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Pharmacological inhibition of JAK3 enhances the antitumor activity of imatinib in human chronic myeloid leukemia

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ABSTRACT

Imatinib (IMA) is the standard treatment for CML; however, stopping IMA sometimes results in disease relapse, which suggests that leukemic stem cells (LSCs) remain in such patients, even after complete molecular remission has been achieved. Therefore, new strategies will be required to eradicate LSCs. The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is part of the BCR-ABL signaling network, and it is activated in CML, especially in LSCs. JAK2 is known to be associated with CML survival, but the role of JAK3 in CML remains unknown. The antitumor effects of IMA and a JAK3 inhibitor, tofacitinib were examined using the MTT assay in K562 and KCL22. To investigate the mechanisms of action of IMA and the JAK inhibitors in CML cells, we examined apoptosis, the cell cycle, and JAK-STAT signaling using flow cytometry, immunofluorescent microscopy, and Western blotting. The pharmacological inhibition of JAK3 by tofacitinib synergistically enhanced the antitumor effects of IMA in CML cells. Furthermore, the administration of IMA plus a JAK inhibitor reduced the expression of stem cells markers, such as ABCG2 and ALDH1A1. Co-blocking JAK3 with IMA and a JAK3 inhibitor might represent a new treatment strategy for eradicating LSCs and preventing CML relapses.

1. Introduction

Chronic myeloid leukemia (CML) is a clonal malignant hematopoietic stem cell disorder that is characterized by an acquired cytogenetic abnormality resulting from a translocation between chromosomes 9 and 22, which generates the famous Philadelphia chromosome and BCR-ABL fusion gene. The BCR-ABL fusion gene is found in Philadelphia chromosome-positive CML and acute lymphoblastic leukemia, and its tyrosine kinase activity is essential for leukemogenesis (Calderón-Cabrera et al., 2013; Grosso et al., 2009; Carlesso et al., 1996; Savage and Antman, 2002). Imatinib (IMA) is an inhibitor of ABL kinases that targets the platelet-derived growth factor receptor and c-KIT (Savage and Antman, 2002; Apperley et al., 2002). IMA was reported to be markedly effective against newly diagnosed chronic-phase CML (Hughes et al., 2010). Most CML patients who receive IMA treatment achieve a long-term complete cytogenetic response and even a complete molecular response (CMR) (Branford et al., 2007). Thus, IMA has revolutionized the treatment of CML, but it is acknowledged that some patients who stop TKI remain free of CML and appear to be cured however this occurs in a minority of patients diagnosed (Rousselot et al., 2007). Some patients have not relapsed after stopping IMA upon achieving a CMR, therefore, in some patients it appears that IMA may indeed eliminate the leukemic stem cells (LSCs). Although some patients may be cured of CML with IMA alone, this is the minority of patients and new efforts need to be identified to eradicate the LSC for the majority of patients. (Takeishi et al., 2013; Chomel et al., 2011). Also, it has been demonstrated that IMA therapy should only be stopped in patients whose BCR-ABL transcript levels are at least 4-log lower than those seen at the initial diagnosis (M Mahon et al., 2010). Therefore, new strategies for eliminating LSCs are needed. For example, we hypothesize that the survival of LSCs might not be affected by the inhibition of BCR-ABL kinase activity during IMA treatment. Therefore, it might be necessary to inhibit other signaling pathways in addition to BCR-ABL kinase activity (Corbin et al., 2011; Colavita et al., 2013).

BCR-ABL activates several signaling pathways, such as the phosphoinositide 3-kinase (PI3K), receptor-associated factor (RAF), extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK)/ERK kinase (MEK), and Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathways (Steelman et al., 2004). The Janus family of non-receptor tyrosine kinases is involved in cytokine signaling and transduces signals via STAT. JAK play pivotal roles in cell proliferation and differentiation in leukemia (Warsch et al.,

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2013; Hoelbl et al., 2010; Nair et al., 2012b). There are four JAK: JAK1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2) (Giordanetto and Kroemer, 2002).

BCR-ABL can activate STAT3/5 via mechanisms other than the JAK pathway. In addition, constitutive activation of STAT3/5 is observed in CML (Hoelbl et al., 2010; Nair et al., 2012b). Therefore, inhibition of the JAK/STAT pathway might represent a good target during the development of drugs for CML. It has been reported that JAK2 inhibitors, such as ruxolitinib (RUX), are effective against CML (Colavita et al., 2013; Quintarelli et al., 2014), and JAK inhibitors reinforced cell death within the bone marrow microenvironment in a mouse model (Nair et al., 2012a; Traer et al., 2012). Thus, the simultaneous inhibition of BCR-ABL and JAK2 might be a useful therapeutic strategy for overcoming tyrosine kinase inhibitor (TKI) resistance (Quintarelli et al., 2014; Traer et al., 2012). However, it remains unclear whether BCR-ABL and JAK3 have synergistic effects on CML. Furthermore, JAK3 expression is only found in hematopoietic cells (Pattison et al., 2012). Tofacitinib (TOF) is a new JAK3 inhibitor, and it is currently approved for the treatment of rheumatoid arthritis in the US. Therefore, we examined the effects of combined treatment with TOF and IMA on CML cell lines.

2. Materials and methods

2.1. Reagents

RPMI1640medium, fetal bovine serum, and phosphate-buffered saline (PBS) were purchased from Life Technologies (CA, USA). IMA, a TKI, was obtained from BioVision, Inc. (CA, USA). TOF, a selective JAK3 inhibitor, and RUX, a selective JAK1/2 inhibitor, were obtained from Selleckchem (TX, USA). The IMA and JAK inhibitors were dissolved in dimethyl sulfoxide.

2.2. Cell cultures

K562 and KCL22 cells (CML cells) were obtained from the RIKEN BioResource Center (Ibaraki, Japan) and the American Type Culture Collection (VA, USA), respectively. The human acute myeloid leukemia cell line THP-1 was obtained from the RIKEN BioResource Center (Ibaraki, Japan) and used as a BCR-ABL-negative control. The K562, KCL22, and THP-1 cells were grown in RPMI1640 supplemented with 10% fetal bovine serum and 10 mg/ml of 1% (v/v) penicillin-streptomycin (Sigma-Aldrich, MO, USA). All cells were incubated at 37 °C in a humidified atmosphere containing 5% (v/v) CO2.

2.3. Cell survival assay

Cell survival assay was performed using 3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide (MTT) (DOJINDO, Kumamoto, Japan), according to the manufacturer's standard protocol, as described previously. Briefly, cells (1 × 105 cells/well) were plated onto 96-well microplates and treated with or without IMA (0.06–1.0 μM) and/or TOF (10–1000 nM) or RUX (10–1000 nM) for 72 h at 37 °C in a humidified 5% (v/v) CO2 atmosphere. The medium (200 μl) was then incubated with 10 μl of 5 mg/ml MTT solution for 4 h at 37 °C. After being centrifuged at 352 g for 5 min, the culture medium was removed, and 100 μl of dimethyl sulfoxide were added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Model 680 microplate reader; Bio-Rad Laboratories, Inc., Hercules, CA). The results are expressed as percentages; i.e., as the ratio of the absorbance of treated cells to that of the control (100%). Percentage survival was calculated using the following formula: percentage survival = (absorbance of treated wells − absorbance of blank wells) / (absorbance of untreated wells − absorbance of blank wells) × 100.

2.4. Western blot analysis

Cells were cultured with 0.25 μM IMA for 24 h followed by 100 nM of the relevant JAK inhibitor for 1 h before being examined. For the total protein extraction, cells were lysed in RIPA buffer (Sigma-Aldrich, MO, USA) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail 2 (Wako, Osaka, Japan). Cell lysates were centrifuged at 12,000 × *g* for 15 min at 4 °C, and their protein concentrations were determined using the DC protein assay reagent (BIO-RAD Laboratories, Inc.). Fifty μg of total protein were heated for 5 min at 95 °C and then loaded onto 4–20% SDS polyacrylamide gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA), before being transferred to PVDF membranes (Hybond P, GE Healthcare, Buckinghamshire, UK) and incubated with primary antibodies (Supplemental Table 1). After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody, specific chemiluminescent signals were visualized using the ECL Western blotting detection system (GE Healthcare, UK). Images were obtained using an LAS-3000 FUJIFILM luminescent image analyzer (FUJIFILM, Tokyo, Japan).

2.5. Nuclear morphological examination/fluorescent microscopy

The effects of the reagents on nuclear morphology were investigated using Hoechst 33342 staining (Wako, Osaka, Japan). Briefly, K562 cells (density: 1.0 × 105 cells/well) were seeded onto a 96-well plate and treated with 100 nM TOF or 100 nM RUX, with or without 0.25 μM IMA for 72 h, and then stained with Hoechst 33342 (1 mg/ml of PBS) for 30 min at room temperature. Nuclear morphology was examined with a BZ-8000 fluorescent microscope (KEYENCE, Osaka, Japan)

2.6. Analysis of the cell cycle and the apoptotic sub-G0 population

The cell cycle and sub-G0 population were analyzed using flow cytometry. K562 cells were plated on 6-well plates at a density of 1 × 105 cells/well and then were incubated with 100 nM TOF or 100 nM RUX, and/or 250 μM IMA. After 72 h, the cells were collected and washed with PBS, before being resuspended in 70% ice-cold ethanol at − 20 °C overnight. After cells had been centrifuged, the ethanol was discarded, and cells were resuspended in 50 μl of PBS containing 0.1 mg/ml RNase (F. Hoffmann-La Roche, Ltd., Basel, Swiss) for 30 min at 37 °C. After being washed with PBS, the cell pellet was gently resuspended in 100 μl of propidium iodide (PI) solution (5 μg PI and 0.1% Triton X-100 in PBS) at 4 °C in a darkened environment for 10 min. Cell cycle analysis was performed using a FACS Calibur (Becton, Dickinson and Company, NJ, USA). Doublets, cell debris, and fixation artifacts were gated out, and cell cycle analysis was conducted using the Flow Jo software (FLOWJO, LLC, OR, USA).

2.7. Statistical analyses

All experiments were independently repeated a minimum of three times, and the resultant data are expressed as mean ± standard deviation (S.D.) values. Cell survival data and the frequency of apoptosis were analyzed using Student's *t*-test. Combined effect data were analyzed using two-way analysis of variance (ANOVA) followed by the Tukey test. P-values of < 0.05 were considered to indicate statistical significance.

3. Results

3.1. JAK/STAT inhibition enhances the effects of IMA on CML

K562, KCL22, and THP-1 cells were exposed to different doses of IMA or JAK inhibitors for 72 h to quantify the effects of TKI activity. Cell growth inhibition was then evaluated using the MTT assay. The

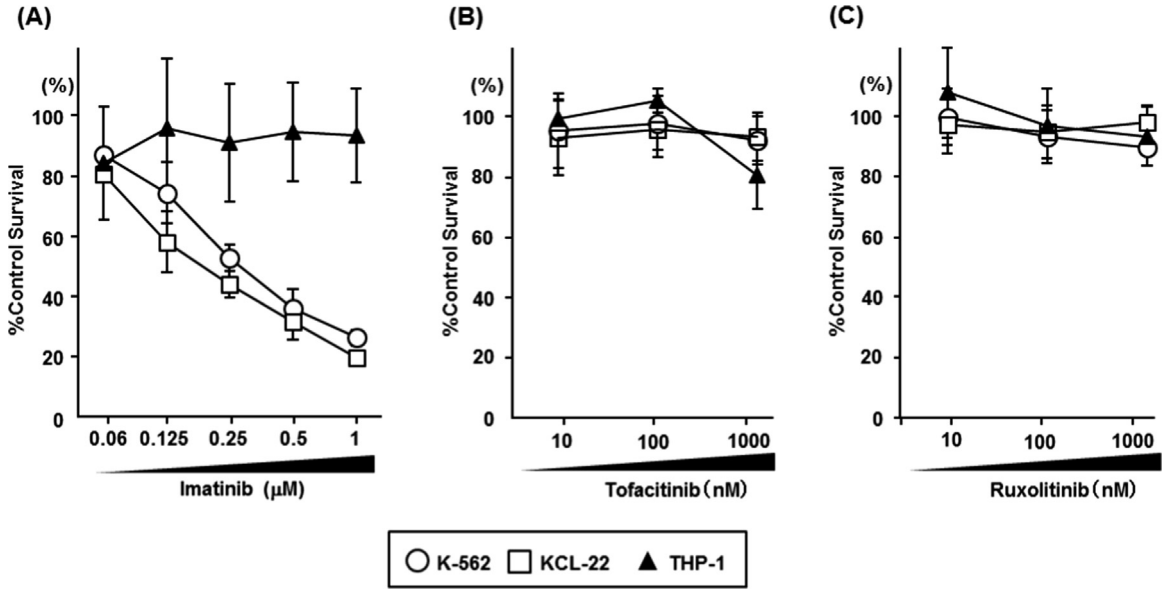

Fig. 1. Antitumor effects of IMA, TOF, and RUX in CML cells. K562 and KCL22, which are BCR-ABL-positive CML cell lines, and THP-1, which is a BCR-ABL-negative acute myelocytic leukemia cell line, were used to examine drug sensitivity. Cells (density: 1 × 105 cells/well) were plated in 96-well plates with (A) IMA (63–1000 nM), (B) TOF (10–1000 nM), or (C) RUX (10–1000 nM). After 72 h incubation, cell survival was determined using the MTT assay. The error bars represent the mean ± S.D. (n = 6).

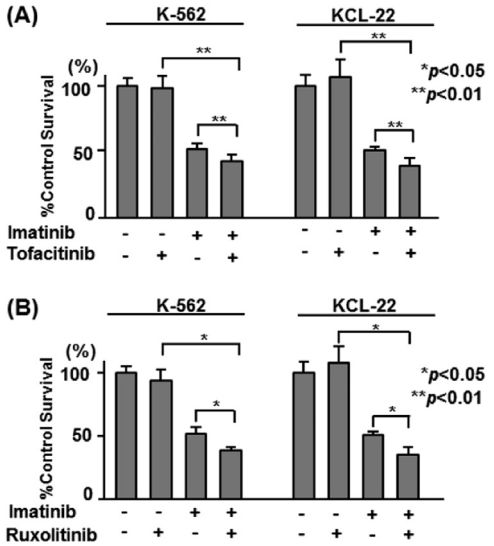

Fig. 2. JAK inhibitors enhanced the antitumor effects of IMA in BCR-ABL-positive CML cells. K562 and KCL22 cells (density: 1 × 105 cells/well) were plated in 96-well plates, and medium containing (A) 100 nM TOF or (B) 100 nM RUX, with or without 250 nM or 125 nM IMA was added after 24 h. After 72 h incubation, cell survival was determined using the MTT assay. The error bars represent the mean ± S.D. (n = 6). *P < 0.05, **P < 0.01, statistically significant when compared with the control group.

proliferation of K562 and KCL22 cells, but not THP-1 cells, was inhibited by IMA in a concentration-dependent manner (Fig. 1). The IC50 value of IMA was 0.28 μM for K562 and 0.17 μM for KCL22. Although treatment with TOF or RUX alone did not suppress cell proliferation, both JAK inhibitors made the K562 and KCL22 more sensitive to IMA (Fig. 2). The synergistic effects of TOF and IMA seemed to be similar to those of RUX and IMA.

Furthermore, we also detected apoptosis after combined treatment with IMA and either JAK inhibitor. Neither JAK inhibitor induced apoptosis or reduced the frequency of IMA-induced apoptosis (Fig. 3A and B). IMA induced poly-(ADP-ribose) polymerase (PARP) 1 and caspase 3 expression; however, neither JAK inhibitor induced the expression of these proteins (Fig. 3B).

3.2. The activation status of the JAK/STAT pathway

It has previously been reported that the JAK/STAT pathway plays an important role in CML cell survival (Carlesso et al., 1996; Nair et al., 2012b). We revealed that both RUX and TOF enhanced the effects of IMA on CML cell survival (Fig. 2). Thus, we investigated the phosphorylation status of the JAK/STAT pathway after co-treatment with TOF or RUX, and IMA.

Although the BCR-ABL signal was very strong, IMA markedly inhibited the phosphorylation of Abl, JAK2, JAK3, and STAT3. Treatment with either JAK inhibitor alone had little effect on Abl, JAK2/3, or STAT3/5 phosphorylation due to the strong effects of BCR-ABL. However, combined treatment with IMA plus a JAK inhibitor resulted in the downregulation of JAK/STAT tyrosine kinase phosphorylation. In the presence of IMA, TOF did not inhibit JAK2 phosphorylation, but did reduce JAK3 phosphorylation, whereas RUX inhibited the phosphorylation of JAK2 and JAK3. Compared with IMA alone, the phosphorylation of STAT3/5 was reduced by combined treatment with IMA and either JAK inhibitor. In particular, IMA did not suppress STAT5 phosphorylation; however, combined treatment with IMA plus a JAK inhibitor suppressed STAT5 phosphorylation (Fig. 4). We have illustrated these results in Fig. 5.

3.3. The effects of JAK inhibitors on LSCs

IMA induced cell cycle arrest at the sub-G1 phase by inhibiting BCR-ABL expression. Changes in the cell cycle were found to be involved in the resistance of LSCs to chemotherapy (Lakshmikuttyamma et al., 2008; Kim et al., 2013). In addition, the LSC exhibited resistance to TKI, such as IMA or dasatinib, which is considered to be a cause of relapse. (Copland et al., 2006). LSCs strongly express aldehyde dehydrogenase 1 family member A1 (ALDH1A1) and ATP-binding cassette sub-family G member 2 (ABCG2), which are related to drug resistance (Zhou et al., 2001; Brendel et al., 2007). Therefore, changes in the cell cycle and reductions in the expression of ALDH1A1 and/or ABCG2 seem to make cells more sensitive to IMA. Thus, we evaluated the effects of TKI on the cell cycle and the expression of stem cell markers in K562 cells. Each of the JAK inhibitors increased the frequency of sub-G1 phase cells, and treatment with IMA plus a JAK inhibitor increased the frequency of sub-G1 phase cells to an even greater extent (Fig. 6A). In addition, the

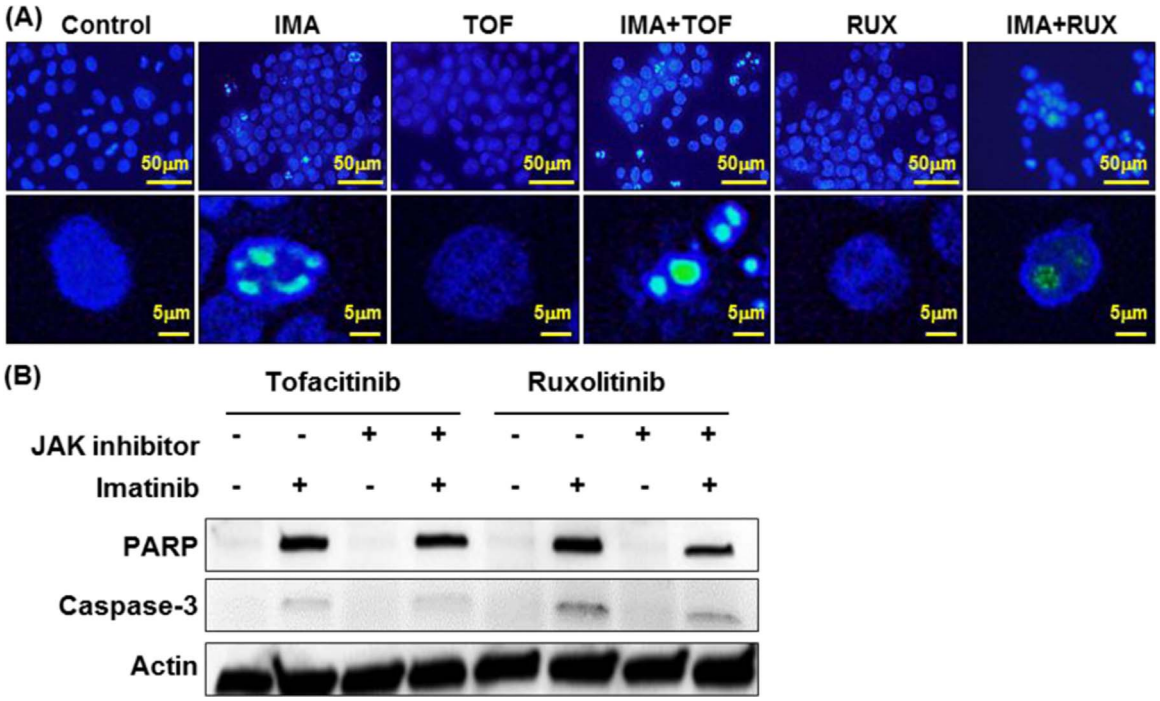

Fig. 3. Effects of JAK inhibitors on IMA-induced apoptosis in BCR-ABL-positive CML cells. Apoptosis was assessed based on nuclear morphology and Western blotting. K562 cells were used as BCR-ABL-positive CML cells. (A) Nuclear morphological changes were detected by fluorescence microscopy after Hoechst 33342 staining. Cells were treated with 100 nM TOF or 100 nM RUX, and/or 250 nM IMA for 72 h prior to being stained. (B) The cleavage of PARP1 and caspase-3 was assessed by Western blotting after treatment with 100 nM TOF or 100 nM RUX, and/or 250 nM IMA for 1 h.

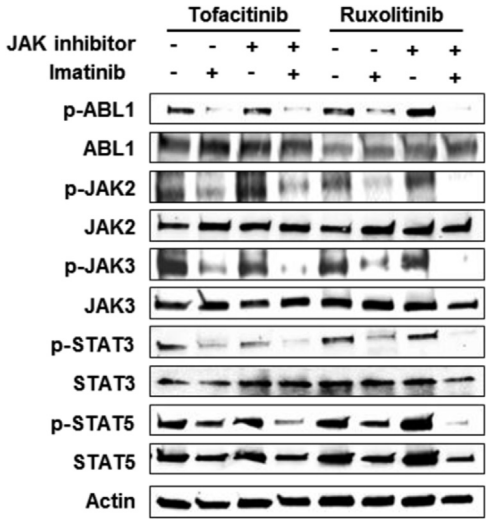

Fig. 4. JAK/STAT signaling profiles observed after treatment with JAK inhibitors with or without IMA. The changes in the levels of phosphorylated and total ABL1, JAK2, JA3, STAT3, and STAT5 seen following treatment with 100 nM TOF or 100 nM RUX for 1 h, and/or 250 nM IMA for 24 h was determined by Western blotting.

expression level of ABCG2 was decreased by treatment with IMA or either JAK inhibitor alone. Moreover, a marked reduction in the expression of ALDH1A1 was observed after treatment with a combination of IMA and JAK inhibitors; however, ALDH1A1 expression was not suppressed by treatment with any of these substances alone (Fig. 6B).

4. Discussion

Although IMA has changed the treatment of CML, the development of primary and acquired resistance is a problem, even for second-generation TKI agents (Nambu et al., 2010). Even in cases in which BCR-ABL transcripts are no longer detectable by the PCR after IMA treatment, some patients suffer relapses after the cessation of IMA therapy, which suggests that quiescent CML stem cells that are resistant to IMA might be present in these patients (Essers and Trumpp, 2010). The activation of signaling pathways that are independent from BCR-ABL, such as the JAK/STAT, RAF/MEK/ERK, and PI3K pathways, seems to be one of the causes of resistance, especially in LSCs, and therapeutic approaches to CML-LSCs eradication are still under investigation (Steelman et al., 2004; Essers and Trumpp, 2010). Currently, another therapeutic approach have also been studied by targeting autophagy using mechanism different from inhibition of the signaling pathways (Moosavi et al., 2016). Activation of the JAK/STAT pathway is associated with stem cell survival (Quintarelli et al., 2014; Nair et al., 2012a; Traer et al., 2012), and CML stem cells exhibit reduced sensitivity to TKI (Hoelbl et al., 2010; Nair et al., 2012b). During IMA treatment, RUX, a JAK2 inhibitor, effectively prevented CML recurrence in a mouse model (Nair et al., 2012a). However, little is known about the combined effects of JAK3 and BCR-ABL inhibitors. In our study, TOF promoted the antitumor effects of IMA to a similar extent to RUX; however, the use of JAK inhibitors alone did not affect the survival of CML cells (Fig. 2). The therapeutic effects of combined treatment with IMA and TOF seem to be equal to those of combined treatment with IMA and RUX.

In addition, some reports have suggested that JAK activation is dependent on BCR-ABL, whereas others did not (Carlesso et al., 1996; Colavita et al., 2013; Hoelbl et al., 2010; Nair et al., 2012b; Coppo et al., 2006). Furthermore, it was reported that JAK inhibitors were only effective against CML when BCR-ABL was inhibited by nilotinib (Coppo et al., 2006). If BCR-ABL were active in the absence of TKI, JAK inhibitors would have little effect on JAK phosphorylation (Gallipoli et al., 2014). In our study, JAK inhibitors were only effective against CML when combined with IMA (Fig. 2), and treatment with a JAK inhibitor alone did not effectively inhibit JAK phosphorylation in CML cells (Fig. 4), which is consistent with the findings of previous studies (Gallipoli et al., 2014). This might be explained by the result that BCR-ABL strongly activates downstream signals, including the JAK/STAT

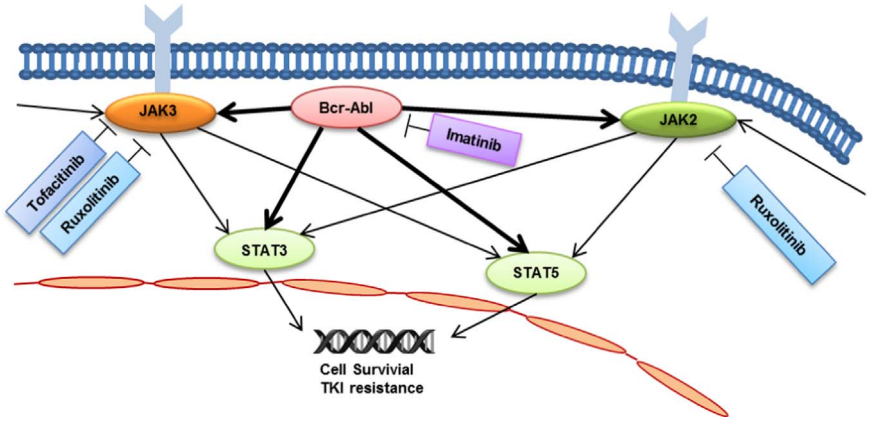

Fig. 5. Model of the links between BCR-ABL and JAK/STAT signaling in CML cells. Model of the links between BCR-ABL and JAK/STAT signaling in CML cells. Targeting both BCR-ABL and JAK3 pathways would interfere with these essential survival signals.

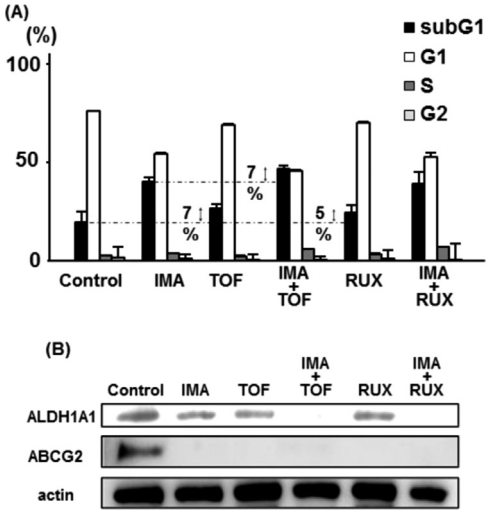

Fig. 6. Combined effects of IMA and JAK inhibitors on the cell cycle and stem cells. JAK inhibitors enhanced IMA-induced cell cycle arrest and decreased the expression of the stem cell marker ALDH1A1 in BCR-ABL-positive CML cells. Cell cycle arrest was detected via PI staining and fluorescence-activated cell sorting analysis. (A) Cell cycle arrest was assessed using PI staining and fluorescence-activated cell sorting analysis after the cells had been treated with the control substance or 100 nM TOF or 100 nM RUX, and/or 250 nM IMA for 72 h. A representative result is shown. Percentages of cells in the sub-G0/1, -G0/1, S, and G2/M phases: control: 19.4, 76.3, 2.8, and 1.5, respectively; IMA: 40.8, 54.7, 3.6, and 0.9, respectively; TOF: 27.4, 69.5, 2.3, and 0.8, respectively; IMA + TOF: 47.4, 45.8, 6.1, and 0.7, respectively; RUX: 25.3, 70.2, 3.5, and 1.0, respectively; IMA + RUX: 39.3, 52.9, 7.1, and 0.7, respectively. (B) ALDH1A1 and ABCG2 expression were assessed using Western blotting after treatment with the control substance or 100 nM TOF or 100 nM RUX for 1 h, and/or 250 nM IMA for 24 h.

pathway (Steelman et al., 2004). On the other hand, JAK activity was markedly inhibited by 100 nM of RUX in BCR-ABL-negative cells (Chase et al., 2013), and RUX was found to inhibit JAK signaling in BCR-ABL-negative cells, but not in BCR-ABL-positive cells (Changelian et al., 2003). In addition, IMA-resistant CML cells were able to survive when BCR-ABL signaling cascades were inhibited (Lee et al., 2007; Donato et al., 2003), which suggests that IMA resistance might be independently associated with BCR-ABL and partially associated with the JAK/STAT pathway, which can be activated by other kinases. Our data showed that the co-administration of either of the examined JAK inhibitors resulted in enhanced JAK/STAT inhibition compared with that induced by treatment with IMA alone. Moreover, in the presence of IMA, TOF inhibited JAK3 to an equivalent extent to RUX, but it did not inhibit JAK2 to the same degree as RUX (Fig. 4). Therefore, we propose that JAK3 might contribute to the survival of CML cells.

Strong expression of ALDH1A1 and ABCG2 is associated with IMA resistance, and these molecules are highly expressed in LSCs (Zhou et al., 2001; Brendel et al., 2007; Gerber et al., 2011; Fleischman, 2012). In this study, the expression of ALDH1A1 was decreased by treatment with a combination of IMA and a JAK inhibitor although treatment with IMA or a JAK inhibitor alone had little effect (Fig. 6). Our study showed that JAK inhibitors adversely affected LSCs when BCR-ABL was inactivated by IMA. Therefore, the inhibition of ALDH1A1 expression with IMA plus JAK inhibitors might result in the eradication of LSCs. The administration of IMA plus a JAK inhibitor, such as TOF or RUX, might represent a new treatment strategy for CML.

In conclusion, JAK3 contributes to the mechanism responsible for CML cell survival, and combined treatment with IMA and a JAK3 inhibitor, such as TOF, reduced the survival of CML cells. The present study indicated that combined treatment with TOF and IMA or RUX and IMA might represent a new therapeutic strategy for human CML.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2018.02.022.

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