CX-5461 potentiates imatinib-induced apoptosis in K562 cells by stimulating *KIF1B* expression

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Abstract. The selective RNA polymerase I inhibitor CX-5461 has been shown to be effective in treating some types of leukemic disorders. Emerging evidence suggests that combined treatments with CX-5461 and other chemotherapeutic agents may achieve enhanced effectiveness as compared with monotherapies. Currently, pharmacodynamic properties of the combination of CX-5461 with tyrosine kinase inhibitors remain to be explored. The present study tested whether CX-5461 could potentiate the effect of imatinib in the human chronic myeloid leukemia cell line K562, which is p53-deficient. It was demonstrated that CX-5461 at 100 nM, which was non-cytotoxic in K562 cells, potentiated the pro-apoptotic effect of imatinib. Mechanistically, the present study identified that the upregulated expression of kinesin family member 1B (*KIF1B*) gene might be involved in mediating the pro-apoptotic effect of imatinib/CX-5461 combination. Under the present experimental settings, however, neither CX-5461 nor imatinib alone exhibited a significant effect on *KIF1B* expression. Moreover, using other leukemic cell lines, it was demonstrated that regulation of *KIF1B* expression by imatinib/CX-5461 was not a ubiquitous phenomenon in leukemic cells and should be studied in a cell type-specific manner. In conclusion, the results suggested that the synergistic interaction between CX-5461 and imatinib may be of potential clinical value for the treatment of tyrosine kinase inhibitor-resistant chronic myeloid leukemia.

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Abbreviations: CML, chronic myeloid leukemia; Ph, Philadelphia chromosome; PI3K, phosphatidylinositol 3-kinase; TKI, tyrosine kinase inhibitor; NSR, nucleolar stress response; Pol I, RNA polymerase I; KIF1B, kinesin family member 1B

Key words: RNA polymerase I inhibitor, CX-5461, kinesin family member 1B, imatinib, synergy, kinesin family member 1B, apoptosis

Introduction

Chronic myeloid leukemia (CML) and Philadelphia Chromosome (Ph)-positive acute lymphoblastic leukemia are caused by expression of the oncogenic fusion protein Bcr-Abl, which is a constitutively active tyrosine kinase (1). Malignant transformation of affected cells by Bcr-Abl is mediated by a number of signaling mechanisms, including pathways of PI3K/Akt, mitogen-activated protein kinases, RhoA-Rac, and JAK/STAT, leading to dysregulation of cell survival, proliferation, differentiation and metabolism (2,3). Tyrosine kinase inhibitors (TKIs) such as imatinib and dasatinib, which target the binding of Bcr-Abl with ATP, have been successfully used in the treatment of CML. However, a major challenge associated with the use of these drugs in the clinic is the development of resistance (4). It has been shown that primary or acquired resistance occurs in >20% of CML patients undergoing imatinib treatment (5).

TKI resistance can be mediated by Bcr-Abl-dependent and -independent mechanisms. Bcr-Abl-dependent resistance is primarily caused by mutations (e.g. the well characterized T315I mutation) in the kinase domain of Abl (4). By comparison, the mechanisms of Bcr-Abl-independent resistance remain to be elucidated. It has been recognized that compensatory activation of the Akt/mTOR pathway and/or inactivation of the p53 gene are involved in this process (6,7). In fact, Bcr-Abl-independent resistance has particular clinical significance, because in the presence of effective kinase inhibition, Bcr-Abl-independent resistance may be a major contributor to the maintenance of minimal residual disease and relapse of CML (4). Supporting this notion, evidence shows that reactivation of the p53 pathway, either directly or indirectly, may boost the eradication of CML leukemia stem cells, which are thought to be TKI resistant (8-10). Clinical studies have revealed that progression of CML into blastic crisis is often associated with mutations or loss of the p53 gene (11-13). It is estimated that ≤30% of CML cases are associated with inactivation of the p53 gene (14). Therefore, an intriguing question is whether there is a means to enhance the therapeutic efficacy of TKIs in the absence of functional p53.

Cancer cells are generally associated with a phenotype of oncogene and/or non-oncogene addiction, which results in a status of enhanced levels of various cellular stresses (15). Emerging evidence suggests that exploiting these stress pathways by stress overload might be used to enhance the efficacy of current anti-cancer therapies (16,17). In eukaryotic cells, the nucleolus may act as a signaling hub involved in mediating cellular stress responses (18,19), while inhibiting ribosomal (r)DNA transcription or inhibiting rRNA processing induces a unique cellular stress response termed nucleolar stress response (NSR) (18,19). NSR can be induced by RNA polymerase I (Pol I) inhibitors. Of the available small molecule Pol I inhibitors, CX-5461 is the first-in-class selective Pol I inhibitor with an IC_{50} value at the 100 nM range (20). Currently, CX-5461 has entered into clinical studies to treat blood malignancies (21).

The conventional NSR pathway involves stabilization and accumulation of the tumor suppressor p53, through disruption of the association between p53 and the E3 ubiquitin ligase Mdm2, leading to decreased p53 ubiquitination and degradation (18,19). Indeed, the cancer killing activity of CX-5461 appears to be largely attributable to this mechanism (22). Nonetheless, there is evidence suggesting that induction of NSR may also lead to p53-independent consequences (19). Currently, the pharmacodynamic properties of combined treatment with CX-5461 and TKI in CML cells remain to be explored. The present study, therefore, tested whether non-cytotoxic concentrations of CX-5461 can potentiate the efficacy of imatinib to induce apoptosis. Especially, in order to clarify whether CX-5461 can enhance the activity of imatinib in a setting of p53 loss-of-function, study was performed in the human CML cell line K562, which is p53-deficient (23-26).

Materials and methods

Reagents. CX-5461 and BMH-21 were obtained from Selleck Chemicals. Imatinib mesylate was from Cayman Chemical Company. The compounds were initially dissolved in DMSO and a series of 200X stock solutions were prepared according to proposed final concentrations. Drug treatment was performed by adding 5 μl of the stock solution in each ml of culture medium. DMSO was dissolved 1:200 in the culture medium as vehicle control. Primary antibodies used were: Anti-Kif1b (cat. no. A6638; ABclonal Biotech Co., Ltd.) and anti-Rbfox2 (cat. no. A05389-1; Wuhan Boster Biological Technology, Ltd.). The dilution ratios of anti-Kif1b and anti-Rbfox2 used in western blotting experiments were 1:500 and 1:1,000 respectively. The secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. SA00001-2; Wuhan Sanying Biotechnology; 1:5,000).

Cell cultures. K562 ($p53^-/Ph^+$) and NALM-6 ($p53^+/Ph^-$) cells were originally obtained from American Type Culture Collection. The THP-1 cell line ($p53^-/Ph^-$) was obtained from National Collection of Authenticated Cell Cultures. All of the cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (all from Thermo Fisher Scientific, Inc.), in a humidified environment with 5% CO_2 at 37˚C. Untreated cells were maintained at a density of below $1x10^6$ cells/ml. Medium renewal was performed every 2 to 3 days.

Cell proliferation and viability assay. The number of viable cells was assessed using a colorimetric Enhanced Cell Counting Kit-8 (cat. no. C0042; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, 100 μl aliquots of cell suspension were transferred to a 96-well plate, and 10 μl of the CCK-8 solution was added to each well. After mixing, the cells were placed back to the cell incubator and further incubated for 2 h at 37˚C. Cell number was assessed by measuring absorbance at 450 nm using a microplate reader (EMax Plus; Molecular Devices, LLC).

Flow cytometry. Cell apoptosis was assessed using a FITC-Annexin V Apoptosis Detection kit (BD Biosciences). Cells were washed with cold PBS and resuspended in 1X Binding Buffer at a concentration of $1x10^6$ cells/ml. Then 100 μl aliquot of the cell suspension was transferred to a 5-ml test tube, and working solutions (5 μl each) of Annexin V and propidium iodide (PI), prepared according to the manufacturer's protocols, were added. After 15 min of incubation at room temperature in the dark, 400 μl of the Binding Buffer was added to each tube. Flow cytometry analysis was performed using a BD Accuri C6 Plus machine (BD Biosciences). The following controls were used to set up the quadrants: Unstained cells, cells stained with Annexin only, and cells stained only with PI. Cells in both early stage ($Annexin^+/PI^-$) and late stage ($Annexin^+/PI^+$) apoptosis were equally counted as apoptotic cells. Data were analyzed with WinMDI (version 2.9; The Scripps Institute; http://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm) and FlowJo (version 10; FlowJo LLC) software.

Transcriptome sequencing. Cells were divided into three treatment groups (with 3 independent biological replications in each group): control, imatinib alone, and imatinib + CX-5461. Total RNA was extracted using TRIzol® (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. RNA integrity was confirmed with an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). Qualified total RNA was further purified by RNAClean XP Kit (Beckman Coulter, Inc.) and RNase-Free DNase Set (Qiagen GmbH). RNA concentration was determined using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Library construction was performed using VAHTS Stranded mRNA-seq Library Prep Kit for Illumina (Vazyme Biotech Co., Ltd.). RNA sequencing was performed using a NextSeq Illumina550 platform (Illumina, Inc.) in a paired-end manner. RNA processing, library construction and sequencing services were provided by Shanghai Biotechnology Corporation. The complete dataset of the raw count values and fragments per kilo base per million mapped reads (FPKM) values are provided in Table SI. Differentially expressed genes were defined by the false discovery rate (q value) <0.05 and fold change >2. The reproducibility of the sequencing assay was confirmed by the degree of correlation between biological duplicate samples (see ENCODE Guidelines and Best Practices for RNA-Seq; https://www.encodeproject.org/about/experiment-guidelines) (27).

Reverse transcription-quantitative (RT-q) PCR. Around $5x10^5$ cells were homogenized in TRIzol® (Thermo Fisher Scientific, Inc.) and total RNA was isolated according to

the manufacturer's instructions. The RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesized from 50 ng of total RNA using the PrimeScript RT-PCR Kit (cat. no. RR037A; Takara Bio, Inc.) according to the manufacturer's instructions. Reverse transcription was carried out using 100 μM of random 6mers, under a thermocycler condition of 37˚C for 15 min followed by 85˚C for 5 sec. The real-time PCR reaction was carried out using the UltraSYBR Mixture kit (cat. no. CW0957M; CWBio) according to the manufacturer's instructions in a LightCycler 96 Instrument (Roche Diagnostics). The primer sequences used in the study are listed in Table SII. The reaction was carried out using 5 ng of cDNA in a 50-μl reaction volume, containing 0.2 μM of primers. The following thermocycler settings were applied: 95˚C for 10 min; 40 cycles of 95˚C for 16 sec and 60˚C for 1 min. Human GAPDH was used as the housekeeping gene. Fold changes were determined using the $2^{-\Delta\Delta Cq}$ method, where ΔCq=Cq target gene-Cq housekeeping gene; ΔΔCq=ΔCq test sample-ΔCq calibrator (control) sample (see 'Real-time PCR handbook' at https://www.thermofisher.com/content/dam/LifeTech/global/Forms/PDF/real-time-pcr-handbook.pdf) (28).

Small interfering (si)RNA transfection. siRNA constructs targeting *RBFOX2* (sense sequence CCGGAGUUAUAU GCAGCAUTT; anti-sense AUGCUGCAUAUAACUCCG GTT) and *KIF1B-β* (sense GCCAUCCUCUCCCUAAAU ATT; anti-sense UAUUUAGGGAGAGGAUGGCTT) were obtained from Shanghai GenePharma Co., Ltd. The *RBFOX2* siRNA was designed using *RBFOX2* transcript variant 1 mRNA (Reference Sequence accession: NM_001031695.4) as the template, targeting the 54-nucleotide exon which was conserved in transcript variants 1-18. The *KIF1B-β* siRNA was designed using *KIF1B* transcript variant 1 mRNA (Reference Sequence accession: NM_015074.3) as the template, targeting the nucleotides 4108 to 4126. A universal non-targeting siRNA was used as control (sense UUCUCCGAACGUGUC ACGUTT; anti-sense ACGUGACACGUUCGGAGAATT). K562 cells were re-suspended in antibiotic-free Opti-MEM I Reduced Serum medium (cat. no. 31985-062 from Thermo Fisher Scientific, Inc.) at $1x10^6$ cells per ml, and transfected using Lipofectamine® RNAiMAX (cat. no. 13778-075; Thermo Fisher Scientific, Inc.) with a final siRNA concentration of 200 nM at 37˚C. Fresh medium was replenished after 6 h of incubation with siRNA, and the cells were used for experimentation at 48 h after transfection.

Western blotting. Total proteins were extracted using RIPA lysis buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) containing Protein Phosphatase Inhibitors cocktail (cat. no. P1260; Beijing Solarbio Science & Technology Co., Ltd.). The protein concentration was determined using a BCA protein assay kit (cat. no. P0010; Beyotime Institute of Biotechnology). Samples were boiled in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.02% bromophenol blue). Protein samples (25 μg per lane) were loaded onto precast 4-20% gradient Bis-Tris PAGE gel (Dalian Meilun Biotechnology Co., Ltd.), separated by electrophoresis, and transferred to Immobilon-NC nitrocellulose membrane (Merck KGaA). The membrane was blocked with 5% non-fat milk for 2 h at room temperature, and then incubated with primary antibodies overnight at 4˚C. After washing, the membrane was probed with horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1-2 h at room temperature. The bands were developed by enhanced chemiluminescence using SuperKine West Femto Maximum Sensitivity Substrate (cat. no. BMU102-CN; Abbkine Scientific Co., Ltd.), and visualized with a Tanon 3500 Gel Imaging System (Tanon Science and Technology Co., Ltd.). Band densitometry analysis was performed using ImageJ software (version 1.46r) (National Institutes of Health).

Caspase 3 activity assay. Caspase 3 activity was measured with a colorimetric assay kit (cat. no. C1115; Beyotime Institute of Biotechnology), which detected the cleavage of substrate (Ac-DEVD-p-nitroanilide) to the yellow-colored product p-nitroaniline, according to the manufacturer's protocol. Briefly, $4x10^5$ cells were collected by centrifugation (600 x g for 5 min), washed with PBS, and incubated in 200 μl of the lysis buffer supplied on ice for 15 min with vortexing. The sample was then centrifuged at 16,000 x g 4˚C for 15 min. The supernatant was transferred to a test tube, mixed with 0.2 mM substrate, and incubated at 37˚C overnight. The absorbance at 405 nm was measured using a SUNRISE microplate reader (Tecan Group, Ltd.).

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Data analysis was performed with unpaired t-test or one-way analysis of variance followed by post hoc Tukey's test as appropriate. All of the *n* values represented the number of independent biological replicates. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CX-5461 potentiates imatinib-induced cytotoxicity and apoptosis. In order to determine the potential synergy between imatinib and CX-5461, the combination subthresholding approach was adopted, an effect-based method which detects additivity by showing that combination of non-effective doses of two drugs yields a significant effect (29). Following this principle, the synergy is determined by showing that neither [drug A] nor [drug B] has a significant effect individually, while the effect of [drug A]/[drug B] combination is significant. According to Picard *et al* (30), the effective plasma concentration of imatinib in humans is between 300-3,000 nM. Based on this information, 100 nM was selected as the reference concentration for imatinib in the following experiments. The rationale for using 100 nM of imatinib was that this concentration represented a non-effective one for single treatment, thereby allowing the present study to test drug synergy with the subthresholding approach. First, it was confirmed that at a concentration of 100 nM, neither imatinib nor CX-5461 individually had any significant effect on the viability of K562 cells after 48 h of treatment (Fig. 1). In comparison, under the same experimental conditions, combined treatment with CX-5461 and imatinib, both at 100 nM (for 48 h), significantly decreased the cell viability (Fig. 2A). To precisely define the nature of the synergistic effect of CX-5461 on imatinib-induced

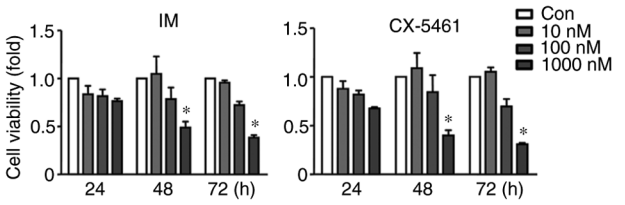

Figure 1. Concentration-dependent effects of IM and CX on the viability of K562 cells measured at different time points. Data were expressed as mean ± standard error of the mean. *P<0.05 vs. Con, one-way analysis of variance, *n*=3. IM, imatinib; CX, CX-5461; Con, control.

cell toxicity, concentration-response analysis was performed in the absence or presence of 100 nM CX-5461. As shown in Fig. 2B, CX-5461 increased the Emax (maximum effect) of imatinib (83.4±3.1 vs. 70.8±1.9%, P=0.026, unpaired t-test, *n*=3), but had no significant effect on the EC_{50} value (concentration producing 50% of the maximum effect; 0.23±0.02 vs. 0.20±0.009 mM; P=0.215). Furthermore, treatment with imatinib/CX-5461 combination (for 48 h) increased the number of apoptotic cells as compared with CX-5461 or imatinib mono-treatment (Fig. 2C).

BMH-21 does not mimic the actions of CX-5461. To clarify whether the synergistic action of CX-5461 with imatinib was due to inhibition of rDNA transcription, the cells were treated with another unrelated Pol I inhibitor BMH-21. It was found that treatment with BMH-21 at 1 μM for 48 h moderately reduced the cell viability (Fig. S1A). At this concentration, however, a synergistic effect of BMH-21 on imatinib-induced apoptosis was not observed (Fig. S1B). These data indicated that the synergistic effect of CX-5461 with imatinib was unlikely to be a direct result of the reduced rDNA transcription.

Imatinib/CX-5461 synergy is not related to changed expressions of various apoptosis regulators. To clarify whether the synergistic effect of CX-5461 on imatinib-induced apoptosis was related to changed expressions of essential apoptosis-regulating factors, the mRNA levels of *FAS*, *BAX*, *BIM*, *BCL-2* and *BCL-XL* genes were examined. As shown in Fig. 3, CX-5461 exhibited no significant effects on the expression of these genes either in the absence or presence of imatinib co-treatment.

Transcriptome sequencing analysis of the effects of imatinib/CX-5461 in K562 cells. To further explore the possible mechanism underlying the synergy between CX-5461 and imatinib, genome-wide RNA sequencing experiments were performed in cells which were untreated, treated with imatinib alone, and treated with imatinib/CX-5461 combination respectively. Technically, the reproducibility of the assay was confirmed by the high degree of correlation (coefficient >0.9) between biological duplicate samples (27). The specific correlation coefficient values between sample pairs are shown in Fig. 4A. Consistent with the sub-efficacious concentration of imatinib used in this experiment, it was shown that only a small number of gene expressions were altered by imatinib treatment alone (Fig. 4B). Specifically, nine genes were upregulated and 16 genes were downregulated significantly by imatinib. Similarly, it was demonstrated that in the presence of imatinib, only a minor group of genes were responsive to CX-5461 co-treatment (Fig. 4B and Table I). Specifically, nine genes were upregulated and five genes were downregulated significantly. As the transcription factor p53 has a central role in mediating the cellular effects of Pol I inhibition-induced NSR (18,19), in K562 cells (without functional p53) the CX-5461-responsive genes did not contain typical p53 targets (see Table I). Moreover, it was shown that under the present experimental settings, CX-5461-responsive genes in K562 cells were totally distinct from those responsive to imatinib (Fig. 4C). Among the differentially expressed genes responsive to CX-5461, special attention was paid to the *RBFOX2* gene (downregulated), which encodes a RNA splicing factor (31-33). Rbfox2 mediates the splicing of *KIF1B* mRNA, while reduced Rbfox2 expression has been shown to facilitate the preferential expression of the *KIF1B-β* isoform (over *KIF1B-α*), which has a pro-apoptotic role in solid tumor cells (34-40).

Imatinib/CX-5461 combination modulates KIF1B expression in K562 cells. The present study examined whether changed expression of *KIF1B* was involved in the pro-apoptotic effect of imatinib/CX-5461 combination. Using qPCR, it was found that CX-5461 treatment significantly upregulated the expression levels of both *KIF1B-α* and *KIF1B-β* in the presence of imatinib (Fig. 5A); however, CX-5461 alone had no significant effect on *KIF1B* expression in the absence of imatinib (Fig. S2). Similarly, imatinib alone did not significantly affect the expression of *KIF1B* (Fig. S2). To further confirm the finding that imatinib/CX-5461 could upregulate the expression of *KIF1B*, two additional PCR primers were designed targeting the *N*-terminal part of Kif1b, which was 100% identical for Kif1b-α and Kif1b-β. As shown in Fig. 5B, the mRNA level of total *KIF1B* was significantly increased in imatinib/CX-5461-treated cells. Moreover, it was confirmed that imatinib/CX-5461 significantly increased the protein level of total Kif1b as compared with the imatinib mono-treatment (Fig. 5B). Remarkably, the present study did not detect any significant change in the *KIF1B-α* to *KIF1B-β* ratio following imatinib/CX-5461 treatment (Fig. 5A), which seemed to be in contrast to the reported effect of Rbfox2 (38). To clarify this question, gene silencing for *RBFOX2* was performed (Fig. 5C), and it was demonstrated that knocking down the *RBFOX2* expression in K562 cells did not change the expressions of *KIF1B-α* or *KIF1B-β*, nor did it affect the ratio between the two isoforms (Fig. 5D). These results suggest that there is no association between Rbfox2 and *KIF1B* mRNA splicing in K562 cells under the present experimental settings.

KIF1B-β has a pro-apoptotic role in K562 cells. As *KIF1B-α* was not implicated in modulating cell apoptosis (34,40), it was then tested whether *KIF1B-β* was involved in mediating the pro-apoptotic action of imatinib/CX-5461 combination in K562 cells. For this purpose, K562 cells were transfected with *KIF1B-β* siRNA, of which the gene silencing effect was confirmed by western blotting (Fig. 6A). Flow cytometry assays, demonstrated that in cells transfected with the control siRNA, CX-5461 in the presence of imatinib increased the apoptotic response; by contrast, in cells transfected with *KIF1B-β* siRNA, the pro-apoptotic effect of CX-5461 was blunted (Fig. 6B). To further corroborate the above flow

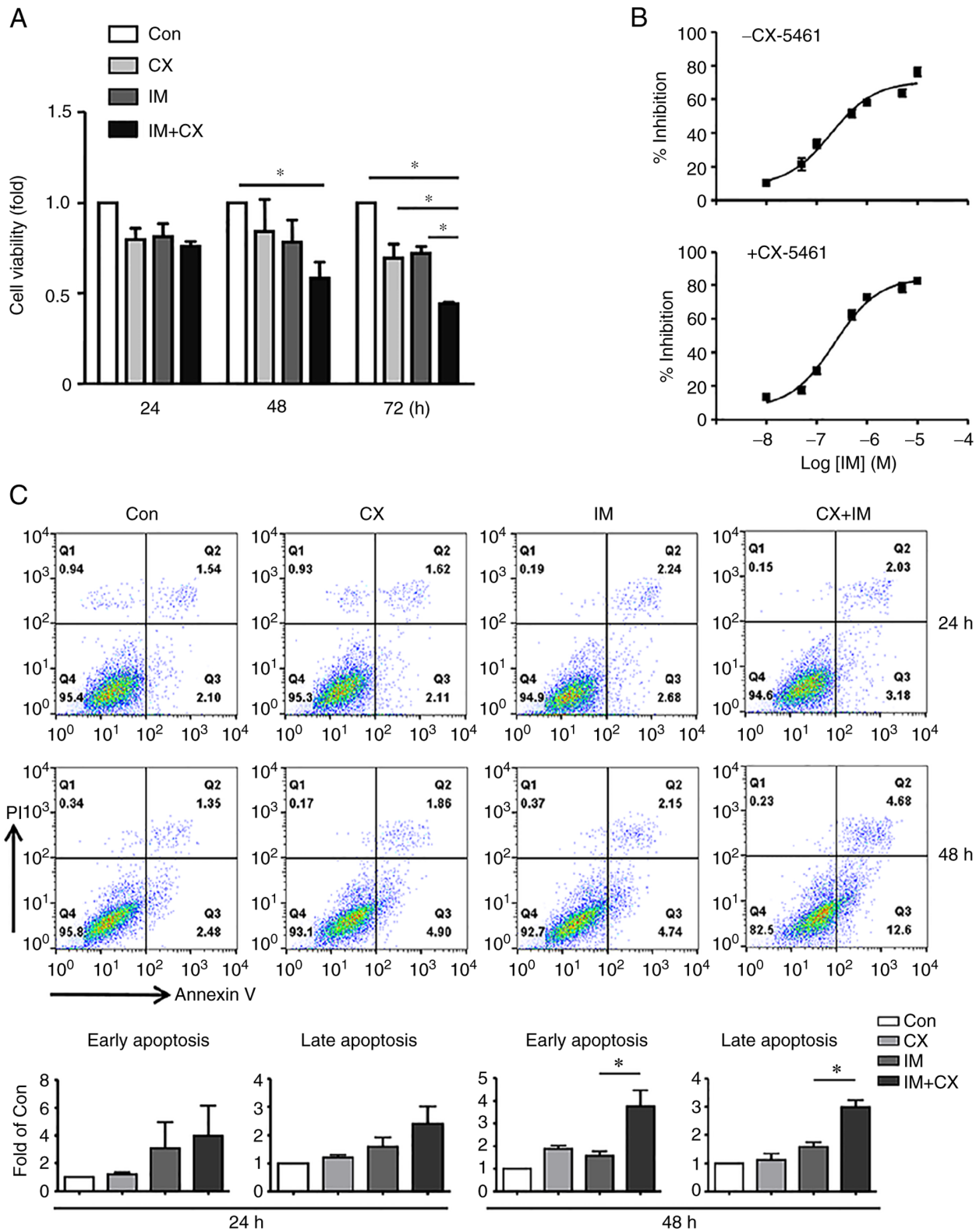

Figure 2. Synergistic effects of CX on IM-induced cytotoxicity and apoptosis in K562 cells. (A) Effects of CX (100 nM), IM (100 nM), and their combination on cell viability. (B) Concentration-response curves of imatinib-induced inhibition of cell viability in the absence or presence of CX (100 nM, treatment for 48 h). (C) Representative flow cytometry results and quantitative data showing the effects of CX (100 nM), IM (100 nM) and their combination on cell apoptosis. Early-stage apoptosis and late-stage apoptosis were shown separately. Data were expressed as mean ± standard error of the mean. *P<0.05, one-way analysis of variance, *n*=3 (for panel A), 3 (for panel B), 4 (for the 24 h experiments in panel C), and 3 (for the 48 h experiments in panel C). CX, CX-5461; IM, imatinib; Con, control.

cytometry results, caspase 3 activity assay was performed in K562 cells. In cells transfected with the control siRNA, imatinib/CX-5461 increased the level of caspase 3 activation as compared with imatinib mono-treatment, whereas in cells transfected with *KIF1B-β* siRNA, the effect of CX-5461 was diminished (Fig. 6C).

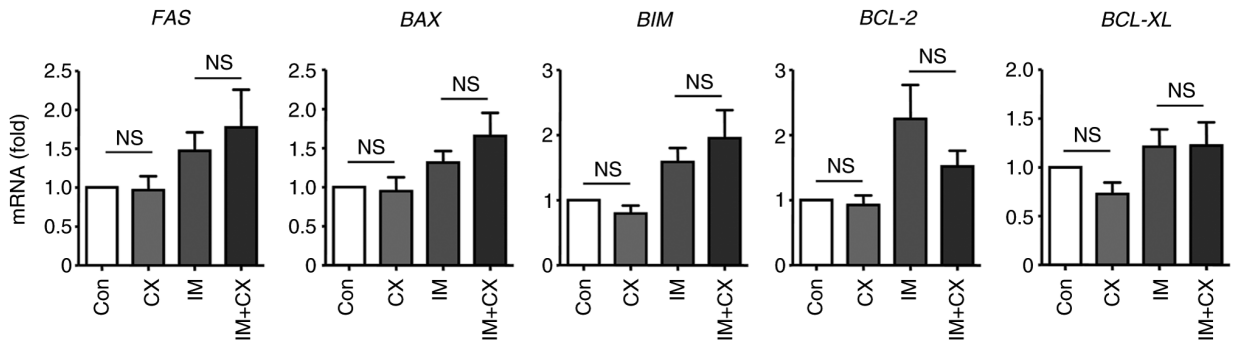

Figure 3. Reverse transcription-quantitative PCR results showing that CX (100 nM for 48 h), in the absence or presence of IM (100 nM), had no effects on the expression of various apoptosis-related genes in K562 cells. Data were expressed as mean ± standard error of the mean, *n*=10 for *FAS*, 8 for *BAX*, 9 for *BIM*, 10 for *BCL-2* and 10 for *BCL-XL*. NS, non-significant; CX, CX-5461; IM, imatinib; Con, control.

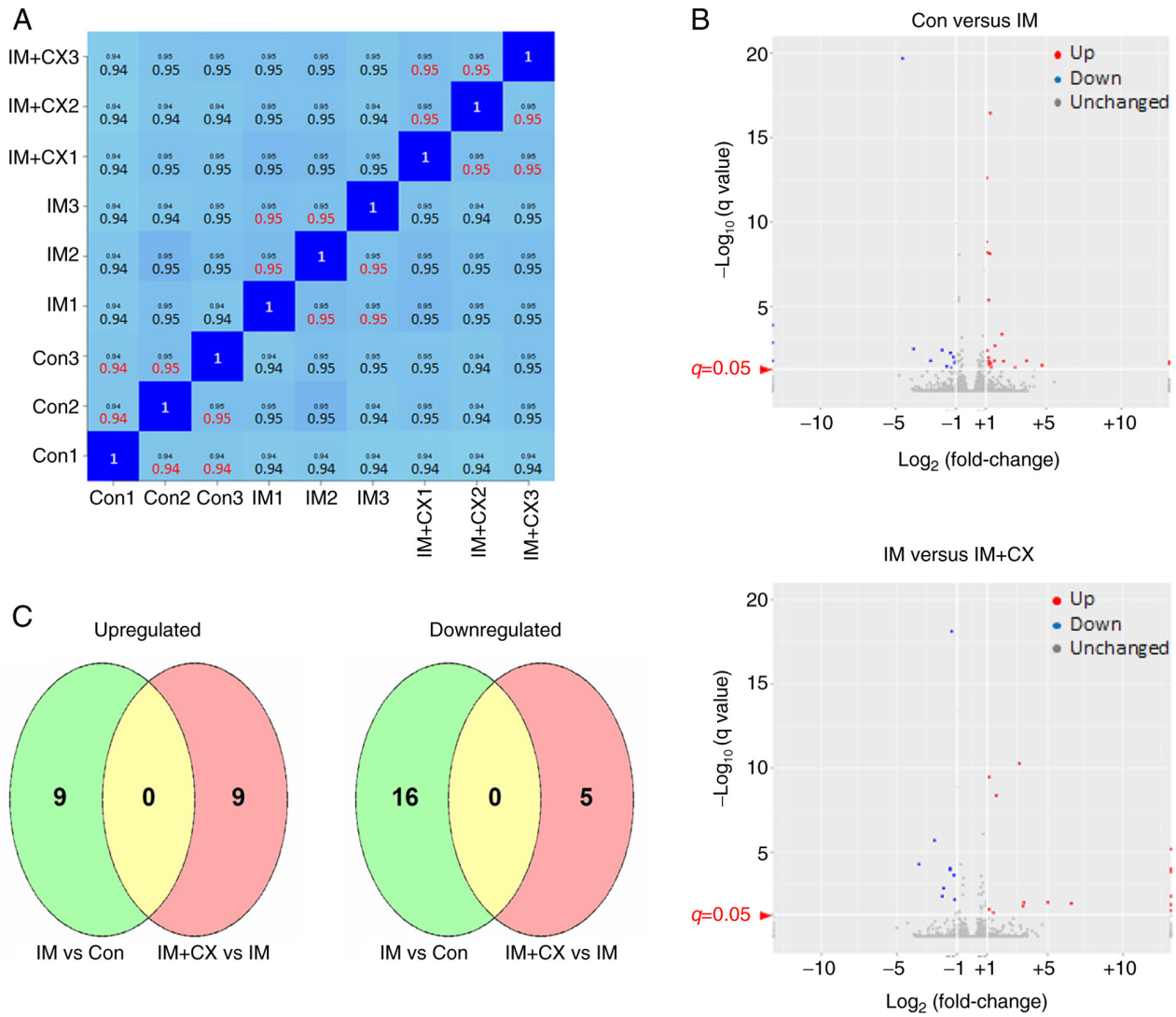

Figure 4. RNA sequencing analysis of the effects of IM/CX in K562 cells. Cells were divided into three treatment groups with three independent biological replications: Con, IM and IM + CX (I + CX). (A) High degree of gene expression correlations between biological duplicate sample pairs (shown in red). The numbers shown were specific correlation coefficient values. (B) Volcano plots showing the profiles of differentially expressed genes regulated by imatinib/CX-5461. (C) Venn diagrams showing the numbers of genes (with definitive functional annotations) upregulated and downregulated in response to imatinib/CX-5461. IM, imatinib; CX, CX-5461; Con, control.

Regulation of KIF1B-β expression by imatinib/CX-5461 in other cell lines. The effects of imatinib/CX-5461 on *KIF1B* expression in NALM-6 (human acute lymphoblastic leukemia cell line; $p53^+/Ph^-$) and THP-1 (human monocytic leukemia cell line; $p53^-/Ph^-$) cells was also tested. In NALM-6 cells, it was shown that only imatinib/CX-5461 combination significantly

Table I. Genes regulated by CX-5461 in the presence of IM.

Ensembl ID	Gene name	Fold change	P-value	*q*-value	Change in regulation vs. IM alone
ENSG00000180354	*MTURN*	0.21	$8.35x10^{-29}$	$2.11x10^{-24}$	Down
ENSG00000166532	*RIMKLB*	2.56	$9.02x10^{-23}$	$7.62x10^{-19}$	Up
ENSG00000167842	*MIS12*	0.46	$8.11x10^{-14}$	$3.42x10^{-10}$	Down
ENSG00000100320	*RBFOX2*	0.33	$1.35x10^{-12}$	$4.28x10^{-9}$	Down
ENSG00000198932	*GPRASP1*	5.65	$7.84x10^{-10}$	$1.99x10^{-6}$	Up
ENSG00000102780	*DGKH*	2.76	$4.90x10^{-8}$	$8.87x10^{-5}$	Up
ENSG00000131779	*PEX11B*	2.76	$7.00x10^{-8}$	$1.11x10^{-4}$	Up
ENSG00000125841	*NRSN2*	2.30	$1.64x10^{-7}$	$2.31x10^{-4}$	Up
ENSG00000116731	*PRDM2*	3.71	$1.29x10^{-6}$	$1.37x10^{-3}$	Up
ENSG00000112242	*E2F3*	2.23	$7.98x10^{-6}$	$6.52x10^{-3}$	Up
ENSG00000158985	*CDC42SE2*	0.46	$4.00x10^{-5}$	$2.47x10^{-2}$	Down
ENSG00000138942	*RNF185*	0.37	$6.93x10^{-5}$	$3.99x10^{-2}$	Down
ENSG00000102524	*TNFSF13B*	2.05	$8.39x10^{-5}$	$4.52x10^{-2}$	Up
ENSG00000171574	*ZNF584*	2.01	$8.39x10^{-5}$	$4.52x10^{-2}$	UP

Statistical results were comparisons between the CX plus IM group vs. the IM alone group (n=3 biological replications). Only genes with defined functional annotations were included. Statistical significance was determined as $q<0.05$. CX, CX-5461; IM, imatinib.

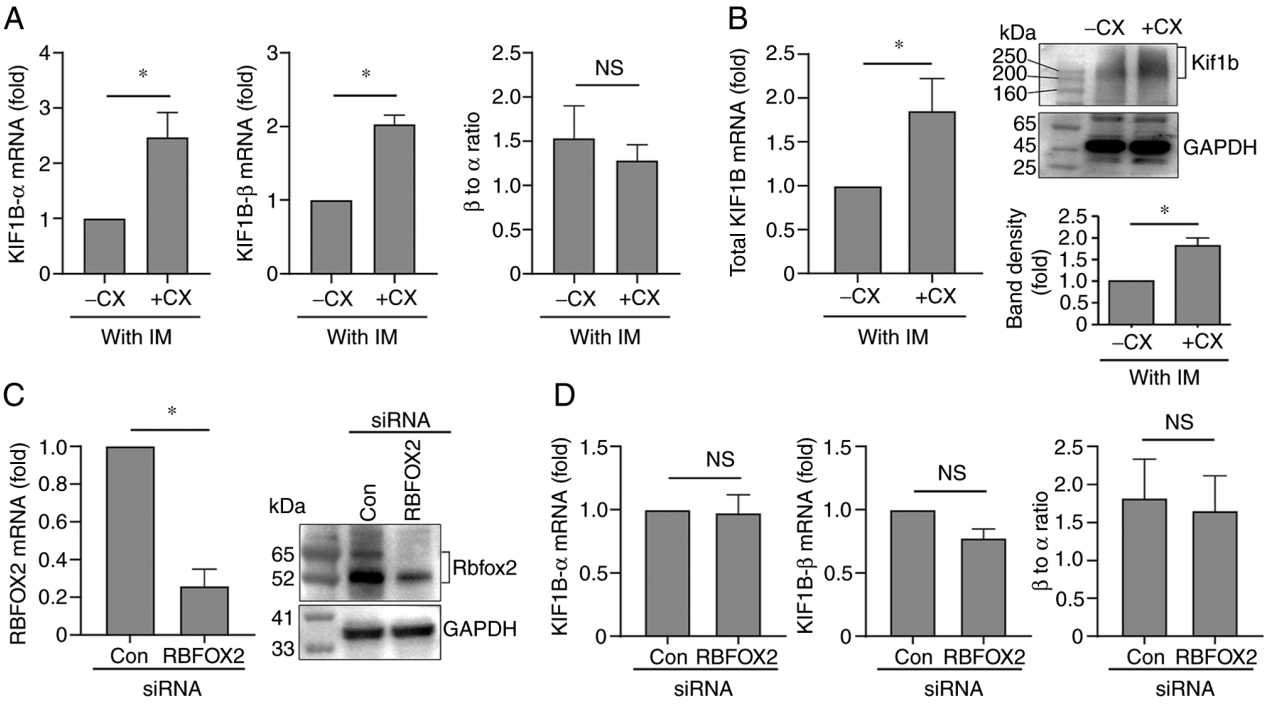

Figure 5. Regulation of the expression of *KIF1B* isoforms in K562 cells. (A) Reverse transcription-quantitative PCR results showing the effects of CX (100 nM for 48 h in the presence of 100 nM IM) on the levels of *KIF1B-α*, *KIF1B-β*, and their relative ratio. Con. groups of untreated cells and cells treated with CX or IM separately were not included in this experiment. Instead, for results of the control experiments with untreated, CX-5461 alone-treated, and imatinib alone-treated cells, see Fig. S2. (B) Effects of IM/CX combination on the mRNA (left panel) and protein (right panel) levels of total *KIF1B* as compared with the IM mono-treatment. (C) Reverse transcription-quantitative PCR (left panel) and western blotting (right panel) results showing the gene silencing efficiency of the *RBFOX2* siRNA. (D) Effects of *RBFOX2* gene silencing on the levels of *KIF1B-α*, *KIF1B-β*, and their relative ratio. Data were expressed as mean ± standard error of the mean. *P<0.05, unpaired t-test, *n*=8 (A), 7 (left panel of B), 3 (right panel of B), 5 (left panel of C), 2 (right panel of C), 5 (left panel of D) and 4 (middle and right panels of D). NS, non-significant; CX, CX-5461; CX, CX-5461; Con, control, si, small interfering.

increased the expression of *KIF1B-α* and *KIF1B-β* (Fig. S3), whereas the effects of imatinib or CX-5461 alone did not reach a statistical significance, although the moderate increases induced by CX-5461 were significant using unpaired t-test. In THP-1 cells, however, imatinib and CX-5461, either alone or in combination, had no significant effects on the expression

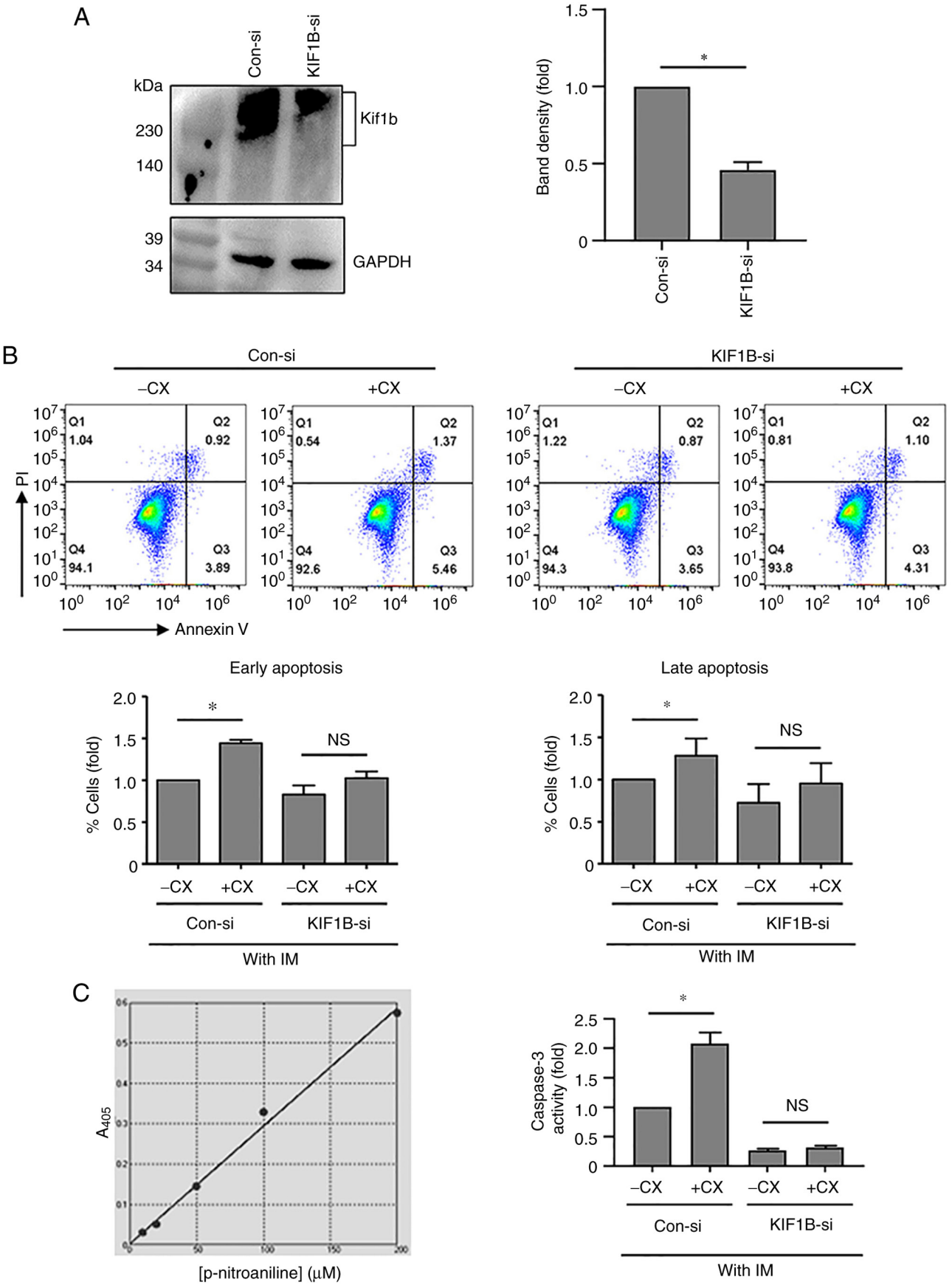

Figure 6. Role of *KIF1B-β* in mediating the pro-apoptotic action of IM/CX in K562 cells. (A) Representative western blot images and the quantitative densitometry data confirming the gene silencing effect of *KIF1B-β* siRNA (KIF1B-si) in untreated cells. (B) Flow cytometry data showing that *KIF1B-β* gene silencing blunted the synergistic pro-apoptotic effect of IM/CX combination (all 100 nM for 24 h) in K562 cells. Early-stage apoptosis and late-stage apoptosis are shown. (C) Measurements of caspase 3 activity in K562 cells showing that *KIF1B-β* gene silencing blunted the pro-apoptotic effect of IM/CX combination. The assay standard curve is shown on the left (r^2=0.993). Data are mean ± standard error of the mean. *P<0.05, unpaired t-test for (A) and one-way analysis of variance for (B and C), *n*=4 for all experiments. IM, imatinib; CX, CX-5461; si, small interfering; Con, control; Con-si, non-targeting control siRNA.

of *KIF1B* isoforms (Fig. S3). The partial effect of imatinib in NALM-6 cells (lacking Bcr-Abl) may be due to the off-target activities of the drug (41).

Discussion

Selective targeting of Pol I-dependent rDNA transcription is a conceptually novel strategy to treat cancers. Being the first selective Pol I inhibitor, CX-5461 shows therapeutic effects on a spectrum of solid and blood cancers in both pre-clinical and clinical investigations (20,21,42). At present, the precise molecular mechanisms underlying the pharmacological effects of CX-5461 are still under debate (20,43-46). Indeed, most of the evidence suggests that the CX-5461 effects are mainly attributable to the induction of a non-canonical DNA damage response, rather than direct impairment of the ribosome biogenesis (42). The effects of CX-5461 may be either p53-dependent or p53-independent, depending on the specific cellular context (42). The major finding from the present study is that CX-5461 at a non-cytotoxic concentration can potentiate the pro-apoptotic effect of imatinib in K562 cells in the absence of functional p53, via an unrecognized pathway, i.e., upregulation of the *KIF1B-β* expression. This pharmacological property of CX-5461 had not been reported previously, to the best of the authors' knowledge. Bcr-Abl-targeting TKIs including imatinib have significantly improved the prognosis of CML patients; however, the occurrence of adverse events remains to be a critical issue in the clinic for long-term TKI treatments (47). Moreover, p53 loss-of-function may compromise the anti-leukemic efficacy of imatinib (7) and may contribute to the evolution from chronic phase CML to blast crisis (48). Therefore, the synergistic interaction between CX-5461 and imatinib may be of potential value in the clinical management of refractory CML.

Following the initial success of CX-5461 single therapy in cancer treatment, emerging evidence from several studies has indicated that combined treatment with CX-5461 and other chemotherapeutic agents may achieve enhanced effectiveness as compared with single therapies. For example, CX-5461 has been shown to have synergistic effects in conjunction with the DNA topoisomerase 1 inhibitor topotecan (49), the mammalian target of rapamycin complex (mTORC1/2) inhibitor INK128 (50), the mTORC1 inhibitor everolimus (51), the poly (ADP-ribose) polymerase inhibitor talazoparib (52) and the pan-PIM kinase inhibitor CX-6258 (53), in different cancer cells/models. In addition, it has been demonstrated that CX-5461, at a concentration not sufficient to inhibit rDNA transcription, sensitizes tumor cells to irradiation (54). In this regard, the results of the present study have provided new information about a synergistic effect of combining CX-5461 with imatinib. Given the favorable safety profile of CX-5461 as revealed by a Phase I clinical trial (55), it is probable that imatinib/CX-5461 combination may represent a useful strategy to alleviate the adverse effects caused by TKIs through dose reduction (47).

Current evidence suggests that there is not a unified mechanism to explain the synergy between CX-5461 and other inhibitors, depending on the molecular target involved and the status of p53 gene (49-53). The RNA sequencing test in K562 cells identified only a small number of differentially expressed genes; these genes included no typical p53 targets. These data are consistent with the notion that p53 is the predominant transcription factor mediating the cellular effects of Pol I inhibition-induced NSR (18,19). The weak genomic response to CX-5461 treatment also explains the strong correlation between inter-group samples (Fig. 4A). It was noted that CX-5461 downregulated the expression of *RBFOX2*, which encodes a RNA-binding protein with important roles in regulating pre-mRNA splicing (31,32). It has been shown that Rbfox2 is involved in mediating the alternative splicing events in B lymphoma cells (33). Moreover, there is evidence suggesting that Rbfox2 regulates the alternative splicing of the mRNA of kinesin family member 1B (KIF1B), resulting in the expression of two splicing variants, KIF1B-α and KIF1B-β (34,35). It has been well documented that KIF1B-β has potent pro-apoptotic functions (34-39). Notably, a study demonstrated that reduction in Rbfox2 expression resulted in the preferential expression of KIF1B-β (40). However, the present study failed to detect significant alterations in the ratio between two isoforms in K562 cells, either in response to imatinib/CX-5461 treatment or to *RBFOX2* gene silencing, suggesting that Rbfox2 protein was unlikely to have a major role in mediating *KIF1B* mRNA splicing in CML cells. Instead, it was observed that CX-5461 significantly upregulated the levels of both *KIF1B-α* and *KIF1B-β*. This unanticipated finding is notable because there is evidence suggesting that mutations in the *KIF1B* gene may be associated with the pathogenesis of acute lymphoblastic leukemia (56). Hence, the *KIF1B* gene is likely to have important functional roles in leukemic cells. Consistent with previous findings of the pro-apoptotic property of *KIF1B-β* in solid tumor cells, the present study confirmed that *KIF1B* gene silencing (presumably reducing both *KIF1B-α* and *KIF1B-β*) in K562 cells blunted the pro-apoptotic effect of imatinib/CX-5461. Based on these observations, it is hypothesized that the synergistic pro-apoptotic effect of imatinib/CX-5461 in K562 cells is mediated by upregulating the expression of *KIF1B-β*.

Currently it is not understood how CX-5461 regulates *KIF1B* expression. In a previous study by Ochiai *et al* (57), it was shown that in neuroblastoma cells, the transcription factor N-Myc could repress the expression of *KIF1B-β* by upregulating the expression of Bmi1, a component of the Polycomb transcriptional repressor complexes. Notably, N-Myc is overexpressed in ~50% of T-cell acute lymphoblastic leukemia patients and is implicated in promoting the survival of leukemic cells (58). Moreover, there is evidence indicating that CX-5461 may downregulate the expression of *N-MYC* gene, thereby suppressing the tumor growth (59). Taking these lines of data together, it is suggested that CX-5461 may possibly stimulate *KIF1B* expression in K562 cells by downregulating the expression of *N-MYC* (57). This hypothesis warrants further study. The inconsistent effects of imatinib/CX-5461 in K562, NALM-6 and THP-1 cells indicate that the regulation of *KIF1B* expression is not a ubiquitous phenomenon in leukemic cells and should be examined in a cell type-specific manner in future studies. On the other hand, the similar effects in K562 (Ph^+) and NALM-6 (Ph^-) cells suggest that imatinib/CX-5461-induced upregulation of *KIF1B* does not strictly depend on the presence of Bcr-Abl. This phenomenon may be explained by the fact that imatinib has limited target

specificity; in addition to Bcr-Abl, other tyrosine kinases such as platelet-derived growth factor receptor and c-kit can also be inhibited by imatinib (41). The relative independence on Bcr-Abl and p53 of the action of imatinib/CX-5461 combination may have two advantages: i) This strategy may be useful in CML patients with TKI resistance caused by p53 loss-of-function (6,7); ii) the combined treatment with imatinib/CX-5461 is likely to be efficacious in CML cells with resistance-causing mutations in Bcr-Abl (4). K562 cells represent the prototypical cell culture model of CML; however, a limitation of the present study was that no experimentation in additional Ph^+ CML cell lines as well as hypothetically sensitive Ph^- non-CML cell lines was conducted. The *BCR-ABL* translocation in CML cells may occur via a number of different junction points. In addition, CML is a disease of hematopoietic stem cells and, as a result, can give rise to multiple lineages of tumor cells (60). Therefore, it would be of scientific interest to further test the actions of imatinib/CX-5461 combination in additional Ph^+ CML cell lines such as LAMA-84 and CML-T1 (60).

In summary, the present study demonstrated that the selective Pol I inhibitor CX-5461, at a non-cytotoxic concentration, potentiates the pro-apoptotic effect of imatinib in the CML cell line K562 in a p53-independent manner. This effect of CX-5461 is mainly mediated by upregulating the expression of *KIF1B-β*, which is pro-apoptotic in K562 cells. This pharmacological property of CX-5461 may be of potential clinical value in the treatment of refractory CML.

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Availability of data and materials

The original RNA sequencing data (in fastq format) are freely accessible at Mendeley Data repository with the following URL links: i) https://data.mendeley.com/datasets/n6jjyjbb76/1 (R1 data for Con-1/2/3 and IM-1 samples); ii) https://data.mendeley.com/datasets/b77ncvnb9d/1 (R1 data for IM-2/3 and IM+CX-1/2 samples); iii) https://data.mendeley.com/datasets/xfbrfhtt6p/1 (R1 data for IM+CX-3 sample and R2 data for Con-1/2/3 samples); iv) https://data.mendeley.com/datasets/576tbt8m4b/1 (R2 data for IM-1/2/3 and IM+CX-1 samples); v) https://data.mendeley.com/datasets/ft27d96y63/1 (R2 data for IM+CX-2/3 samples).

Note: R1 and R2 represented the two files generated by paired-end sequencing (i.e., 5' to 3' sequencing and 3' to 5' sequencing respectively) for each sample.

The complete list of the raw count values and FPKM values are presented in Table SI. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CD was involved in experimentation, data collection, and data analysis. JW was involved in experimentation and data collection. XC was involved in experimental design and data interpretation. BD was involved in analysis of the genomic data, creating the illustrations, and manuscript revision. HG was involved in data interpretation and project supervision. MC was involved in experimental design, data interpretation, and project supervision. FJ was involved in study conception, data interpretation, manuscript writing, revision and approval. All authors read and approved the final manuscript. CD and FJ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

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Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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