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Kinesin spindle protein inhibitor SB743921 induces mitotic arrest and apoptosis and overcomes imatinib resistance of chronic myeloid leukemia cells

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Abstract
Inhibition of the cell mitotic pathway may provide a novel means for therapeutic intervention in chronic myeloid leukemia (CML). Kinesin spindle protein (KSP), a microtubule-associated motor protein which is essential for cell cycle progression, is overexpressed in bcr–abl+CML cells. Retrovirus-mediated bcr–abl transduction increases KSP expression in cord blood CD34+ cells. SB743921 is a selective KSP inhibitor which is being investigated in ongoing clinical trials for treatment of myeloma, leukemia and solid tumors. Treatment of CML cells with SB743921 resulted in reduced proliferation and colony forming cell (CFC) formation ability. SB743921 also actively blocked cell cycle progression, leading to apoptosis in both primary CML cells and cell lines. KSP inhibition sensitized CML cells to imatinib-induced apoptosis. Importantly, SB743921 inhibited the proliferation of various CML cells including T315I mutation-harboring cells. Furthermore, we demonstrated that SB743921 treatment suppressed ERK and AKT activity in CML cells. These data indicate that SB743921 may become a novel treatment agent for patients with CML.

Keywords: Apoptosis, CML, KSP, imatinib resistance, proliferation, SB743921

Introduction
Chronic myeloid leukemia (CML) is a lethal hematological malignancy resulting from the clonal expansion of transformed primitive hematopoietic stem cells caused by constitutive activity of BCR–ABL tyrosine kinase [1]. Current CML target therapy relies heavily on the tyrosine kinase inhibitors (TKIs). Imatinib, a specific inhibitor of BCR–ABL, has dramatically improved long-term survival rates of patients with CML [2]. Several other TKIs such as Sprycel (dasatinib), Tasigna (nilotinib), Iclusig (ponatinib) or Bosulif (bosutinib) have been successfully developed as second-line therapeutic agents [3–7]. However, some patients with CML still become resistant to TKIs.

New drug development provides more treatment options for patients with TKI-resistant CML. Novel targets in the mitotic pathway required for cell division have been focused on. Kinesin spindle protein (KSP, Eg5) is a microtubule-associated motor protein that is expressed only during mitosis [8,9]. It controls the formation of a functional mitotic spindle by hydrolyzing adenosine triphosphate (ATP) [10,11]. Inhibition of KSP leads to mitotic arrest followed by cell death in malignant cells [12,13]. KSP is also highly expressed in acute leukemia cell lines and most samples of acute myeloid leukemia (AML) blasts [14]. Functional studies in mice have shown that Eg5 is involved in the leukemogenesis of B-cell leukaemia [15]. It is suggested that KSP is a potential anti-leukemia target.

KSP inhibitors provide new opportunities for the development of anti-leukemia therapeutics. Some of them, such as ispinesib (SB715992), ARRY-520 and MK-0731, are being studied in clinical trials in patients with acute leukemia, CML or advanced myelodysplastic syndromes and solid tumors (www.ClinicalTrials.gov) [16–18]. Unfortunately, ispinesib has demonstrated lack of, or very modest, activity in patients with solid tumors [19]. SB743921, a novel KSP inhibitor, showed promising activity in a clinical trial in patients with relapsed and refractory lymphoma [20]. However, little is known about the effects of KSP inhibitors on bcr–abl+CML cells and their potential in treatment of patients with CML. In this study, we investigated the inhibitory effect of SB743921 on CML cells and its mechanisms.
Materials and methods

Patients and donors
Bone marrow samples were obtained from untreated patients with CML and healthy donors at Peking University First Hospital. After written informed consent from healthy donors or patients with CML was obtained, bone marrow (BM) was collected according to standard procedures.

Isolation of normal or CML CD34 + cells
Mononuclear cells were isolated by Ficoll-Paque™ (GE Healthcare, Uppsala, Sweden) density gradient separation for 30 min at 400 × g and washed twice in phosphate-buffered saline (PBS). Then CD34 + cells were collected from BM mononuclear cells by magnetic separation according to the manufacturer’s instructions (EasySep kit; STEMCELL Technologies, Vancouver, BC, Canada). The purity of the CD34 + cells was higher than 90% as determined by flow cytometry.

Transfection of cord blood CD34 + cells
CD34 + cells isolated from cord blood were cultured in serum-free medium (STEMCELL Technologies) containing growth factors (interleukin-3 [IL-3, 50 ng/mL]; IL-6 [100 ng/mL]; Flt-3 ligand [100 ng/mL]; stem cell factor [SCF, 50 ng/mL] and thrombopoietin [TPO, 100 ng/mL]) at 37 °C in 5% CO2 for 48 h and exposed to retroviral vectors expressing BCR-ABL (MIGR1-p210) or green fluorescent protein (GFP) alone (MIG-R1) (multiplicity of infection, MOI = 20). Cells were labeled 48 h later with anti-CD34 – allophycocyanin (APC) antibodies (eBioscience, Cleveland, OH) and CD34 + GFP + cells were selected using a BD FACSARiaTM cell sorter (Becton Dickinson, Franklin Lakes, NJ). Next the sorted CD34 + cells were cultured in serum-free expansion medium (SFEM) with low concentrations of growth factor (GF), similar to those present in long-term BM culture stroma-conditioned medium (granulocyte-macrophage colony stimulating factor [GM-CSF, 200 pg/mL], leukemia inhibitory factor [LIF, 50 pg/mL], granulocyte colony stimulating factor [G-CSF, 1 ng/mL], SCF [200 pg/mL], macrophage inflammatory protein-1α [MIP-1α, 200 pg/mL] and IL-6 [1 ng/mL]), for 48 h and 72 h. The expression of KSP was determined by quantitative polymerase chain reaction (Q-PCR) and Western blot.

Real-time quantitative PCR
The KSP gene expression of CD34 + cells at the mRNA level in healthy donors and patients with CML was identified by real-time reverse transcription (RT)-PCR analysis. Total RNA was isolated using the RNeasy method, according to the manufacturer’s protocol (Qiagen, Valencia, CA). Four micrograms of total RNA from each sample were subjected to RT using a Superscript first strand cDNA synthesis kit (Thermo, Vilnius, Lithuania) according to the manufacturer’s instructions. Real-time PCR reactions were then carried out in a total 15 μL reaction mixture containing: 2.5 μL of cDNA, 7.2 μL of 2 × SYBR Premix Ex Taq (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China), 0.3 μL of ROX-II, 1.0 μL each of 10 μM forward and reverse primers and 4.0 μL of H2O. The PCR program was initiated by 30 s at 95°C, before 40 thermal cycles each of 3 s at 95°C and 30 s at 60°C. Data were analyzed according to the comparative Ct method and were normalized by β-actin expression in each sample. Melt curves for each PCR reaction were generated to ensure the purity of the amplification product.

Colonies forming assay
CD34 + cells were counted using trypan blue and suspended in GEMM (granulocyte, erythroid, macrophage, megakaryocyte) medium (Iscove’s Modified Dulbecco’s Medium [IMDM] supplemented with fetal bovine serum [FBS] 30%, erythropoietin [EPO] 50 IU/mL, IL-3 5 μg/mL, SCF 5 μg/mL, G-CSF 20 μg/mL, GM-CSF 20 μg/mL) containing 1% methylcellulose. A total of 1000 CD34 + cells were seeded in triplicate in 24-well plates. Cells were incubated for 14 days at 37°C under a 5% CO2 atmosphere, and colony forming cells (CFCs) were then counted.

Cell cycle analysis
CD34 + cells were suspended in SFEM (STEMCELL Technologies) containing 50 ng/mL SCF, 100 ng/mL TPO, 100 ng/mL Flt-3, 50 ng/mL IL-3 and 100 ng/mL IL-6 at a density of 2.5 × 104. A total of 5 × 105 cells were plated and treated with dimethylsulfoxide (DMSO) control, imatinib or SB743921 (Selleck Chemicals, Houston, TX) at indicated concentrations. After being cultured in a 5% CO2 atmosphere at 37°C for 24 h, cells were collected, washed in PBS and then fixed in ice-cold 70% ethanol. Cells were washed with PBS again and stained with propidium iodide solution (20 μg/mL propidium iodide and 0.2 mg/mL RNase A in PBS). Data were acquired on a FACSCalibur instrument and analyzed using the FlowJo software package (Tree Star, Inc., Ashland, OR).

Apoptosis assay
CD34 + cells were suspended in SFEM containing 0.2 ng/mL SCF, 1.0 ng/mL G-CSF, 0.2 ng/mL GM-CSF and 1.0 ng/mL IL-6. A total of 1.0 × 105 cells were plated and treated with DMSO control, imatinib, SB743921 or ERK inhibitor SCH772984 (Selleck Chemicals) at indicated concentrations. After being cultured in a 5% CO2 atmosphere at 37°C for 24 h, the cells were stained with an Annexin V Apoptosis Kit (eBioscience) according to the manufacturer’s instructions. Briefly, cells were collected and washed in PBS, then resuspended in 100 μL of 1 × binding buffer with 5 μL Annexin V-fluorescein isothiocyanate (FITC). After being incubated at room temperature for 20 min, samples were stained with propidium iodide and detected by flow cytometry within 1 h.

Cell growth assay
K562 and KCL22 cells were seeded in six-well plates at a number of 5 × 105 in 2 mL RPMI-1640 medium supplemented with 10% FBS and treated with 2% DMSO, 50 nM imatinib, 2 nM SB743921 and 50 nM imatinib + 2 nM SB743921, respectively. Cell number and viability were determined every 24 h. Results were plotted for live cells against time to generate a growth curve.
Western Blot analysis
Protein was extracted from cells by suspending in RIPA buffer (1× PBS, 1% Nonidet NP-40, 0.1% sodium dodecyl sulfate [SDS]) containing a cocktail of protease inhibitors for 30 min on ice. Lysates were clarified by centrifugation and assayed for protein concentration. Thirty micrograms of total protein in cell lysates were separated by electrophoresis on a SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane and blotted with 5% milk in Tris-buffered saline plus Tween 20 (TBST). The membranes were incubated with primary antibodies anti-ERK (1:1000), anti-ERK Phospho (1:1000), anti-AKT (1:1000), anti-AKT Phospho (1:1000), anti-KSP (1:1000) and anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (1:1000), and subsequently incubated with secondary antibodies conjugated with peroxidase. The signal was then detected using a chemiluminescent detection system.

Statistical analysis
Analysis of variance and Student’s t-tests were performed using Graphpad Prism 5 software (Graphpad Software, Inc., La Jolla, CA). Differences were considered statistically different if \( p < 0.05 \).

Results
KSP is highly expressed in CML CD34+ cells
The expression of KSP was detected by quantitative PCR. Compared with normal samples, KSP expression increased in CML CD34+ cells [Figure 1(A)]. We further investigated whether KSP overexpression is associated with BCR-ABL in CML cells. Cord blood CD34+ cells were transduced with retrovirus carrying the bcr-abl gene and sorted. RT-PCR and Western blot results showed that retrovirus-mediated bcr-abl transduction increased KSP expression in cord blood CD34+ cells cultured in SFEM medium with low growth factors [Figures 1(B) and 1(C)]. This indicates that KSP high expression in CML CD34+ cells is dependent on BCR-ABL.

SB743921 inhibits growth of bcr-abl+ leukemia cells
SB743921 is a specific inhibitor of KSP kinase. Treatment of K562 and CML CD34+ cells with SB743921 resulted in growth inhibition. As shown in Figures 2(A) and 2(B), SB743921 showed dose-dependent anti-leukemic activity in K562 cells and primary CD34+ cells. To assess the specificity of SB743921 on the growth of leukemia cells, we tested its effect on in vitro colony-forming ability of both CML and normal human bone marrow progenitors. At a concentration of 1 nM, SB743921 could significantly inhibit CFC formation of CML primary cells, whereas it showed slight inhibitory effects on the colony-forming ability of normal bone marrow progenitors [Figure 2(C)].

SB743921 induces apoptosis and cell cycle arrest of CML cells
SB743921 was found to induce apoptosis in K562 and KCL22 cells. The characteristic morphological features of apoptosis such as cell shrinkage, chromatin condensation, DNA fragmentation and membrane blebbing were observed in these cells treated with imatinib and SB743921 [Figure 3(A)]. We further observed the apoptotic effect of SB743921 on normal or CML primary CD34+ cells. Treatment of CML CD34+ cells with SB743921 at concentrations of 1 and 3 nM

![Figure 1](https://example.com/figure1.png)

![Figure 2](https://example.com/figure2.png)

![Figure 3](https://example.com/figure3.png)
showed a significantly additive anti-proliferative effect. A similar additive anti-proliferative effect of SB743921 and imatinib on primary CML CD34+/H11001 cells was observed [Figure 5(B)]. These data indicate that SB743921 treatment sensitizes CML progenitor cells to imatinib.

SB743921 overcomes imatinib resistance in CML cells
TF-1 leukemia cells harboring bcr-abl T315I mutation were shown to be imatinib resistant [Figure 6(A)]. TF-1 and TF-1-T315I cells were treated with various doses of SB743921 for 48 h and induction of apoptosis was measured using the Annexin V assay. As shown in Figure 6(B), SB743921 induced apoptosis of both TF-1 and TF-1-T315I cells [Figure 6(B)]. SB743921 treatment resulted in morphological changes of typical apoptosis in these cells [Figure 6(C)]. We also examined the effects of SB743921 on the proliferation of TF-1-T315I cells. Cells were exposed to different doses of SB743921 for 48 h. TF-1-T315I cells showed dose-dependent inhibition with SB743921 (1 nM, 3 nM) [Figure 6(D)]. This indicates that SB743921 overcomes imatinib resistance in CML cells.

SB743921 inhibits MEK/ERK and AKT signaling in CML cells
The underlying mechanism whereby SB743921 induces apoptosis after prolonged mitotic arrest is not completely understood. We detected that KSP protein was decreased in K562 cells or KCL22 cells treated with SB743921 [Figures 7(A)] showed a significantly additive anti-proliferative effect. A similar additive anti-proliferative effect of SB743921 and imatinib on primary CML CD34 + cells was observed [Figure 5(B)]. These data indicate that SB743921 treatment sensitizes CML progenitor cells to imatinib.

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SB743921 inhibits MEK/ERK and AKT signaling in CML cells
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resulted in increased apoptosis, whereas SB743921 treatment slightly induced apoptosis of normal CD34 + cells [Figure 3(B)]. Similarly, cord blood CD34 + cells were transduced with bcr-abl (p210) and treated with SB743921, and apoptosis of transduced cord blood CD34 + cells was significantly induced [Figure 3(C)].

K562 cells were incubated with SB743921 at a concentration of 2 nM for 24 h and then the cells were analyzed for cell cycle distribution by means of flow cytometry. Cell cycle analysis demonstrated that SB743921 induced a depletion of cells in the G1 phase and a concomitant accumulation of cells in the G2/M phase (Figure 4). The fraction of hypodiploid cells which indicated that these cells had undergone apoptosis was increased in K562 cells treated with SB743921. These results suggest that SB743921 induced G2/M cell cycle arrest followed by apoptosis in K562 cells.

**SB743921 sensitizes CML progenitor cells to imatinib**

It is known that imatinib exerts a toxic effect on CML CD34 + cells. We further tested the efficacy of SB743921 combined with imatinib on the growth of CML CD34 + cells in vitro. As shown in Figure 5(A), exposure to imatinib at a low concentration of 50 nM did not demonstrate a significant inhibitory effect on the growth of KCL22 and CML CD34 + cells. SB743921 alone at a concentration of 2 nM impaired KCL22 cell growth. Interestingly, combined treatment of these cells with 50 nM imatinib and 2 nM SB743921
Apoptosis induction in CML by SB743921

Figure 3. SB743921 induces apoptosis of CML cells. (A) Morphological changes of K562 and KCL22 cells treated with SB743921 (3 nM), imatinib (50 nM) or their combination. (B) CML CD34+ cells (n = 3) and normal CD34+ cells (n = 3) were exposed to SB743921 (1 nM, 3 nM) for 24 h. Apoptosis was analyzed by Annexin V-FITC labeling. Results represent the mean ± SEM of separate experiments. Representative flow cytometry plots are shown below the chart. (C) Cord blood CD34+ cells transduced with MIRG1 and p210 were exposed to SB743921 (1 nM, 3 nM) for 48 h. Apoptosis was analyzed by Annexin V-APC labeling. **p < 0.01, compared with untreated controls.

Figure 4. SB743921 induces cycle arrest of CML CD34+ cells. (A) Donor CD34+ cells (n = 3) and (B) CML CD34+ cells (n = 3) were incubated with SB743921 (3 nM) for 24 h as indicated. Cell cycle was analyzed using propidium iodide staining.

Discussion

TKI therapy has dramatically improved long-term survival rates of patients with CML. However, some patients still become resistant to TKIs. Novel compounds modulating the expression or activity of molecular targets besides BCR-ABL could be a new approach in the treatment of CML resistant to TKIs. KSP is essential for the proper separation of spindle poles during mitosis, and has emerged as a target for cancer therapeutics [21]. Targeting KSP may provide a novel route for manipulation of the cell cycle and overcome the drug resistance in CML therapy. The present study demonstrates anti-leukemia activity of a novel KSP inhibitor, SB743921, against bcr-abl+ CML cell lines and primary CD34+ cells.

and 7(B)]. SB743921 treatment also decreased AKT and ERK phosphorylation of both K562 cells and KCL22 cells. Similarly, ERK inhibition with SCH772984 induced the apoptosis of K562 cells [Figure 7(C)]. These results clearly demonstrate that inhibition of AKT and ERK coincided with the mitotic cell death of SB743921 treated CML cells.
We observed that bcr-abl transduction induces the up-regulation of KSP in cord blood CD34+ cells cultured in SFEM medium with low growth factors. SB743921 treatment reduces the KSP protein level and shows cytotoxic activity in BCR-ABL-expressing cells. Treatment of CML cells with SB743921 results in reduced viability, cell cycle blockage, proliferation inhibition and apoptosis. Interestingly, KSP inhibition with SB743921 sensitizes CML cells to apoptosis and overcomes the imatinib resistance dependent on T315I mutation. KSP inhibitors selectively act on cells undergoing cell division, which means that KSP inhibitors are mitosis-specific drugs and may inhibit the growth of normal hematopoietic cells. Due to the high sensitivity of CML cell lines to SB743921-induced growth inhibition, the effect of SB743921 on normal progenitor cell proliferation was evaluated. SB743921 slightly inhibits CFC formation of normal human CD34+ cells isolated from bone marrow, suggesting that the sensitivity of CML cells to SB743921 is associated with high KSP levels in CML cells.

TKI resistance often emerges, and limits their utility in CML therapy. The bcr-abl-independent signaling pathways are known to be involved in imatinib resistance in CML cells [22]. Constitutive up-regulation of KSP might play a key role in the biology of CML. Thus KSP inhibition with SB743921 is a validated approach to overcome TKI resistance in CML cells. In vitro studies showed that SB743921 acted additively with imatinib to inhibit growth and induce apoptosis of CML cells. Importantly, SB743921 alone could induce apoptosis of both imatinib resistant and non-resistant cells. T315I is the most frequent mutation that causes imatinib resistance in patients with advanced CML or Philadelphia chromosome positive (Ph+) ALL [23]. TF-1 leukemia cells harboring bcr-abl T315I mutation were shown to be imatinib resistant. SB743921 treatment could overcome the imatinib resistance is a validated approach to overcome TKI resistance in CML cells. In vitro studies showed that SB743921 acted additively with imatinib to inhibit growth and induce apoptosis of CML cells. Importantly, SB743921 alone could induce apoptosis of both imatinib resistant and non-resistant cells. T315I is the most frequent mutation that causes imatinib resistance in patients with advanced CML or Philadelphia chromosome positive (Ph+) ALL [23]. TF-1 leukemia cells harboring bcr-abl T315I mutation were shown to be imatinib resistant. SB743921 treatment could overcome the imatinib resistance
of TF-1-T315I cells, suggesting that it is potentially a useful novel agent in the treatment of imatinib-resistant CML.

KSP inhibitors are more selective microtubule-targeting agents that only affect spindle microtubules. The exact mechanisms by which these compounds induce cell death are less understood. Constitutively active AKT and ERK expression was demonstrated in CML cell lines and primary CML CD34+ cells [24]. We detected the changes of AKT...

Figure 7. SB743921 inhibits MEK/ERK and AKT signaling in CML cells. Western blot analysis was performed on lysates from KCL22 (A) or K562 (B) cells treated with SB743921 (0.5 nM, 1 nM, 3 nM) to examine levels of ERK phosphorylation in comparison to levels of total ERK, and AKT phosphorylation in comparison to levels of total AKT. GAPDH was used as a control to ensure equivalent protein loading. (C) K562 cells were exposed to ERK inhibitor SCH772984 (1 μM, 2 μM, 5 μM) for 48 h. Apoptosis was analyzed by Annexin V-APC labeling.
and ERK signaling in K562 and KCL22 cells treated with SB743921. SB743921 induced a marked decrease in signal transducer ERK and AKT activation in these cells. Moreover, inhibiting ERK with SCH772984 had a similar effect. These results clearly demonstrate that inhibition of AKT and ERK coincided with mitotic cell death.

Several new KSP inhibitors have been identified in recent years, and some of them have entered clinical trials. Some KSP inhibitors have been studied in hematopoietic malignant tumors. ARL-520 could strongly inhibit tumor growth of HL-60 and MV4-11 xenograft without apparent toxicity in SCID (severe combined immune deficiency) mice. It is also effective in monotherapy as well as in combination with dexamethasone in treatment of multiple myeloma [25,26]. Another potent KSP inhibitor, CF(3)-STLC, could induce mitotic arrest and apoptosis of K562 cells in a caspase-3-independent manner [27,28]. KSP inhibitors are a promising class of agents to treat leukemias and related diseases.

In conclusion, we have elucidated that pharmacological inhibition of KSP induces mitotic arrest and apoptosis and overcomes imatinib resistance of chronic leukemia cells. SB743921 may become a novel treatment agent for patients with CML.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/al.

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