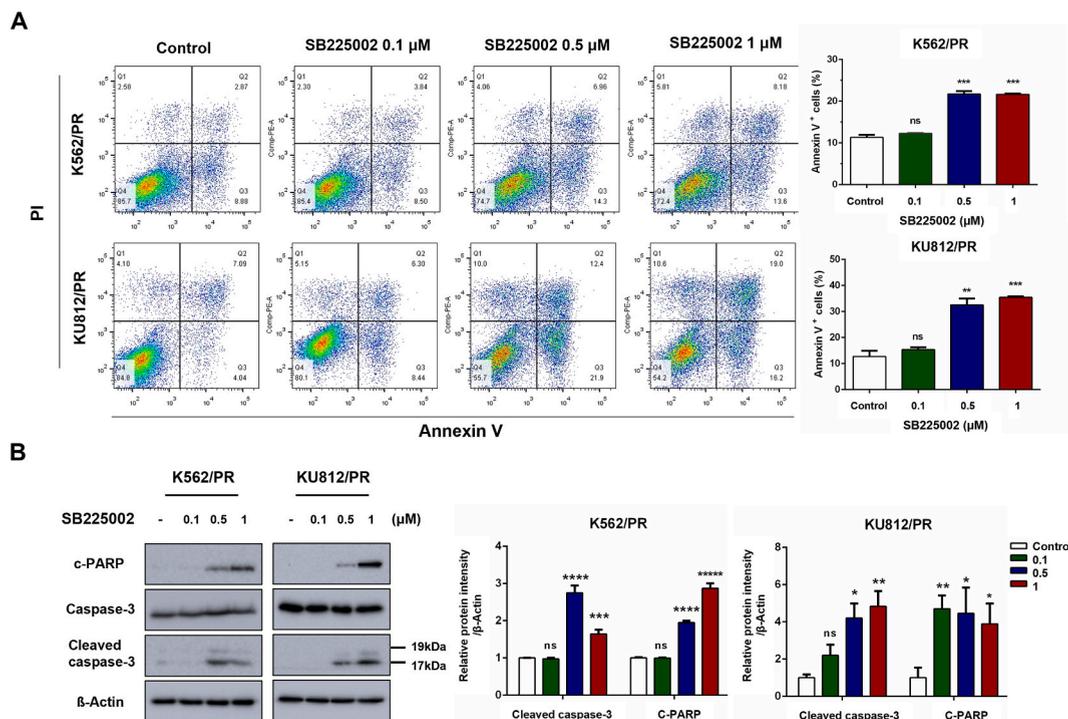
Fig. 5. Effect of cell death upon SB225002 treatment in ponatinib-resistance CML cell lines. (A) Ponatinib-resistant CML cells were treated with SB225002 for 48 h, and the percentage of Annexin V-positive cells was determined using Annexin V/PI staining. The bar graph shows the percentage of Annexin V-positive cells. (B) Western blot analysis was used to detect the expression levels of c-PARP and cleaved caspase-3 in K562/PR and KU812/PR cells after treatment with SB225002. The results indicate that SB225002 treatment induced apoptosis in ponatinib-resistant CML cells. The significance levels are indicated as **P < 0.01, ***P < 0.001, ****P < 0.0001, and ns (no significance). The complete, unaltered images of the band are depicted in Supplementary Fig. 5.

a substantial increase in γH2AX levels (Fig. 4A). It is well known that DNA damage response pathways impact the influence of reactive oxygen species (ROS). For this reason, we employed MitoSOX superoxide indicators to quantify mitochondrial superoxide production, thereby assessing the impact of ROS following SB225002 treatment. Our results indicated that ponatinib-resistant cells treated with SB225002 exhibited significantly elevated levels of mitochondrial ROS (Fig. 4B).

To investigate the potential association between mitochondrial depolarization and the observed increase in mitochondrial ROS, we assessed mitochondrial membrane potential in ponatinib-resistant CML cells. As a positive control, we employed the mitophagy inducer CCCP to disrupt mitochondrial potential and stimulate ROS production. Furthermore, double fluorescence staining of mitochondria with JC-1 was employed to explore whether SB225002 could induce mitophagy via ROS. JC-1 staining enables the detection of mitochondrial membrane potential as JC-1 monomers or J-aggregates, and the results were analyzed using flow cytometry. Our findings indicated that SB225002 treatment led to a downregulation of mitochondrial membrane potential and an increase in oxidative stress (Fig. 4C). Collectively, these findings highlight the association between SB225002, a CXCR2 inhibitor, ROS formation, and impaired mitochondrial function.

3.5. Induction of apoptotic cell death through CXCR2 inhibition in ponatinib-resistant CML

Accumulation of ROS in cancer cells has been closely associated with either chemoresistance or the initiation of apoptosis [29,30]. To investigate whether the CXCR2 antagonist could induce apoptosis in ponatinib-resistant cells, we employed Annexin V/PI staining. Our results demonstrated a significant increase, ranging from 20 to 30 %, in the proportion of Annexin V-positive cells following treatment with SB225002, at dosages exceeding 0.5 μM (Fig. 5A). Furthermore, SB225002 treatment led to a dose-dependent upregulation of proapoptotic markers, including cleavage-PARP and cleavage caspase-3 (Fig. 5B). These observations suggest that the inhibition of CXCR2 may indeed play a pivotal role in inducing apoptosis in ponatinib-resistant CML cells.

3.6. Effect of CXCR2 activation and suppression on pluripotency markers and cell proliferation

Whole-genome sequencing was employed to investigate potential resistance mechanisms to ponatinib in cells. Notably, no mutations were detected in the well-known mutation sites of the kinase domain, including the A-loop, P-loop, and ATP-binding sites. However, mutations were observed in the untranslated regions and introns of the genome of ponatinib-resistant cells (Table 2), indicating that the resistance mechanisms were BCR-ABL1-independent.

Leukemic stem cells (LSCs) in CML are highly stem-like and resistant to TKI-based chemotherapy, necessitating further exploration of the association of CXCR2 activation with pluripotency transcription factors in ponatinib-resistant CML cells. Stemness markers known to regulate embryonic and induced pluripotent stem cells (iPSCs) and CSCs were analyzed to examine this potential correlation. K562/PR and KU812/PR cells exhibited heightened CXCR2 stimulation when compared to TKI-sensitive CML cells, along with elevated expression of stemness markers, including OCT4, Nanog, SOX2, and c-Myc (Fig. 6A). Activating CXCR2 using shRNA in TKI-sensitive cells resulted in increased protein expression of pivotal pluripotency markers, particularly SOX2 and c-Myc, with a significant increase in the pluripotency marker Nanog while OCT4 remained undetectable (Fig. 6B). Moreover, CXCR2 activation led to a significant increase in cell proliferation in the K562 and KU812 cell lines (Fig. 6C). To further elucidate the intricate interplay between CXCR2 signaling and pluripotency markers, along with its effects on cell growth, experiments were conducted using ponatinib-resistant cells. CXCR2 suppression resulted in a noticeable decrease in c-Myc and SOX2 signaling, particularly following SB225002 treatment (Fig. 6D). These observations were further substantiated through the application of shRNA CXCR2 lentiviral particles (Fig. 6E). Additionally, the targeted CXCR2 silencing led to a significant reduction in cell growth in the K562/PR and KU812/PR cell lines, in contrast to the observed growth increase associated with shCXCR2 activation (Fig. 6F).

Considering the relevance of the PI3K/Akt/mTOR signaling pathway in BCR-ABL-independent TKI resistance [31] and its correlation with the expression of CSC markers, we investigated whether inhibiting CXCR2 would impact PI3K/Akt/mTOR signaling in

Table 2
BCR-ABL kinase mutations in ponatinib-resistant cells.

Cell line	Chromosome base pair position	Reference	Alternative	Effect of variant	HGVS.c
K562/PR	23,313,032	G	A	Intron variant	c.3457 + 11G > A
	23,316,021	C	G	3 prime UTR variant	c.*499C > G
	23,316,029	C	A	3 prime UTR variant	c.*507C > A
	23,316,087	T	TTTC	3 prime UTR variant	c.*568_*570dupCTT
	23,316,158	G	A	3 prime UTR variant	c.*636G > A
	23,316,174	T	C	3 prime UTR variant	c.*652T > C
	23,316,973	G	A	3 prime UTR variant	c.*1451G > A
	23,317,335	G	C	3 prime UTR variant	c.*1813G > C
	23,317,807	T	C	3 prime UTR variant	c.*2285T > C
KU812/PR	23,186,017	C	A	Intron variant	c.1279 + 3778C > A
	23,313,430	G	A	Intron variant	c.3457 + 409G > A
	23,313,452	C	G	Intron variant	c.3457 + 431C > G
	23,317,070	GCT	G	3 prime UTR variant	c.*1551_*1552delCT
	23,317,335	G	C	3 prime UTR variant	c.*1813G > C
	23,317,437	C	G	3 prime UTR variant	c.*1915C > G

(caption on next page)

Fig. 6. The CXCR2 antagonist, SB225002, exerts an effect on SOX2, c-Myc, and the PI3K/Akt/mTOR signaling pathways. (A) Relative expression of stemness marker in ponatinib-sensitive and -resistance CML cells. (B) Protein levels of CXCR2, c-Myc, SOX2, and Nanog in K562 and KU812 cells following treatment with a shRNA CXCR2 activator. (C) Assessment of CML cell growth after introducing the CXCR2 lentiviral activator, monitored over 96 h using the WST-1 assay. (D) The protein expression levels of CXCR2, c-Myc, SOX2, and Nanog in ponatinib-resistant cells following treatment with 1 μM of SB225002 for 24 h. (E) Western blot analysis depicting the levels of CXCR2, c-Myc, and SOX2 expression after the inhibition of CXCR2 signaling using shRNA lentiviral particles. (F) Assessment of CML cell growth upon transduction with CXCR2 lentivirus, specifically in ponatinib-resistant cells, monitored over a 96-h period using the WST-1 assay. (G) Evaluation of protein expression levels of CXCR2, phosphorylated PI3K, PI3K, phosphorylated Akt, Akt, phosphorylated mTOR, and mTOR after treatment with SB225002 in K562/PR and KU812/PR cells, conducted through Western blot analysis. Data are presented as the mean ± standard deviation. Statistical significance is denoted as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, no significant difference. The complete, unaltered images of the band are depicted in Supplementary Fig. 6.

ponatinib-resistant CML cells. The findings demonstrated that inhibiting CXCR2 led to a decrease in PI3K/Akt/mTOR signaling (Fig. 6G).

These results underscore the intricate and multifaceted regulatory role of CXCR2 in modulating pluripotency markers and its consequential impact on cell proliferation through the PI3K/Akt/mTOR signaling cascades.

3.7. Identification of CD26 as a specific LSC marker in ponatinib-resistant CML

Activated PI3K/Akt/mTOR signaling pathways are widely recognized for their pivotal roles in cancer progression, poor prognosis,

Fig. 7. Expression of CD26 marker and its modulation in ponatinib-resistance CML cells. (A) Flow cytometry was utilized to evaluate the expression levels of CML leukemic stem cell (LSC) markers in ponatinib-resistant cells. (B) Bar graphs represent mean fluorescence intensity (MFI) values of CML cells, each normalized against its respective isotype control. (C) Western blot analysis illustrates CXCR2 and CD26 protein expression levels in ponatinib-sensitive and -resistant cells, both in the presence and absence of 1 μM SB225002 treatment. Data are presented as the mean ± standard deviation. Statistical significance is indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, no significant difference. The complete, unaltered images of the band are depicted in Supplementary Fig. 7.

and drug resistance [32]. Recent studies have emphasized the significance of these pathways in reinforcing the cancer stem cell (CSC) phenotype and regulating stemness maintenance in solid cancers [33–35]. To assess the impact of PI3K/Akt/mTOR signaling cascades on CML LSCs, we conducted an expression analysis of CML LSC markers that distinguish them from normal hematopoietic stem cell (HSC) markers. These markers included CD25, CD36, CD93, and interleukin-1 receptor accessory protein (IL-1 RAcP) (Fig. 7A). When comparing the expression of LSC markers with TKI-sensitive cells, we observed a significant increase in CD26 expression; however, not all these markers showed elevation in K562/PR and KU812/PR cells (Fig. 7B).

To ascertain whether CXCR2 activation contributed to the elevated CD26 activity in acquired ponatinib-resistant cells, we confirmed protein expression with or without SB225002 treatment (Fig. 7C). Interestingly, both CXCR2 and CD26 markers exhibited increased activity in acquired ponatinib-resistant cells, and these activities were downregulated after treatment with the CXCR2 antagonist. These findings suggest that ponatinib resistance leads to increased CD26 expression, and inhibiting CXCR2 expression can induce the inhibition of this marker.

4. Discussion

This study sheds light on the formidable challenge of treating BCR-ABL-dependent chronic myeloid leukemia (CML) arising from tyrosine kinase inhibitor (TKI) resistance and the persistence of CML leukemic stem cells (LSCs). The study elucidates that CXCR2 and its ligands exhibit a correlation with chemoresistance after the development of ponatinib resistance. Notably, CXCR2 activation through the PI3K/AKT/mTOR pathway, its role in regulating pluripotency markers, and its association with CD26 are identified as critical factors in ponatinib resistance and the presence of CML stem-like cells. The emergence of the CML LSC marker CD26 in cells resistant to ponatinib suggests that targeting CXCR2 holds the potential to overcome this resistance and eradicate CML stem/progenitor cells.

The role of highly expressed cytokines and their chemokine receptor CXCR2 in tumor growth, metastasis, and chemoresistance in solid tumors is well-established [22,25,36–40]. Exposure to chemotherapeutic drugs often triggers the expression of cytokines such as IL-8 and GRO-α, which can lead to chemotherapeutic resistance, representing an adaptive response of cancer cells to chemical stress [41,42]. However, the function of CXCR2 and its cytokine activation in promoting resistance to TKI treatment in CML has been poorly understood thus far. The current study fills this knowledge gap by demonstrating that inhibiting CXCR2 signaling with IL-8 and GRO-α can overcome resistance to treatment in CML cells that have developed ponatinib resistance. The sustained increase in CXCR2 expression in ponatinib-resistant CML cells, even in the presence of ponatinib, suggests that CXCR2 and its ligands may significantly contribute to the development of chemoresistance in CML. This study unveils that TKI chemotherapeutic stress induces CXCR2 stimulation after the onset of ponatinib resistance through a BCR-ABL-independent pathway. Based on these findings, targeting CXCR2 emerges as a promising therapeutic strategy for CML patients who have developed TKI resistance.

In our previous study, we established a link between CXCR2 stimulation and pluripotency-related transcription factors OCT4, SOX2, and Nanog in human embryonic stem cells and iPSCs [43,44]. Cancer stem cells (CSCs) share key characteristics with stem cells, including self-renewal capacity and multilineage differentiation potential, which drive cancer progression and therapeutic resistance [44,45]. The elimination of CSCs is essential for effective cancer treatment, particularly in CML, wherein LSCs contribute to TKI resistance and relapse, hindering the achievement of treatment-free remission [14,46,47]. This study unveils that ponatinib-resistant CML cells are linked to CXCR2 upregulation, which results in increased expression of pluripotency markers SOX2 and c-Myc. Both SOX2 and c-Myc are transcription factors that, when overexpressed in tumor cells, promote tumor growth, metastasis, chemoresistance, and stemness, thereby contributing to the acquisition of CSC properties [48,49]. Importantly, SOX2-expressing cancer cells often exhibit elevated levels of CSC markers and are associated with poor prognoses [50–54]. Similarly, c-Myc overexpression is observed in various cancers, driving cancer stem cell-like traits and aggressive disease progression [55,56]. Notably, CML LSCs are highly sensitive to alterations in c-Myc expression, which is required for the maintenance of CML leukemia-initiating cells [57,58]. Although the relationship between c-Myc and SOX2 in cancer cells remains unclear, some studies suggest that they co-localize within a protein complex [59,60]. Specifically, c-Myc has been shown to regulate the expression of the SOX2 gene in breast cancer-derived CSCs [61], implying that c-Myc may modulate the expression of other transcription markers to regulate cancer stemness. Our findings reveal that the activation of the CXCR2 pathway is associated with pluripotent stem cell markers in ponatinib-resistant CML cells. Furthermore, with the development of ponatinib resistance, stemness markers increase in tandem with CXCR2 activation. Activation of shCXCR2 specifically results in increased levels of SOX2 and c-Myc in TKI-sensitive CML cells. Collectively, these findings suggest that the CXCR2 signaling pathway plays a crucial role in enhancing LSC properties via the c-Myc and SOX2 pathways.

Moreover, it is crucial to highlight the close association of the PI3K/Akt/mTOR signaling pathway with hematopoietic malignancies, especially its substantial impact on LSCs. This signaling pathway has been shown to significantly influence LSC quiescence and resistance to chemotherapy in acute myeloid leukemia (AML) [62]. Another study has demonstrated that a dual inhibitor targeting the PI3K/mTOR pathway can impact CML LSCs through the PI3K/Akt/mTOR pathway cascades [63]. However, one study revealed that while mTOR inhibition had no effect on CML stem cells (CD34$^+$/CD38$^-$), PI3K inhibition restored cell line sensitivity to nilotinib, a second-generation TKI [64]. Therefore, further research is warranted to understand the precise role of the PI3K/Akt/mTOR pathway in CML LSCs. Nevertheless, the PI3K/Akt/mTOR signaling pathway plays a critical role in CML LSC biology.

In our study, we elucidated the potential of CXCR2 modulation in addressing chemoresistance and the dynamic behavior of CML LSCs. In our previous study, we explored the inhibitory potential of CXCR2 in imatinib and nilotinib-resistant CML cell lines using *in vitro* and *in vivo* experiments. However, the present research focuses exclusively on *in vitro* investigations. Consequently, further comprehensive studies are necessary to validate the viability of CXCR2 signaling inhibition in clinical settings.

In conclusion, the findings of this study underscore the significance of targeting CXCR2 as a pivotal factor in the survival and

elimination of CML LSCs that have developed resistance to ponatinib. This highlights the potential of CXCR2 inhibitors as promising adjunctive or alternative therapeutic modalities for patients grappling with the challenge of TKI resistance, including ponatinib, in the context of CML. These therapeutic avenues, informed by the intricate interplay of signaling pathways within the realm of leukemia stem cells, hold potential for advancing the management of CML patients navigating the complex terrain of TKI resistance.

CRediT authorship contribution statement

Ji-Hea Kim: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Ka-Won Kang:** Resources. **Yong Park:** Resources. **Byung Soo Kim:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22091.

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