HS-438, a new inhibitor of Imatinib-resistant BCR-ABL T315I mutation in chronic myeloid leukemia

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Abstract
Imatinib is a selective breakpoint cluster region–Abelson (BCR-ABL) tyrosine kinase inhibitor (TKI) that has significantly improved the prognosis of patients with chronic myeloid leukemia (CML). However, T315I gene mutations of the BCR-ABL kinase domain have been shown to confer resistance to Imatinib. In the present study, we synthesized a novel BCR-ABL inhibitor, HS-438, and identified its anti-leukemic effects in vitro and in vivo. We found that HS-438 strongly inhibited the expression of BCR-ABL signaling pathways in wild-type BCR-ABL (BaF3/WT) cells as well as T315I-mutated BCR-ABL (BaF3/T315I) cells with resistance to Imatinib. HS-438 induced cell cycle arrest, particularly during the G0/G1 cell cycle phase, and induced apoptosis. In BaF3/T315I xenograft models, HS-438 significantly delayed tumor growth, unlike Imatinib. In summary, we suggest that HS-438 may be a novel drug candidate with the therapeutic potential to target BCR-ABL and overcome Imatinib resistance in patients with CML.

Introduction
Leukemia is a type of cancer of the bone marrow or blood characterized by an immoderate increase of immature white blood cells. Chronic myeloid leukemia (CML), classified as an incurable disease only 20 years ago, now accounts for 10–20% of all adult leukemia. The incidence of CML in the United States is about 5,000 cases per year, and its prevalence has been increasing annually since 2000 owing to low annual mortality rates of 1–2%. Approximately 80,000 new cases were predicted for 2013, and prevalence will reach about 180,000 new cases by 2030 [1].

The cause of CML is the Philadelphia chromosome carrying the chimeric breakpoint cluster region-Abelson (BCR-ABL) oncogene. It is generated by a chimeric reciprocal translocation of chromosomes 9 and 22, and is specifically designated t(9;22). A segment of the c-ABL gene on chromosome 9q34 is transposed onto the BCR gene on chromosome 22q11, creating a BCR-ABL fusion protein on the shorter chromosome 22 [2]. It encodes a cytoplasmic protein tyrosine kinase with elevated and dysregulated enzymatic activity that plays a vital role in the pathogenesis and progression of CML [3]. This fusion protein is found in ~95% of patients with CML and 30% of adult patients with acute lymphoblastic leukemia (ALL) [4]. Several mechanisms are involved in the malignant transformation orchestrated by the BCR-ABL fusion oncoprotein. First, since BCR-ABL activates a number of cell cycle controlling enzymes and proteins, the speed of cell division is accelerated. Subsequently, the BCR-ABL fusion oncoprotein constitutively activates mitogenic signaling pathways such as the janus kinase (JAK)/signal transduction and transcription (STAT) pathway, phosphatidylinositol-3 (PI3) kinase pathway, RAS/mitogen-activated protein kinase (MAPK) pathways, and the myc pathway [5].

The best of potential CML therapy is targeting Philadelphia chromosome using BCR-ABL tyrosine kinase inhibitor. Imatinib (Gleevec) was developed as an ABL-selective tyrosine kinase inhibitor in 1993 and showed significant effect that improved drug response rate and survival rate in chronic phase. The Imatinib competitively binds to the adenosine triphosphate (ATP) binding site on the ABL tyrosine kinase domain [6], thereby the signaling by this oncogenic protein is prevented. As a result, the growth of BCR-ABL positive leukemia cell is inhibited [7]. However, despite its impressive efficacy, disease relapse has been observed after...
initial response to Imatinib. Indeed, the formation of mutation in ABL kinase domain inhibits the action of Imatinib to ABL, resulting in acquired resistance to Imatinib in some patients. Drug resistance during Imatinib treatment is mostly related to point mutations occurring within the kinase domain of BCR-ABL. Among all mutations on ABL Kinase domain, T315I mutation accounts for 15–20% of mutations of the ABL kinase domain, and E255K and M351T mutations are also highly prevalent [8]. Imatinib strongly inhibits phosphorylation of tyrosine in wild type BCR-ABL whereas does not act on BCR-ABL with T315I mutations [9]. To overcome this resistance, second-generation ABL kinase inhibitors such as Nilotinib and Dasatinib have been developed. Although the second-generation BCR-ABL inhibitors have overcome several types of mutations, Nilotinib and Dasatinib have not demonstrated effectiveness against T315I mutations [10,11].

The aim of the present study was to identify whether a novel BCR-ABL TKI, HS-438 selectively inhibits BCR-ABL activity in vitro and in vivo, and could be used to overcome the limitations associated with Imatinib in CML.

Materials and methods

Cells and materials

BaF3 cells were kindly provided by Dr. Deininger (Huntsman Cancer Institute, Salt Lake City, UT). BaF3/WT and BaF3/T315I cells, which inducibly expressed wild-type BCR-ABL and BCR-ABL with the T315I mutation, respectively, were derived by retroviral transfection using the mammalian expression vector pSRα as described previously [12]. BaF3/WT and BaF3/T315I cells were grown in Roswell Park Memorial Institute Media 1640 (RPMI 1640) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. RPMI 1640, FBS, and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY). Imatinib was purchased from LC laboratories (Woburn, MA), and sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) assays were purchased from Welgene Inc. (Daegu, Korea). Antibodies against STAT5, p-crk avian sarcoma virus CT10 oncogene homolog-like [pCrkl (Tyr207)], pBCR-ABL (Tyr177), pCrkl, pSTAT5 (Tyr694), cleaved caspase-3, caspase-9, PARP, Bcl-2, Bax, XIAP, Survivin, McI-1, Cycclin D1, p27, p21, and β-actin were all purchased from Cell Signaling Technology Inc. (Danvers, MA).

Measurement of cell proliferation

Cellular antiproliferation effects of corresponding compounds were determined by XTT assay. Cells were seeded onto 96-well plates at a density of 2.5 × 10^3 cells per well and incubated at 37 °C for 24 h. Then, the cells were treated with corresponding compounds or control at the indicated concentrations (0.001–5 μM) for 48 h. Then, 10 μl of XTT labeling mixture (1 ml of XTT/20 μl of phenazinemethosulfate [PMS]) was added to each well. After incubation for 2 h, optical density (OD) was determined using a microplate reader by measuring absorbance at wavelength of 540 nm (OD_540) and 620 nm (OD_620). Absorbance rates for each well were calculated as OD_540/OD_620.

Western blotting

After the cells were treated with various concentrations of either 1-(2-hydroxyethyl)-3-(6-(2-methoxyphenyl)benzo[d]thiazol-2-yl)urea (HS-438) or Imatinib and incubated at 37 °C for either 1 h or 24 h, they were collected and washed with cold phosphate-buffered saline (PBS). Then, the cells were lysed by adding a buffer containing 1% Triton X-100, 1% Nonidet P-40 (NP-40), and protease and phosphatase inhibitor cocktails (GenDEPOT, Barker, TX). Cell lysates were collected by dissolving cells in 1× sodium dodecyl sulfate (SDS) sample lysis buffer and then boiling. Equal amounts of cell lysates were loaded onto 12% SDS gel and separated by electrophoresis. Separated proteins were then electro-transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). After being blocked with 1% bovine serum albumin (BSA), the membranes were incubated with the primary antibody at 4 °C overnight. After they were washed with 1× Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% bovine serum albumin (BSA) membranes were incubated with the primary antibody at 4 °C for overnight. After they were washed with 1× Tris-buffered saline (TBS) containing 0.1% Tween-20, the membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. Protein

Fig. 1. Structure and predicted binding mode of HS-438 with ABL/T315I. (A) Structure of HS-438, 1-(2-hydroxyethyl)-3-(6-(2-methoxyphenyl)benzo[d]thiazol-2-yl)urea. (B) The putative binding mode of HS-438 in the BCR-ABL model.
lanes were visualized using an enhanced chemiluminescence (ECL) plus system (Amersham Biosciences, Piscataway, NJ). Primary monoclonal antibodies against the following factors were used: pBCR-ABL (Tyr<sup>177</sup>), pSTAT5 (Tyr<sup>694</sup>), pCrkl (Tyr<sup>207</sup>), STAT5, Crkl, PARP, cleaved caspase-9, c-leaved caspase-3, Bcl-2, Bax, XIAP, Survivin, Mcl-1, Cyclin D1, p27, p21, and β-actin (Cell Signaling Technology Inc. Danvers, MA). Secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ).

Immunofluorescence

BaF3/T315I cells were plated in 12-well plates with RPMI medium and treated with 1 μM HS-438 for 1 h. The cells were then suspended on poly-L-lysine-coated slides, followed by cytospin for 5 min at 500 rpm (Shandon Cytospin 3, Shandon, Germany). Thereafter, the cells were washed twice with PBS and fixed in an acetone:methanol solution (1:1) for 10 min at -20°C. The cells were blocked in 1.5% horse serum in PBS for 30 min at room temperature, and then incubated overnight in a humidified chamber at 4°C with primary antibodies including rabbit anti-pBCR-ABL (Tyr<sup>177</sup>) and pSTAT5 (Tyr<sup>694</sup>) (Cell Signaling Technology, Danvers, MA). After washing twice with PBS, the cells were incubated with rabbit tetramethyl rhodamine isothiocyanate (TRITC) secondary antibody (1:100, Dianova, Germany) for 1 h at room temperature. They were also stained with 4,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Slides were then washed twice with PBS and covered with DABCO (Sigma–Aldrich) before viewing with a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (MMP, Δψm) was assessed using tetramethylrhodamine ethyl ester (TMRE), a fluorescent potential-dependent indicator. BaF3/T315I cells were treated with 1 μM HS-438 for 10 h and stained with TMRE for 30 min at 37°C. The cells were then suspended on poly-L-lysine-coated slides, followed by cytospin for 5 min at 500 rpm (Shandon Cytospin 3, Shandon, Germany). The cells were also stained with DAPI to visualize the nuclei. The slides were then washed twice with PBS and covered with DABCO (Sigma–Aldrich) before viewing with a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Hoechst staining and TUNEL staining

BaF3/T315I cells were plated in 6-well plates with RPMI medium and treated with HS-438 (1 μM). The cells were then suspended on poly-L-lysine-coated slides, followed by cytospin for 5 min at 500 rpm (Shandon Cytospin 3, Shandon, Germany). They were then fixed in ice-cold 2% para-formaldehyde (PFA), washed with PBS, and stained with 1 μg/mL Hoechst 33342 (Cell Signaling Technology, Danvers, MA) for 20 min at room temperature. Stained cells were examined under a fluorescence microscope for evidence of nuclear fragmentation. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was subsequently performed using a TUNEL kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions.

Table 1
Results from high-throughput binding assay against a panel of kinases. A panel of 96 kinases was tested at 1 μM concentrations in a high-throughput binding assay. Only representative kinases are shown here. Lower percent of control (POC) values represent stronger hits. Values shown are the mean of duplicate measurements.

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Fig. 2. Effect of HS-438 on viability of BaF3/WT and BaF3/T315I cells. Viability of cell lines with BCR-ABL/WT, BCR-ABL/T315I, or parental BaF3 cell lines. Comparative viability of cells treated with (A) Imatinib and (B) HS-438 was measured using XTT assay. Cells were seeded in 96-well culture plates, and were treated with various concentrations of Imatinib or HS-438, or with a control of 0.1% dimethyl sulfoxide (DMSO). Cells were subjected to an XTT assay following a 48 h incubation period. The composite curve of percentage inhibition of cell viability by Imatinib and HS-438 were plotted using mean values of triplicate experiments.
Cell cycle analysis

BaF3/T315I cells were plated in 6-well plates with RPMI medium and treated with HS-438 at the indicated concentrations (0.1–5 μM). The cells were collected and fixed overnight in cold 70% ethanol at 4 °C. After washing with PBS, the cells were subsequently stained with 50 μg/mL propidium iodide (PI) and 100 μg/mL RNase A for 1 h at room temperature in the dark, and then flow cytometric analysis was performed to determine the percentage of cells in specific phases of the cell cycle (sub-G1, G0/G1, S, and G2/M) using a FACs Calibur flow cytometer (Becton Dickinson, San Jose, CA). Flow cytometric data were analyzed using FlowJo software (Tree Star, Inc.).

Tumor xenograft study

All animal experiments were performed in accordance with the guidelines of the INHA Institutional Animal Care and Use Committee (INHA IACUC) at the Medical School of Inha University under the authority of project number INHA 130318-197. Male nude mice were obtained from Central Lab. Animal Inc. (Seoul, Korea). The mice were fed a diet of standard mouse chow and tap water ad libitum, and maintained under a 12-h dark/light cycle at 21 °C. Six-week-old male nu/nu BALB/c mice weighing 20–22 g were randomly divided into 4 groups: control, HS-438 25 mg/kg, HS-438 50 mg/kg, and Imatinib 25 mg/kg. BaF3/T315I cells were harvested and mixed with PBS (200 μl/mouse) and then inoculated along the flank of each nude mouse (1 × 10⁵ cells). When the tumor size reached approximately 50–100 mm³, the mice were given HS-438, Imatinib, or vehicle only (negative control) once daily for 15 days. Tumor dimensions were measured every 2 days by using a digital caliper, and tumor volume was calculated using the following formula: \( V = \text{length} \times \text{width}^2 \times 0.5 \). All mice were sacrificed at the end of the experiment. Tumors were excised, and fixed in buffered formalin. Remaining tissues were stored at 70 °C for further analysis.

Immunohistochemistry

After deparaffinization, immunostaining was performed using 8-μm-thick sections of tumor samples. Microwave antigen retrieval was performed in citrate buffer (pH 6.0) for 10 min prior to peroxidase quenching with 0.3% hydrogen peroxide (H₂O₂) in PBS for 10 min. Sections were then washed in water and preblocked with normal goat or horse serum for 10 min. Next, the tissue sections were incubated overnight at 4 °C in 1:50 dilutions of cleaved caspase-3 (Cell Signaling Technologies). The sections were then incubated with biotinylated secondary antibodies (1:200) for 1 h. After washing with PBS, streptodavidin-HRP was applied. Finally, sections were developed with diaminobenzidine tetrahydrochloride substrate for 10 min, and counterstained with hematoxylin. Slides were observed under a confocal laser scanning microscope (Olympus, Tokyo, Japan). At least 3 random fields of each section were examined at 400 × magnification and analyzed using a computer image analysis system (Media Cybernetics, Silver Spring, MD).

Statistical analysis

Data were expressed as the mean ± standard deviation (S.D.). Statistical analysis was performed using ANOVA and unpaired Student’s t-tests. Differences were considered statistically significant when \( p < 0.05 \).

Results

Synthesis of HS-438 and its binding mode to BCR-ABL

We identified a novel BCR-ABL inhibitor, 1-(20 hydroxyethyl)-3-(6-(2-methoxyphenyl)benzo[d]thiazol-2-yl)urea or HS-438,
which was synthesized as described in our previous study [13]. As shown in Table 1, HS-438 was further subjected to kinase selectivity profiling against a panel of 96 cancer-related kinases at 1 μM concentrations in a high-throughput binding assay (KINOMEscan®, Ambit Biosciences, San Diego, CA, USA). Percent of control (POC) determinations were also performed. HS-438 displayed only a high degree of selectivity to ABL but not to other kinases such as Src, Aurora, and c-Kit, which are known to have high structural similarity to ABL. The only kinases against which HS-438 demonstrated target activity were ABL (POC 0.85) and ABL/T315I (POC 6.2). Docking simulations were conducted in order to obtain energetic and structural insight into the binding modes. Fig. 1 shows the calculated binding mode of HS-438 in an ATP binding site of the T315I mutated ABL, which has “DFG-in” conformation. X-ray crystal structure of the T315I mutant in a complex with PPY-A (PDB code: 2Z60) was used as a T315I mutant ABL template, and the binding mode with HS-438 was obtained through docking simulations using modified Autodock. The analysis indicated that HS-438 binds to the ATP pocket near the gatekeeper site (Ile313–Asn322) and Gly loop (Leu248–Tyr256). In the hinge region, nitrogen of the benzothiazole core and C2 urea group appear to form hydrogen bonds with the backbone amidic hydrogen and carbonyl oxygen of Met318. The terminal hydroxy group of urea is directed towards a solvent accessible area and appears to form an additional hydrogen bond with carbonyl oxygen of the Thr319 backbone. In addition, 20-methoxy group in the C6 aryl established a hydrogen bond with the
Asp<sup>381</sup> backbone. Interestingly, mutation of Thr<sup>315</sup> to the more bulky Ile<sup>315</sup> did not cause steric hindrance to the inhibitor, and the aryl moiety of HS-438 appears to form favorable hydrophobic interactions established simultaneously in the Gly loop.

**HS-438 inhibits the proliferation of BaF3/WT and BaF3/T315I cells**

XTT assays were performed to evaluate the inhibitory effect of HS-438 on the growth of BaF3/WT and BaF3/T315I cells. Cells were treated with various concentrations of HS-438 and Imatinib for 48 h. As shown in Fig. 2A and B, both Imatinib and HS-438 treatment reduced the cell viability of BaF3/WT cells; IC<sub>50</sub> values were 0.112 μM for Imatinib and 0.138 μM for HS-438. HS-438 significantly reduced cell viability of BaF3/T315I cells (IC<sub>50</sub> 0.301 μM), while Imatinib had little effect (IC<sub>50</sub> > 5 μM). Neither Imatinib nor HS-438 inhibited cell viability of murine normal BaF3 parental cells (IC<sub>50</sub> > 5 μM). These data suggest that HS-438 exhibits potent inhibitory activity in T315I mutated BCR-ABL cells with the resistance to Imatinib as well as BCR-ABL expressing leukemic cells.

**HS-438 inhibits BCR-ABL signaling pathways in BaF3/WT and BaF3/T315I cells**

To determine whether the antiproliferative effects of HS-438 were dependent on the inhibition of BCR-ABL activity, phosphorylation of BCR-ABL and its respective downstream signals, STAT5 and Crkl, were measured by Western blotting. As shown in Fig. 3A, HS-438 strongly inhibited the phosphorylation of BCR-ABL (Tyr<sup>177</sup>) in both BaF3/WT and BaF3/T315I cells. Likewise, phosphorylation levels of STAT5 (Tyr<sup>598</sup>) and Crkl (Tyr<sup>207</sup>) were effectively suppressed in a dose-dependent manner. In contrast, Imatinib did not alter phosphorylation levels of BCR-ABL, STAT5, or Crkl in BaF3/T315I cells. To further confirm these results, we conducted confocal fluorescent microscopy analysis in BaF3/T315I cells. In agreement with the western blotting results, HS-438 obviously reduced the phosphorylation levels of BCR-ABL in BaF3/T315I cells, indicating that HS-438 effectively inhibits BCR-ABL signaling pathways in both BaF3/WT and BaF3/T315I cells (Fig. 3B).

**HS-438 induces apoptotic cell death in BaF3/WT and BaF3/T315I cells**

Several cell-based apoptosis assays were performed to test whether the cytotoxic effect of HS-438 was associated with the induction of apoptosis. Firstly, the apoptotic effect of HS-438 in BaF3/T315I cells was identified by Hoechst staining and TUNEL assay. As shown in Fig. 4A, cells treated with HS-438 presented more prominent morphological features characteristic of apoptotic cells, such as bright nuclear condensation and perinuclear apoptotic bodies identified by Hoechst staining. Similarly, HS-438–induced apoptosis was observed by TUNEL staining. A significant increase in percent TUNEL-positive cells was observed with HS-438 treatment (Fig. 4B). Levels of key proteins involved in apoptosis in BaF3/WT and BaF3/T315I were examined by western blotting, and are shown in Fig. 4C. HS-438 significantly increased expression levels of cleaved PARP, caspase-9 and caspase-3 in BaF3/WT and BaF3/T315I cells. These results were also confirmed by immunofluorescence after treating with 1 μM HS-438 in BaF3/T315I cells for 24 h (Fig. 4B).

**HS-438 induces mitochondria-dependent apoptosis in BaF3/WT and BaF3/T315I cells**

Alteration of MMP is an initial and irreversible step of the apoptosis pathway [14]. Thus, we measured MMP in HS-438–treated BaF3/T315I cells using TMRE staining. As shown in Fig. 5A, HS-438 significantly reduced fluorescence intensity reflecting MMP compared to control. Since mitochondria-mediated apoptosis is
regulated by various protein families, such as inhibitor of apoptosis protein (IAP) and Bcl-2 [14], we investigated expression levels of several IAP family proteins, such as XIAP and Survivin, as well as Bcl-2 family proteins Bax, Mcl-1, and Bcl-2 by western blot (Fig. 5B). HS-438 increased the expression of Bax and decreased the expression of the anti-apoptotic proteins Bcl-2, Survivin, XIAP, and Mcl-1. These results indicated that HS-438 induced apoptosis through mitochondria-dependent pathways in BaF3/WT and BaF3/T315I cells.

**HS-438 inhibits cell proliferation through G0/G1 arrest in BaF3/T315I cells**

In order to better understand the anticancer mechanism of HS-438, its effects on BaF3/T315I cell cycle progression were examined. Cells were incubated with HS-438 for 24 h. Control and treated cells were collected, stained with PI, and analyzed by flow cytometry. The cell cycle data showed that treatment with HS-438 induced cell cycle arrest in the G0/G1 phase (64.9% at 5 μM) (Fig. 6). Expression levels of p21, p27, and Cyclin D1, which typically cause cell cycle arrest in the G0/G1 phase, were also investigated. This was achieved by exposing BaF3/T315I cells to HS-438 for 24 h, followed by protein expression studies by western blotting. As shown in Fig. 6B, treatment with HS-438 (1 and 5 μM) increased expression of p21 and p27, and decreased expression of Cyclin D1 as compared with the control and Imatinib. These findings demonstrated that HS-438 inhibited cell proliferation through the induction of G0/G1 arrest.

**HS-438 suppresses tumor growth in a xenograft models**

Based on the *in vitro* findings, we extended our study to an *in vivo* mouse xenograft model. After inoculation with BaF3/T315I cells, mice were intraperitoneally injected with HS-438 at doses of 25 and 50 mg/kg, and Imatinib at a dose of 25 mg/kg for 15 days. Treatment of HS-438 inhibited tumor growth on day 9. Inhibition of tumor growth with HS-438 was more visible and significant on day 15 as compared with Imatinib (p < 0.05, Fig. 7A). No significant changes in body weight or adverse effect were observed in mice treated with HS-438 as compared to the control (Fig. 7B). To further confirm whether HS-438 inhibits tumor growth through the induction of apoptosis, we identified expression levels of cleaved caspase-3 as well as DNA fragmentation in isolated tumor tissue. As shown in Fig. 7C, treatment with HS-438 increased expression of cleaved caspase-3, and TUNEL-stained cells indicated DNA fragmentation in the HS-438 treated group as compared to the control and Imatinib groups. Taken together, these results demonstrate that HS-438 has potent antitumor efficacy *in vivo*.

**Discussion**

The dysregulated activity of BCR-ABL tyrosine kinase resulting from the t(9;22) chimeric chromosomal translocation has been found to be necessary for the transformed phenotype of CML cells [15,16]. This character has paved the way for the development of novel therapies that selectively target the BCR-ABL gene product. Indeed, patients with advanced CML are less sensitive to

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Fig. 5. Effect of HS-438 on the mitochondria-related apoptosis of BaF3/WT and BaF3/T315I cells. (A) BaF3/T315I cells were treated with HS-438 at 1 μM for 10 h and were stained with tetramethylrhodamine ethyl ester (TMRE) for 30 min at 37 °C and analyzed with an Olympus confocal laser scanning microscope. The loss of accumulation of TMRE staining cells indicated dissipation of membrane potential. (B) The expression of Bax, Bcl-2, Mcl-1, Survivin and XIAP that is related with mitochondrial apoptosis, was assayed by western blot in BaF3/WT and BaF3/T315I cells. The cells were treated with HS-438 and Imatinib at 1 and 5 μM for 24 h.
tyrosine-kinase inhibitor (TKI) therapy and responses are short lived. Moreover, drug resistance due to the emergence of TKI-resistant mutations is serious in advanced phase CML patients. Although Imatinib is the first-generation of drugs that specifically target the BCR-ABL tyrosine kinase [7], it has become apparent that a significant portion of patients chronically treated with Imatinib develop clinical resistance due to BCR-ABL overexpression [9,17], or as a result of mutations (T315I, E255K, and M351T) in the BCR-ABL kinase domain [18]. Also, the second-generation TKIs such as Nilotinib, Dasatinib [19] and SKI-606, INNO-406 and

Fig. 6. Effect of HS-438 on cell cycle in BaF3/T315I cells. (A) BaF3/T315I cells were treated with HS-438 (0.1, 1, 2.5, and 5 μM) for 24 h, stained with propidium iodide (PI), and analyzed by a FACS Calibur flow cytometer. Quantification of the PI staining data was presented as the percentages of distribution through stages of the cell cycle: M₁, sub-G₁; M₂, G₀/G₁; M₃, S; M₄, G₂/M. (B) Quantification of distribution of the cell cycle. Expression of Cyclin D1, p27, and p21, which are related to cell cycle G₀/G₁ arrest, was assayed by western blot in the cells treated with HS-438 and Imatinib at 1 and 5 μM for 24 h.
PD166326 [20], can over-ride some, but not all Imatinib-resistant mutations. Especially, these novel inhibitors effectively inhibited the phosphorylation of the mutated BCR-ABL (E255K, M351T) but not T315I mutated BCR-ABL. In addition, an aurora kinase inhibitor such as VX-680 [21], ON012380 [22], reactive oxygen species (ROS) promoting agents such as PEITC and adaphostin [23,24] and p38 MAP kinase inhibitor such as BIRB-796 [25] have been assessed to overcome the problem of the T315I mutation of BCR-ABL gene. However, satisfied results have not been derived yet [21,26,27]. Therefore, innovative treatment is necessary to over-ride TKI-resistant mutations, or to promote elimination of BCR-ABL harboring cells in patients. In this study, we aimed to identify effective targeted chemotherapy against CML cells carrying T315I-mutant BCR-ABL that confers resistance to Imatinib. To achieve this, we synthesized HS-438, a novel BCR-ABL TKI that demonstrated high affinity for the ATP-binding site, resulting in high specificity to BCR-ABL including T315I-mutated BCR-ABL (Table 1). Based on this result, we investigated whether HS-438 had potent activity on BCR-ABL target inhibition in CML cell lines BaF3-expressing wild-type BCR-ABL versus T315I-mutated BCR-ABL. HS-438 potently inhibited proliferation in both BaF3/WT (IC50 1.138 μM) and BaF3/T315I (IC50 0.301 μM) cells. However, Imatinib failed to inhibit proliferation in BaF3/T315I cells since IC50 values were greater than 5 μM. Also, when we compared with Ponatinib, which is recently approved as the third-generation therapeutic agent for CML, Ponatinib exhibited superior inhibitory effects compared with Imatinib and HS-438 in both BaF3/WT and BaF3/T315I cells (Data not shown). However, Ponatinib exhibited toxicity in parental BaF3 cells (IC50 0.775 μM), whereas little toxicity was associated with HS-438 and Imatinib (Data not shown). Although

Fig. 7. In vivo effect of HS-438 in BaF3/T315I cell-derived mouse xenograft model. (A) Tumor growth of BaF3/T315I xenograft in nu/nu BALB/c mice. (B) Mean body weight of nude mice. All mice were subjected to implantation in the flank by a subcutaneous injection of BaF3/T315I (1 × 10⁶ cells/200 μl PBS). HS-438 (25 and 50 mg/kg) was intraperitoneally injected once daily. Tumor size was measured every 2 days. Data were represented as mean ± S.D. (n = 7 or 8), ***p < 0.05 as compared to Imatinib. (C) In vivo effect of HS-438 on proliferation and apoptosis in isolated tumor from BaF3/T315I xenograft. Tumors were excised and processed for immunostaining to detect cleaved caspase-3. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was subsequently performed (400 × magnification).
HS-438 is associated with a smaller anticancer effect than Ponatinib, it is considered to be a far safer agent for CML patients with the T315I mutation.

Also, HS-438 led to a distinct inhibition of BCR-ABL phosphorylation, indicating decreased ABL-kinase activity as well as a pronounced inhibition of phosphorylation of BCR-ABL downstream target STAT5 in both BaF3/WT and BaF3/T315I cells. Furthermore, phosphorylation of another well-known BCR-ABL downstream target Crkl was obviously reduced after treatment with HS-438. Interestingly, Imatinib inhibited the phosphorylation of BCR-ABL pathways, such as of BCR-ABL, STAT5, and Crkl in BaF3/WT, but not in BaF3/T315I cells. These results revealed that the decrease of phosphorylation of Crkl and STAT5 by HS-438 in cells with the highly resistant mutation T315I was significant, indicating an effective inhibition of BCR-ABL carrying this highly resistant mutation, whereas no inhibition of phosphorylation could be induced by Imatinib treatment.

Reductions in cell growth and proliferation have been associated with intense modulation of cell cycle arrest [28]. Also, the loss of cell cycle control gives rise to tumor development and proliferation. In addition, anticancer agents demonstrate the ability to alter the regulation of the cell cycle, resulting in an arrest of cells in several phases of cell cycle; thereby reducing the growth and proliferation of cancer cells [29]. On the basis of that finding, we carried out a cell cycle analysis of HS-438-treated T315I-mutated BCR-ABL cells. As a result, cell cycle analyses showed a clear arrest of cells in the G0/G1 phase after HS-438 treatments, indicating the inhibition of entry into the S-phase from G0/G1 of cell cycle. Also, HS-438 decreased expression of Cyclin D1 and increased expression of p21 and p27, which are well known for cell cycle related proteins [28,30,31]. Although various factors and pathways are involved in cell cycle regulation, our results showed that HS-438 inhibited cell growth and proliferation by cell cycle arrest of the G0/G1 phase, which may be partly regulated by Cyclin D1, p21 and p27. Considering these results, this led us to conclude that HS-438 inhibits cell proliferation by modulating cell cycle progression through the G0/G1 phase, and regulating the expression of its related proteins via inhibition of the BCR-ABL pathway in T315I mutated BCR-ABL cells.

Also, HS-438 induced apoptosis in association with mitochondria in BaF3/T315I cells but not Imatinib treatment. Mitochondria provide energy in the form of ATP and play an important role in the regulation of apoptosis, which has been shown to be linked to the loss of MMP [14]. Changes in the MMP lead to the creation of pores in the mitochondria, which dissipates the transmembrane potential and leads to the release of cytochrome c into the cytoplasm [32,33]. Also, the release of cytochrome c induces expression of various pro-apoptotic proteins in cytoplasm [34]. Especially, Bcl-2 is localized in the outer membrane of mitochondria [35] to block the release of cytochrome c from the mitochondria to the cytoplasm [36]. Thus, Bcl-2 overexpression inhibits apoptosis induction in response to various apoptosis stimuli [14]. Similarly, IAPs including XIAP and survivin as anti-apoptotic protein family have been reported to bind to caspases to inhibit apoptosis signaling [37]. In the present study, HS-438 down-regulated the expression of Bcl-2, Mcl-1, Survivin, and XIAP whereas up-regulated the expression of Bax. These changes led to increase the expression of caspase-3, caspase-9, and cleavage of PARP. Likewise, Imatinib did not change expression of apoptosis-related molecules in BaF3/T315I cells. Collectively, HS-438 appears to induce apoptosis via an intrinsic mitochondria-dependent pathway in BaF3/WT and BaF3/T315I cells. These events were supported by in vivo results, showing that HS-438 inhibited the tumor growth in nude mouse xenografts models. Also, the apoptosis effect of HS-438 was confirmed by the increased cleaved caspase-3 expression and TUNEL positive cells in tumor tissues of BaF3/T315I cells xenograft mice.

In conclusion, our results demonstrate that the novel selective BRC-ABL TKI, HS-438, has potent anticancer activity against the cells bearing wild type and T315I mutant type BCR-ABL. Based on the mechanism of action and promising activity of HS-438 against Imatinib-resistant CML cells, our results present an important insight to be considered for clinical investigations into how to overcome T315I mutation–induced Imatinib resistance in patients with CML.

Conflict of Interest

None declared.

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