A drug targeting 5-lipoxygenase enhances the activity of a JAK2 inhibitor in CD34+ bone marrow cells from patients with JAK2V617F-positive polycythemia vera *in vitro*

YUAN CHEN, HU ZHAO, JING LUO, YOUPING LIAO, KUI TAN and GUOYU HU

Department of Hematology, The Affiliated Zhuzhou Hospital Xiangya Medical College CSU, Zhuzhou, Hunan 412000, P.R. China

Received July 8, 2020; Accepted February 10, 2021

DOI: 10.3892/ol.2021.12612

Abstract. Janus kinase 2 (JAK2) inhibitors, the first targeted treatments for myeloproliferative neoplasms (MPNs), provide substantial benefits, including a marked reduction in splenomegaly and MPN-associated symptoms. However, these drugs rarely induce molecular remission in patients with MPNs. Zileuton, a 5-lipoxygenase (5-LO) inhibitor, has been demonstrated to selectively deplete hematopoietic stem cells (HSCs) expressing a JAK2 point mutation (JAK2V617F) in mouse models of JAK2V617F-induced polycythemia vera (PV). To determine the potential activity of 5-LO inhibitors in combination with JAK inhibitors against human PV HSCs, the present study first analyzed 5-LO expression in CD34+ bone marrow cells from patients with JAK2V617F-positive PV using western blotting and reverse transcription-quantitative PCR, and then examined the effect of zileuton combined with ruxolitinib on colony formation using a colony formation assay. Furthermore, cell cycle and apoptosis in CD34+ cells from patients with PV and healthy volunteers were determined by flow cytometry. In the present study, 5-LO expression was upregulated in CD34+ cells from patients with PV compared with in CD34+ cells from healthy volunteers. Higher levels of leukotriene B4, a product of the 5-LO signaling pathway, were detected in patients with PV compared with in healthy volunteers. Zileuton treatment suppressed the colony formation of CD34+ cells from patients with PV in a dose-dependent manner. Furthermore, zileuton and ruxolitinib exerted their anticancer effects by suppressing hematopoietic colony formation, inducing apoptosis and arresting the cell cycle of human CD34+ cells from patients with PV. The combination of these two drugs exerted a more beneficial effect than either agent alone. Based on these data, zileuton enhanced the antitumor activity of low-dose ruxolitinib in hematopoietic progenitor cells from patients with PV, providing conceptual validation for further clinical applications of combination treatment with ruxolitinib and zileuton for patients with PV.

Correspondence to: Professor Guoyu Hu, Department of Hematology, The Affiliated Zhuzhou Hospital Xiangya Medical College CSU, 116 Changjiang South Road, Tianyuan, Zhuzhou, Hunan 412000, P.R. China
E-mail: doctor.hgy@163.com

Key words: polycythemia vera, 5-lipoxygenase, ruxolitinib, zileuton, apoptosis, cell cycle

Introduction

Polycythemia vera (PV), essential thrombocythemia and primary myelofibrosis (1) are Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) that originate from hematopoietic stem cells (HSCs). In 2005, an activating point mutation in Janus kinase 2 (JAK2V617F) was identified in the majority of patients with MPNs (2-5), which provided critical insights into MPN pathogenesis and promoted the development of Janus kinase 2 (JAK2) inhibitors. According to clinical studies, JAK2 inhibitors effectively decrease spleen size and relieve MPN-associated symptoms; however, their disease-modifying activity is limited (6,7).

5-Lipoxygenase (5-LO) is an important dioxygenase, since it is the key enzyme that catalyzes the transformation of arachidonic acid into inflammatory leukotrienes (LTs) (8). Accumulating evidence has suggested that the 5-LO signaling pathway is directly involved in cancer development by promoting cell proliferation, angiogenesis, migration and invasion, and inhibiting apoptosis (9-13). As demonstrated in a study by Chen *et al* (14), 5-LO is upregulated in a mouse model of JAK2V617F-induced PV, and inhibition of 5-LO by zileuton, a selective 5-LO inhibitor, attenuates PV development by blocking JAK2V617F-expressing HSCs in mice. Therefore, it may be hypothesized that zileuton could potentially eliminate persistent malignant HSCs in patients with PV. However, to the best of our knowledge, no previous reports have described the role of 5-LO in patients with JAK2V617F-positive PV.

Based on the aforementioned evidence, the combination of zileuton with a JAK2 inhibitor may be a promising treatment strategy for patients with PV. The present study first analyzed 5-LO expression in CD34+ cells from the bone marrow of patients with JAK2V617F-positive PV using western blotting and reverse transcription-quantitative PCR (RT-qPCR). Subsequently, the effects of zileuton combined with ruxolitinib

on colony formation, apoptosis and the cell cycle of $CD34^+$ cells from patients with PV were analyzed *in vitro*.

Materials and methods

Patient specimens and cell preparation. Bone marrow and peripheral blood were donated by 18 patients who were newly diagnosed with PV and 10 healthy adult volunteers at the Affiliated Zhuzhou Hospital Xiangya Medical College CSU (Zhuzhou, China) between August 2017 and April 2019. All patients met the World Health Organization diagnostic criteria for PV (1). Patient characteristics are shown in Table I. The healthy volunteers were eligible if they were 18-69 years of age and in healthy condition without active infections, and serious liver, kidney, heart and other diseases. Bone marrow and peripheral blood from 10 healthy volunteers were used as normal controls. The volunteers included 6 women and 4 men. The mean age was 41.5 years, and the age ranged between 23 and 69 years. All participants provided written informed consent according to the protocol approved by the Medical Ethics Committees of the Affiliated Zhuzhou Hospital Xiangya Medical College CSU (Zhuzhou, China) and in accordance with the principles outlined in the Declaration of Helsinki. Mononuclear cells were separated from bone marrow samples at 440 x g for 30 min at room temperature using Ficoll-Hypaque density gradient centrifugation (GE Healthcare). An EasySep™ CD34-positive selection kit (Stemcell Technologies, Inc.) was used to enrich the $CD34^+$ cell population according to the manufacturer's protocol. $CD34^+$ cells with a purity ≥85% were used in each experiment.

Detection of leukotriene B4 (LTB4). Plasma samples from patients with PV and healthy volunteers were collected to detect LTB4 levels using a leukotriene B4 Express ELISA kit (cat. no. 10009292; Cayman Chemical Company). Briefly, the standard or plasma sample, LTB4 AchE tracer and anti-LTB4 antibody were sequentially added to each well of a 96-well plate. The plates were then incubated for 60-90 min at room temperature before measuring the absorbance at 405 nm using an ELISA microplate reader (Thermo Fisher Scientific, Inc.).

RT-qPCR. Total RNA was extracted from the purified $CD34^+$ cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed using a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (cat. no. RR047A; Takara Bio, Inc.) according to the manufacturer's protocols. Reactions were incubated at 37˚C for 15 min followed by heat inactivation for 5 sec at 85˚C for reverse transcription. The PCR amplification was performed using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (cat. no. RR820A; Takara Bio, Inc.). The primer sequences of the human 5-LO gene and the GAPDH gene are listed in Table II. The thermocycling conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 31 sec. Amplification was performed using an ABI 7300 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The $2^{-\Delta\Delta Cq}$ formula (15) was used to analyze the relative mRNA expression levels of 5-LO, which were normalized to the expression levels of GAPDH.

Western blotting. RIPA lysis solution (cat. no. P0013B; Beyotime Institute of Biotechnology) was used to extract total proteins from purified $CD34^+$ cells. BCA assays (cat. no. 23227; Pierce; Thermo Fisher Scientific, Inc.) were used to determine the protein concentration. An equal amount of total proteins (30 μg/lane) was separated by 10% SDS-PAGE and then transferred onto PVDF membrane (cat. no. IPVH00010; EMD Millipore). The membranes were blocked using TBS with 0.05% Tween-20 (TBST) containing 5% non-fat milk at room temperature for 2 h, and then incubated with primary antibodies against 5-LO (dilution, 1:500; cat. no. sc-136195; Santa Cruz Biotechnology, Inc.) and β-actin (dilution, 1:1,000; cat. no. ab8226; Abcam) overnight at 4˚C. After washing with TBST, the membranes were incubated with secondary antibodies (dilution, 1:2,000; cat. no. 7076; Cell Signaling Technology, Inc.) conjugated to horseradish peroxidase at room temperature for 1 h. Finally, the protein blots were visualized using a Hypersensitive ECL chemiluminescence kit (cat. no. P10018FS; Beyotime Institute of Biotechnology). The results were semi-quantified using ImageJ software (v1.52; National Institutes of Health).

Colony formation assay (CFA). The colony-forming ability of the cells was estimated by inoculating 500 $CD34^+$ cells in MethoCult H4435 (Stemcell Technologies, Inc.). Various concentrations of zileuton (50, 100, 250 and 500 μM; Cayman Chemical Company) and/or 50 nM ruxolitinib (Cayman Chemical Company) were added for 14 days at 37˚C. After 14 days, the presence of colonies (>40 cells) was scored under a light microscope (Zeiss LSM 800 with Airyscan; magnification, x25; Zeiss AG).

Flow cytometry. $CD34^+$ cells ($1x10^6$ cells/well) from patients with PV and healthy volunteers were seeded into a 6-well plate and treated with 100 μM zileuton, 50 nM ruxolitinib or the combination of 100 μM zileuton and 50 nM ruxolitinib for 48 h at 37˚C. After 2 days, $5x10^5$ cells were collected and applied to the flow cytometry detection. The apoptosis assay was performed using an Annexin V-FITC Apoptosis Detection Kit (cat. no. 556547; BD Biosciences). In brief, cells were stained with Annexin V-FITC and PI for 15 min at room temperature, and detected using a flow cytometer. Stained cells were analyzed using a FACSCalibur instrument (BD Biosciences) using a 488 nm excitation wavelength and emission was detected at 530 nm (for FITC) and 575 nm (for PI). The data were analyzed using the BD FACSuite™ version 1.01 (BD Biosciences). The phases of the cell cycle and DNA synthesis activity of $CD34^+$ cells were determined using a FITC BrdU Flow kit (cat. no. 559619; BD Biosciences) according to the manufacturer's protocol. Briefly, cells were incubated with BrdU, a nucleoside analogue of thymidine, and then stained with anti-human CD34-allophycocyanin (dilution, 1:5; cat. no. 560940; BD Biosciences). After fixing in BD Cytofix/Cytoperm Buffer for 30 min on ice and permeabilizing in BD Cytoperm Permeabilization Buffer Plus for 10 min on ice, cells were treated with DNase to expose BrdU epitopes and then incubated with a FITC-conjugated anti-BrdU antibody (provided in kit; BD Biosciences) for 20 min at room temperature. DNA was counterstained with 7-aminoactinomycin D (7AAD; provided in kit;

Table I. Patient characteristics and experiments performed using patient samples.

				Experiments					
Case	Age, years	Sex	JAK2V617F allele burden, %	Leukotriene B4 ELISA	Reverse transcription-quantitative PCR	Western blotting	Hematopoietic progenitor cell assays	FACS for apoptosis assay	FACS for cell cycle analysis
PV1	68	Female	49	Y	N	N	Y	Y	Y
PV2	70	Female	26	Y	Y	N	N	N	Y
PV3	43	Male	43	Y	Y	N	N	Y	Y
PV4	46	Male	75	N	Y	N	Y	N	N
PV5	67	Male	90	Y	Y	N	Y	N	N
PV6	54	Female	80	Y	N	N	Y	Y	Y
PV7	69	Male	28	Y	Y	Y	N	Y	N
PV8	55	Female	70	Y	N	Y	Y	N	N
PV9	72	Female	45	Y	Y	Y	Y	Y	Y
PV10	67	Male	83	N	Y	Y	Y	Y	N
PV11	66	Female	58	Y	N	Y	N	N	Y
PV12	41	Female	47	Y	Y	Y	Y	Y	Y
PV13	61	Male	61	Y	Y	Y	Y	N	N
PV14	48	Female	40	Y	N	Y	N	Y	Y
PV15	67	Male	46	N	Y	N	Y	Y	Y
PV16	68	Male	57	Y	N	N	Y	N	N
PV17	56	Female	79	N	Y	N	N	N	N
PV18	66	Male	84	Y	Y	N	Y	Y	Y

PV, polycythemia vera; JAK2V617F, a mutation resulting in a substitution of valine for phenylalanine at amino acid 617 of the JAK2 protein; Y, yes; N, no.

Table II. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Product length, bp	Primer	Sequence (5'-3')
5-lipoxygenase	168	Forward	GCTGAATGACGACTGGTA
		Reverse	CGGTGTTGCTTGAGAATG
GAPDH	263	Forward	ATGCTGGCGCTGAGTACGTC
		Reverse	GGTCATGAGTCCTTCCACGATA

BD Biosciences) for 15 min at room temperature. Stained cells were then analyzed using a FACSCalibur instrument using a 488 nm excitation wavelength and emission was detected at 530 nm (for FITC-conjugated anti-BrdU) and 610 nm (for 7AAD). The data were analyzed using BD FACSuite™ version 1.01.

Statistical analysis. All experiments were repeated three times. Data are presented as medians and interquartile ranges. Differences in 5-LO expression and LTB4 levels between groups were compared using a two-tailed Mann-Whitney test, while data derived from the same samples after different treatments were analyzed using the Friedman test, and the Nemenyi post hoc test was subsequently used for pairwise comparisons between groups. GraphPad Prism 7 software (GraphPad Software, Inc.) and SPSS 26.0 software (IBM Corp.) were utilized for statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

5-LO expression is increased in CD34⁺ cells from patients with PV. To determine the potential effects of 5-LO inhibitor zileuton in the treatment of human PV, the present study first assessed the basal protein and mRNA expression levels of 5-LO in CD34⁺ cells from the bone marrow of patients with PV and healthy volunteers. Western blot analysis revealed that CD34⁺ cells from patients with PV exhibited higher protein expression levels of 5-LO than those from healthy volunteers (Fig. 1A and B; Table SI). Consistent with the results observed for protein expression, 5-LO mRNA expression was also increased in CD34⁺ cells from patients with PV compared

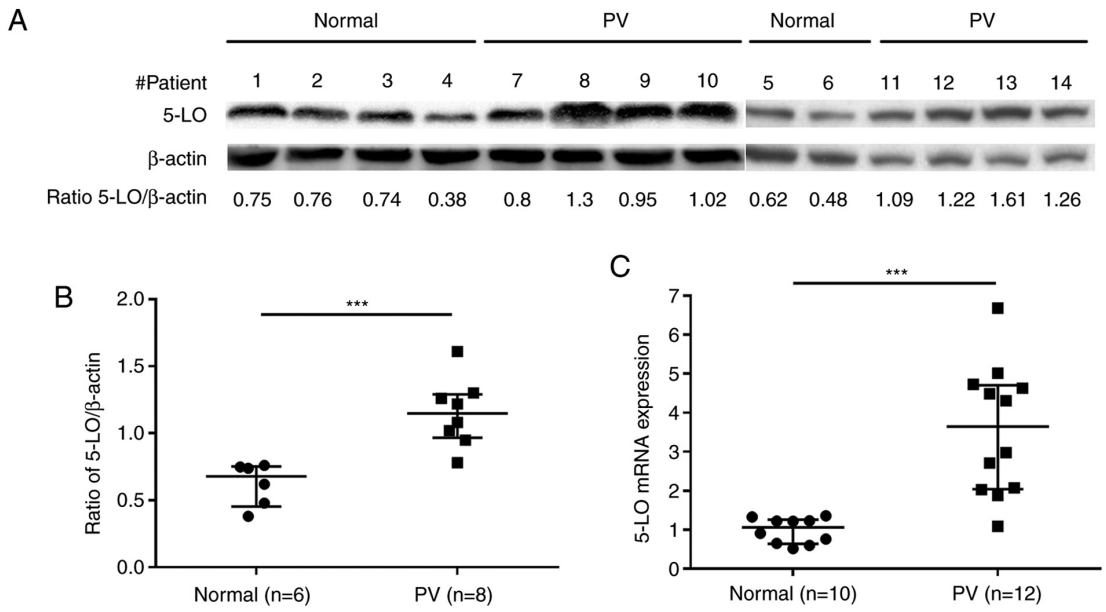

Figure 1. 5-LO mRNA and protein expression in CD34⁺ cells from patients with PV and normal controls. (A) Western blot analysis of 5-LO protein expression in CD34⁺ cells from healthy volunteers (n=6) and patients with PV (n=8). (B) Semi-quantitative analysis of 5-LO protein expression in CD34⁺ cells from healthy volunteers and patients with PV. Ratios of 5-LO/β-actin were calculated. (C) Quantitative analysis of 5-LO mRNA expression in CD34⁺ cells from patients with PV (n=12) and normal controls (n=10) using reverse transcription-quantitative PCR. GAPDH was used as the reference gene. Medians and interquartile ranges are presented on representative plots, and a two-tailed Mann-Whitney test was used to determine significance. ***P<0.001. 5-LO, 5-lipoxygenase; PV, polycythemia vera.

with in those from healthy volunteers, as demonstrated by the results of RT-qPCR (Fig. 1C; Table SII).

LTB4 levels are elevated in patients with PV. Plasma levels of LTB4, a metabolite of the 5-LO signaling pathway, were measured in 14 patients with PV and 10 healthy volunteers using ELISA. Higher LTB4 levels were observed in patients with PV compared with those in healthy volunteers (Fig. 2; Table SIII).

Zileuton suppresses the colony formation of CD34⁺ cells from patients with PV in a dose-dependent manner. A CFA was performed to assess the effect of zileuton treatment on the colony formation of primary CD34⁺ cells *in vitro*. As shown in Fig. 3, zileuton treatment inhibited granulocytes and monocytes (CFU-GM)- and burst-forming unit-erythroid (BFU-E)-derived colony formation by PV CD34⁺ cells in a dose-dependent manner, with an IC_{50} of 460.4 μM for CFU-GM and 233.5 μM for BFU-E (Fig. 3B and C; Table SIV). By contrast, zileuton treatment did not markedly alter the colony formation of the CD34⁺ cells from healthy volunteers (Fig. 3A and C; Table SIV).

Reduction of colony formation by zileuton treatment in PV CD34⁺ cells occurs through a reduction in 5-LO expression. To explore whether the effect of zileuton on colony formation of hematopoietic cells was associated with the levels of 5-LO, 5-LO protein expression in CD34⁺ cells from patients with PV and healthy volunteers was measured with and without treatment with increasing concentrations of zileuton using western blotting. As shown in the Fig. 4, zileuton dose-dependently decreased 5-LO protein expression in CD34⁺ cells from a patient with PV, which exhibited high 5-LO expression, but had no significant effects on 5-LO expression in CD34⁺ cells from a healthy volunteer.

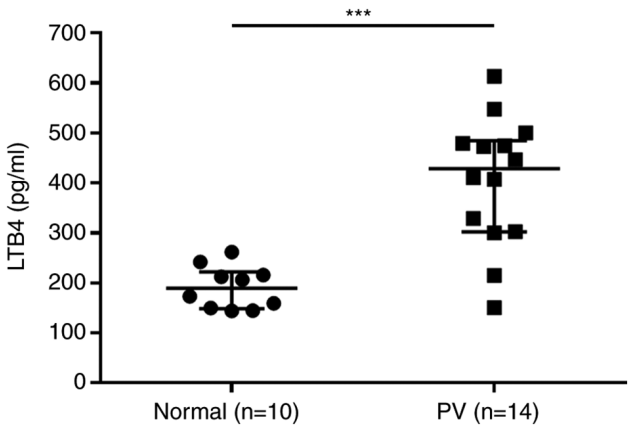

Figure 2. Detection of LTB4 production in patients with PV (n=14) and normal controls (n=10) using an ELISA. Medians and interquartile ranges are presented on representative plots, and a two-tailed Mann-Whitney test was used to determine significance. ***P<0.001. LTB4, leukotriene B4; PV, polycythemia vera.

Combination treatment with zileuton and ruxolitinib synergistically inhibits the colony formation of hematopoietic cells from patients with PV. The present study investigated the effect of combination treatment with zileuton and ruxolitinib on the formation of colonies of primary CD34⁺ cells from 10 patients with PV and 6 healthy volunteers *in vitro*. The doses selected for the present study were 100 μM zileuton and 50 nM ruxolitinib. In the CD34⁺ cells from healthy volunteers, treatment with 100 μM zileuton, 50 nM ruxolitinib or 100 μM zileuton combined with 50 nM ruxolitinib did not alter colony

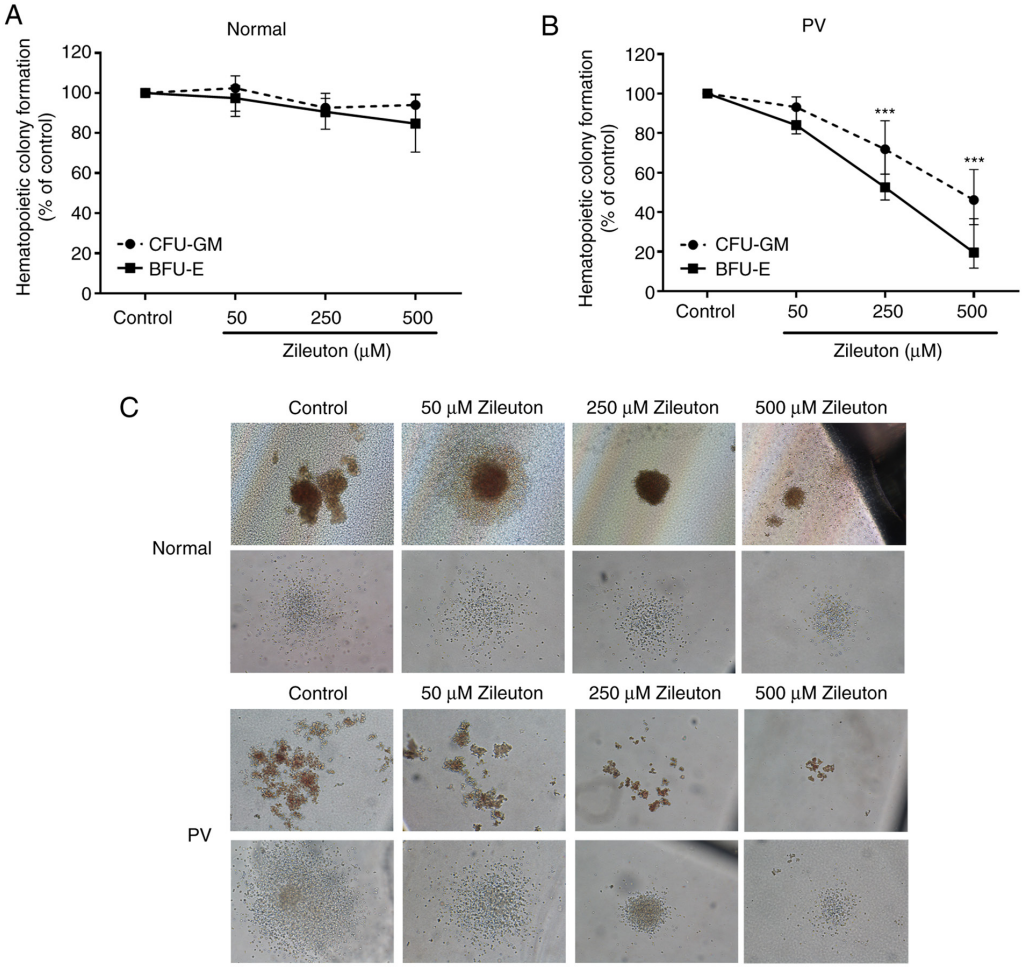

Figure 3. Effects of increasing concentrations of zileuton on the colony formation of CD34+ cells from patients with PV and healthy volunteers. (A) In CD34+ cells from healthy volunteers (n=10), zileuton treatment had little effect on the colony formation compared with the control treatment. (B) In CD34+ cells from patients with PV (n=12), zileuton dose-dependently inhibited the colony formation compared with the control treatment. (C) Effects of increasing concentrations of zileuton on the hematopoietic colony size of CD34+ cells from patients with PV and healthy volunteers. In each panel, the upper colonies are representative BFU-E-derived colonies, and the lower colonies are representative CFU-GM-derived colonies (magnification, x25). Medians and interquartile ranges are presented on representative plots. The Friedman test was used to determine significance and a Nemenyi post hoc test was subsequently used for pairwise comparisons between groups. ***P<0.001 vs. control treatment. BFU-E, burst-forming unit-erythroid; CFU-GM, colony-forming unit for granulocytes and monocytes; PV, polycythemia vera; 5-LO, 5-lipoxygenase.

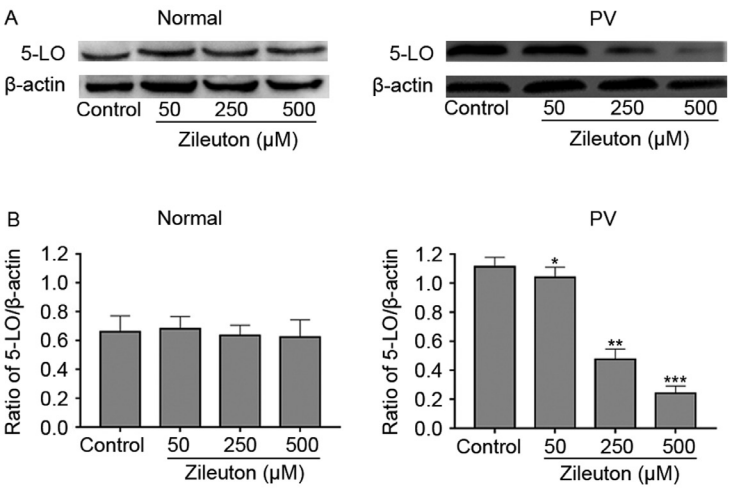

Figure 4. Western blotting results revealing that zileuton dose-dependently decreases 5-LO protein levels in CD34+ cells from a patient with PV. (A) Western blotting for the detection of 5-LO protein expression in CD34+ cells from 1 patient with PV and 1 normal control after treatment with increasing concentrations of zileuton. (B) Ratios of 5-LO/β-actin were calculated. Zileuton treatment dose-dependently decreased 5-LO protein expression compared with the control treatment in CD34+ cells from a patient with PV. All values are shown as medians and interquartile ranges of data from experiments performed in triplicate. The Friedman test was used to determine significance and a Nemenyi post hoc test was subsequently used for pairwise comparisons between groups. *P<0.05, **P<0.01, ***P<0.001 vs. control. PV, polycythemia vera; 5-LO, 5-lipoxygenase.

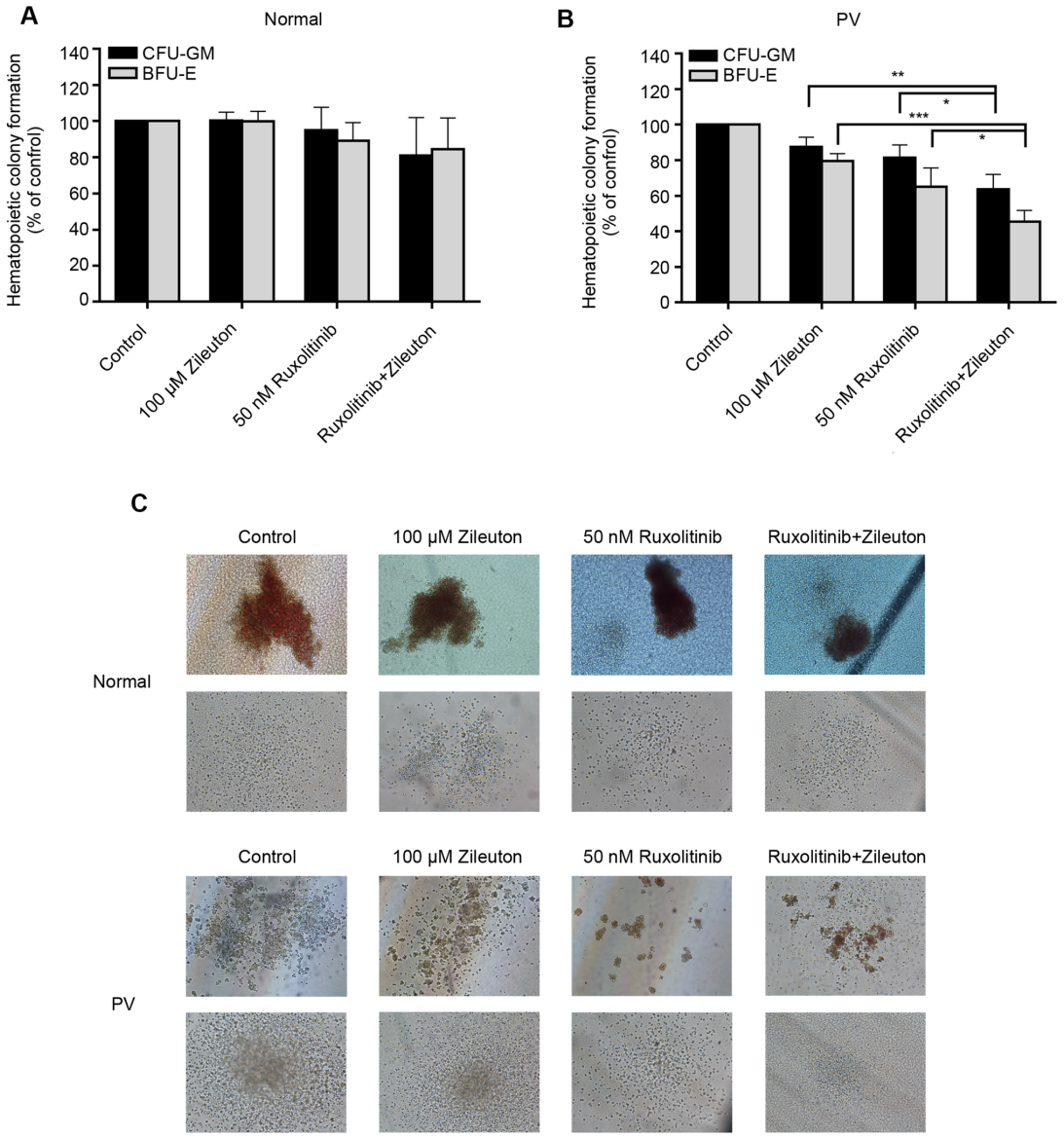

Figure 5. Effects of treatment with 100 μM zileuton and/or 50 nM ruxolitinib on the colony formation of CD34$^+$ cells from patients with PV and normal controls. (A) Zileuton or ruxolitinib alone or in combination exerted little effect on the colony formation of CD34$^+$ cells from healthy volunteers (n=10). (B) In CD34$^+$ cells from patients with PV (n=12), combination treatment of ruxolitinib and zileuton significantly inhibited the colony formation compared with treatment with either individual drug. (C) Effects of treatment with 100 μM zileuton and/or 50 nM ruxolitinib on the hematopoietic colony size of CD34$^+$ cells from patients with PV and healthy volunteers. In each panel, the upper colonies are representative BFU-E-derived colonies, and the lower colonies are representative CFU-GM-derived colonies (magnification, x25). Medians and interquartile ranges are presented on representative plots. The Friedman test was used to determine significance and the Nemenyi post hoc test was subsequently used for pairwise comparisons between groups. *P<0.05; **P<0.01; ***P<0.001. BFU-E, burst-forming unit-erythroid; CFU-GM, colony-forming unit for granulocytes and monocytes; PV, polycythemia vera.

formation (Fig. 5A and C; Table SV). By contrast, the yields of CFU-GM and BFU-E in CD34$^+$ cells from patients with PV treated with 50 nM ruxolitinib were decreased by 19 and 35%, respectively, and the yields of CFU-GM and BFU-E in CD34$^+$ cells from patients with PV treated with 100 μM zileuton were decreased by 12 and 20%, respectively (Fig. 5B and C; Table SV). However, the combination of ruxolitinib with zileuton had a greater effect on the colony formation of CD34$^+$ cells from patients with PV, with a 36% a reduction in CFU-GM and a 55% reduction in BFU-E (Fig. 5B and C; Table SV).

Combination treatment with zileuton and ruxolitinib induces apoptosis in CD34$^+$ cells from patients with PV. The apoptosis rate of CD34$^+$ cells from patients with PV and healthy volunteers after treatment with zileuton and/or ruxolitinib was detected using flow cytometry. The apoptosis rate of CD34$^+$ cells from patients with PV was slightly increased following zileuton or ruxolitinib treatment (Fig. 6; Table SVI). However, combination treatment with zileuton and ruxolitinib induced apoptosis in a greater number of CD34$^+$ cells from patients with PV than treatment with either individual drug. By contrast, neither ruxolitinib nor zileuton alone or in combination induced apoptosis of CD34$^+$ cells from healthy volunteers (Fig. 6; Table SVI).

Treatment with zileuton and ruxolitinib arrests CD34$^+$ cells from patients with PV at the G_0/G_1 phase of the cell cycle. Cell cycle arrest is an important effect of numerous anticancer agents. The present study investigated the effects of zileuton

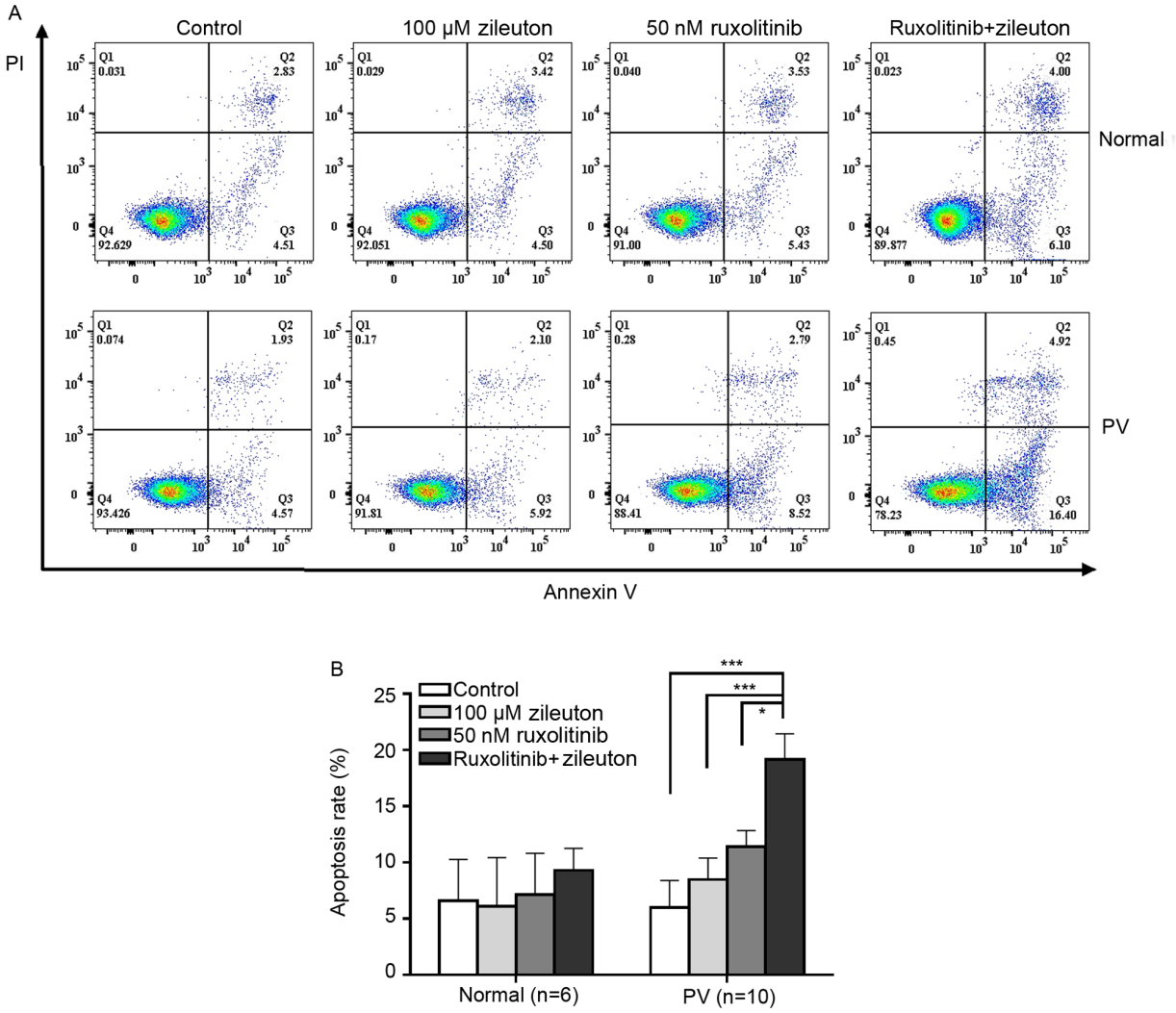

Figure 6. Effects of treatment with 100 μM zileuton and/or 50 nM ruxolitinib on the apoptosis of CD34$^+$ cells from patients with PV and normal controls. (A) Representative flow cytometry plots of apoptosis rates of CD34$^+$ cells from patients with PV and healthy volunteers after treatment with 100 μM zileuton and/or 50 nM ruxolitinib for 48 h. (B) Bar graphs showing apoptosis rates of CD34$^+$ cells from healthy volunteers (n=6) and patients with PV (n=10) after treatment. All values are shown as medians and interquartile ranges of data from experiments performed in triplicate. The Friedman test was used to determine significance and the Nemenyi post hoc test was subsequently used for pairwise comparisons between groups. *P<0.05; ***P<0.001. PV, polycythemia vera.

and/or ruxolitinib on the cell cycle of CD34$^+$ cells from patients with PV *in vitro*. As shown in Fig. 7A and C, treatment with zileuton and ruxolitinib alone slightly increased the number of CD34$^+$ cells from patients with PV that were arrested in the G_0/G_1 phase of the cell cycle, with a concomitant decrease in the percentage of cells in S phase (Table SVII). However, combination treatment with zileuton and ruxolitinib caused more CD34$^+$ cells from patients with PV to be arrested in the G_0/G_1 phase and a significant decrease in the proportion of cells in S phase compared with either monotherapy. By contrast, the percentage of CD34$^+$ cells from healthy volunteers in each phase of the cell cycle did not markedly change, regardless of whether they were treated with zileuton, ruxolitinib or the combination treatment (Fig. 7A and B; Table SVII).

Discussion

PV is the most common Philadelphia chromosome-negative MPN (16). Thrombosis and hemorrhage, as well as myelofibrotic and/or leukemic transformation (17,18), are potential complications occurring in patients with PV. The JAK2V617F mutation in the pseudokinase domain leads to constitutive phosphorylation of JAK2 and overactivation of downstream signaling pathways, ultimately resulting in uncontrolled myeloid cell proliferation (19,20). These findings motivated the clinical development of JAK kinase inhibitors for patients with MPNs. Ruxolitinib was the first selective JAK1/2 inhibitor that was demonstrated to be effective in patients with PV and myelofibrosis (21-23), and it provides substantial benefits, including a marked decrease in splenomegaly and disease symptoms (24). However, treatment with ruxolitinib rarely induces molecular or pathological remission in patients with MPNs (25-27). Furthermore, the dose-dependent hematological toxicity of ruxolitinib represents a major concern for a number of patients (28). Researchers have expressed an interest in identifying novel agents with different mechanisms of action other than targeting the JAK-STAT signaling pathway.

5-LO expression is upregulated in numerous different types of cancer, including pancreatic, breast, prostate, esophageal and colon cancer (29-33). The products of 5-LO, such as

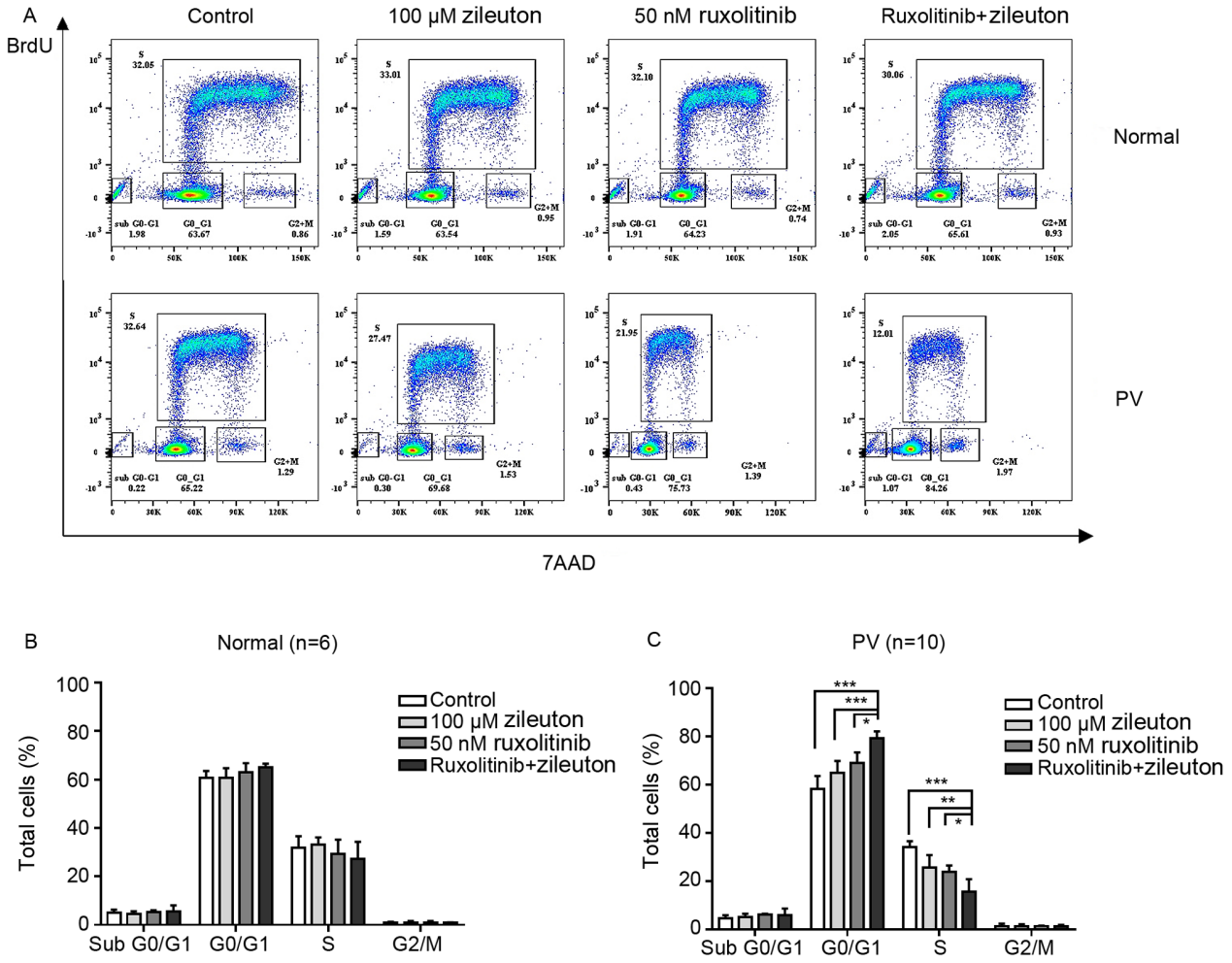

Figure 7. Effects of treatment with 100 μM zileuton and/or 50 nM ruxolitinib on the cell cycle of CD34$^+$ cells from patients with PV and healthy volunteers. (A) Representative flow cytometry plots of alterations in the cell cycle of CD34$^+$ cells from patients with PV and healthy volunteers following treatment with 100 μM zileuton and/or 50 nM ruxolitinib for 48 h. (B) Cell cycle status of CD34$^+$ cells from healthy volunteers (n=6) after treatment. (C) Cell cycle status of CD34$^+$ cells from patients with PV (n=10) after treatment. Medians and interquartile ranges are presented on representative plots. The Friedman test was used to determine significance and the Nemenyi post hoc test was subsequently used for pairwise comparisons between groups. *P<0.05; **P<0.01; ***P<0.001. 7AAD, 7-aminoactinomycin D; BrdU, 5-bromo-2-deoxyuridine; PV, polycythemia vera.

5-hydroxyeicosatetraenoic acid (5-HETE) and LTs, are able to promote cell proliferation, suppress apoptosis, promote angiogenesis and enhance tumor cell invasion (34-36). According to previous studies, epidermal growth factors and neurotensin are involved in the 5-LO-mediated tumor progression in individuals with prostate cancer (37,38). A study of patients with colorectal cancer revealed that 5-HETE stimulates angiogenesis by inducing the expression of VEGF (39,40). Furthermore, increased activities of 5-LO and matrix metalloproteinases are associated with extracellular matrix stiffness (41) and enhance the invasiveness of cancer cells (42,43). The enzyme 5-LO is involved in the development of not only solid malignancies but also certain forms of leukemia (44,45). Recently, the 5-LO gene was shown to be a critical regulator for mouse leukemic stem cells (LSCs) in BCR-ABL-induced chronic myeloid leukemia (CML), and the combination of zileuton and imatinib extends the survival time of mice with CML (46). This finding motivated an initial clinical trial combining zileuton with imatinib as a treatment for CML (clinicaltrials.com; NCT02047149, NCT01130688) with no available efficacy data at present. Zileuton has been revealed to selectively deplete JAK2V617F-expressing HSCs, thereby preventing PV development in mice (14). The molecular mechanism of 5-LO in PV is associated with the β-catenin signaling pathway, which is required for the maintenance of both HSCs (47) and LSCs in individuals with CML (48-50). Based on these results, drugs targeting the 5-LO signaling pathway can eradicate human JAK2V617F$^+$ malignant HSCs and thus are potentially curative treatments for MPNs. However, a previous study reported low expression levels of 5-LO and LTB4 receptor 1 in CD34$^+$ cells from patients with BCR-ABL-positive CML compared with healthy donors (51). Another study reported that 5-LO expression was undetectable in more primitive CML LSCs (52). Therefore, 5-LO has distinct and important regulatory and functional roles in human and murine CML. To the best of our knowledge, no previous reports have described the role of 5-LO in human PV.

In the present study, 5-LO mRNA and protein expression was increased in CD34$^+$ cells from patients with PV compared with in CD34$^+$ cells from healthy volunteers. Higher LTB4 levels were detected in patients with PV compared with healthy volunteers. Based on these results, the 5-LO signaling pathway was upregulated in patients with JAK2V617F-positive

PV, consistent with previous findings from PV mice (14), indicating that 5-LO may be involved in the pathogenesis of human JAK2V617F-positive PV. Zileuton treatment decreased the colony formation of $CD34^+$ cells from patients with PV in a dose-dependent manner by reducing 5-LO expression in PV $CD34^+$ cells. Furthermore, zileuton and ruxolitinib exerted their anticancer effects by suppressing the colony formation of hematopoietic cells, inducing apoptosis and blocking the cell cycle of $CD34^+$ cells from patients with PV. The combination of the two drugs was more effective than either agent alone. Similar effects were not observed in $CD34^+$ cells from healthy volunteers after treatment with zileuton or ruxolitinib, either alone or in combination. Therefore, zileuton enhanced the antitumor activity of ruxolitinib against hematopoietic progenitor cells from patients with PV, suggesting that zileuton and ruxolitinib may represent an effective therapeutic combination. Notably, activities of both 100 μM zileuton and 50 nM ruxolitinib were relatively specific for hematopoietic progenitor cells from patients with PV, while sparing $CD34^+$ cells from healthy volunteers. The latter finding suggests that the combination treatment may exhibit improved safety and low hematological toxicity.

In conclusion, zileuton exerted a synergistic effect with ruxolitinib on $CD34^+$ cells from patients with PV by suppressing cell proliferation, inducing apoptosis and arresting the cell cycle, which provides conceptual validation for further clinical applications of combination therapy with ruxolitinib and zileuton for patients with PV. However, because $CD34^+$ cells collected from some patients were not amplified enough to complete all downstream experiments, only a portion of the samples were analyzed in some experiments. This was a limitation of the present study. Future studies should focus on investigating the molecular mechanisms by which 5-LO inhibitors block colony formation, and induce apoptosis and cell cycle arrest, in patients with PV.

Acknowledgements

Not applicable.

Funding

The present study was funded by the Clinical Medical Technology Innovation Guidance Project of Hunan Province (grant no. 2018SK52803).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GH and YC proposed and designed the current study. JL and YL selected patients and collected samples/clinical data. YC and HZ conducted the experiments. YC, HZ and KT were responsible for acquiring, analyzing and interpreting the data. YC and HZ drafted the initial manuscript. YC, HZ, JL and GH reviewed and edited the manuscript. GH, YC and HZ assessed the authenticity of all the raw data and ensured its legitimacy. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol was established according to the ethical guidelines of the Helsinki Declaration and was approved by the Medical Ethics Committees of The Affiliated Zhuzhou Hospital Xiangya Medical College CSU (Zhuzhou, China). Written informed consent was obtained from each individual.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, Bloomfield CD, Cazzola M and Vardiman JW: The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 127: 2391-2405, 2016.
2. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, Vassiliou GS, Bench AJ, Boyd EM, Curtin N, *et al*: Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 365: 1054-1061, 2005.
3. James C, Ugo V, Le Couédic JP, Staerk J, Delhommeau F, Lacout C, Garçon L, Raslova H, Berger R, Bennaceur-Griscelli A, *et al*: A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 434: 1144-1148, 2005.
4. Jones AV, Kreil S, Zoi K, Waghorn K, Curtis C, Zhang L, Score J, Seear R, Chase AJ, Grand FH, *et al*: Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. Blood 106: 2162-2168, 2005.
5. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M and Skoda RC: A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 352: 1779-1790, 2005.
6. Deininger M, Radich J, Burn TC, Huber R and Verstovsek S: The effect of long-term ruxolitinib treatment on JAK2p.V617F allele burden in patients with myelofibrosis. Blood 126: 1551-1554, 2015.
7. Cervantes F and Pereira A: Does ruxolitinib prolong the survival of patients with myelofibrosis? Blood 129: 832-837, 2017.
8. Needleman P, Turk J, Jakschik BA, Morrison AR and Lefkowith JB: Arachidonic acid metabolism. Ann Rev Biochem 55: 69-102, 1986.
9. Peters-Golden M and Henderson WR Jr: Leukotrienes. N Engl J Med 357: 1841-1854, 2007.
10. Pidgeon GP, Lysaght J, Krishnamoorthy S, Reynolds JV, O'Byrne K, Nie D and Honn KV: Lipoxygenase metabolism: Roles in tumor progression and survival. Cancer Metastasis Rev 26: 503-524, 2007.
11. Wang D and Dubois RN: Eicosanoids and cancer. Nat Rev Cancer 10: 181-193, 2010.
12. Bishayee K and Khuda-Bukhsh AR: 5-lipoxygenase antagonist therapy: A new approach towards targeted cancer chemotherapy. Acta Biochim Biophys Sin (Shanghai) 45: 709-719, 2013.
13. Moore GY and Pidgeon GP: Cross-talk between cancer cells and the tumour microenvironment: The role of the 5-lipoxygenase pathway. Int J Mol Sci 18: 236, 2017.
14. Chen Y, Shan Y, Lu M, DeSouza N, Guo Z, Hoffman R, Liang A and Li S: Alox5 blockade eradicates JAK2V617F-induced polycythemia Vera in mice. Cancer Res 77: 164-174, 2017.
15. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
16. Mehta J, Wang H, Iqbal SU and Mesa R: Epidemiology of myeloproliferative neoplasms in the United States. Leuk Lymphoma 55: 595-600, 2014.
17. Hoffman R, Prchal JT, Samuelson S, Ciurea SO and Rondelli D: Philadelphia chromosome-negative myeloproliferative disorders: Biology and treatment. Biol Blood Marrow Transplant 13 (Suppl 1): S64-S72, 2007.

18. Mascarenhas J: A concise update on risk factors, therapy, and outcome of leukemic transformation of myeloproliferative neoplasms. Clin Lymphoma Myeloma Leuk 16 (Suppl): S124-S129, 2016.
19. Silvennoinen O and Hubbard SR: Molecular insights into regulation of JAK2 in myeloproliferative neoplasms. Blood 125: 3388-3392, 2015.
20. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, Boggon TJ, Wlodarska I, Clark JJ, Moore S, *et al*: Activating mutation in the tyrosine kinase JAK2 in polycythemia Vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 7: 387-397, 2005.
21. Verstovsek S, Mesa RA, Gotlib J, Levy RS, Gupta V, DiPersio JF, Catalano JV, Deininger M, Miller C, Silver RT, *et al*: A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. N Engl J Med 366: 799-807, 2012.
22. Hasselbalch HC and Bjørn ME: Ruxolitinib versus standard therapy for the treatment of polycythemia Vera. N Engl J Med 372: 1670, 2015.
23. Pardanani A, Harrison C, Cortes JE, Cervantes F, Mesa RA, Milligan D, Masszi T, Mishchenko E, Jourdan E, Vannucchi AM, *et al*: Safety and efficacy of fedratinib in patients with primary or secondary myelofibrosis: A randomized clinical trial. JAMA Oncol 1: 643-651, 2015.
24. Kiladjian J, Verstovsek S, Griesshammer M, Masszi T, Durrant S, Passamonti F, Harrison CN, Pane F, Zachee P, Kirito K, *et al*: Results from the 208-week (4-year) Follow-up of RESPONSE Trial, a Phase 3 study comparing ruxolitinib (Rux) with best available therapy (BAT) for the treatment of polycythemia Vera (PV). Blood 130 (Suppl 1): S322, 2017.
25. Verstovsek S, Gotlib J, Mesa RA, Vannucchi AM, Kiladjian JJ, Cervantes F, Harrison CN, Paquette R, Sun W, Naim A, *et al*: Long-term survival in patients treated with ruxolitinib for myelofibrosis: COMFORT-I and -II pooled analyses. J Hematol Oncol 10: 156, 2017.
26. Harrison CN, Vannucchi AM, Kiladjian JJ, Al-Ali HK, Gisslinger H, Knoops L, Cervantes F, Jones MM, Sun K, McQuitty M, *et al*: Long-term findings from COMFORT-II, a phase 3 study of ruxolitinib vs. best available therapy for myelofibrosis. Leukemia 31: 775, 2016.
27. Verstovsek S, Mesa RA, Gotlib J, Gupta V, DiPersio JF, Catalano JV, Deininger MW, Miller CB, Silver RT, Talpaz M, *et al*: Long-term treatment with ruxolitinib for patients with myelofibrosis: 5-year update from the randomized, double-blind, placebo-controlled, phase 3 COMFORT-I trial. J Hematol Oncol 10: 55, 2017.
28. Harrison C, Kiladjian JJ, Al-Ali HK, Gisslinger H, Waltzman R, Stalbovskaya V, McQuitty M, Hunter DS, Levy R, Knoops L, *et al*: JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. N Engl J Med 366: 787-798, 2012.
29. Avis I, Hong SH, Martínez A, Moody T, Choi YH, Trepel J, Das R, Jett M and Mulshine JL: Five-lipoxygenase inhibitors can mediate apoptosis in human breast cancer cell lines through complex eicosanoid interactions. FASEB J 15: 2007-2009, 2001.
30. Melstrom LG, Bentrem DJ, Salabat MR, Kennedy TJ, Ding XZ, Strouch M, Rao SM, Witt RC, Ternent CA, Talamonti MS, *et al*: Overexpression of 5-lipoxygenase in colon polyps and cancer and the effect of 5-LOX inhibitors in vitro and in a murine model. Clin Cancer Res 14: 6525-6530, 2008.
31. Hennig R, Ding XZ, Tong WG, Schneider MB, Standop J, Friess H, Büchler MW, Pour PM and Adrian TE: 5-Lipoxygenase and leukotriene B(4) receptor are expressed in human pancreatic cancers but not in pancreatic ducts in normal tissue. Am J Pathol 161: 421-428, 2002.
32. Hoque A, Lippman SM, Wu TT, Xu Y, Liang ZD, Swisher S, Zhang H, Cao L, Ajani JA and Xu XC: Increased 5-lipoxygenase expression and induction of apoptosis by its inhibitors in esophageal cancer: A potential target for prevention. Carcinogenesis 26: 785-791, 2005.
33. Matsuyama M, Yoshimura R, Mitsuhashi M, Hase T, Tsuchida K, Takemoto Y, Kawahito Y, Sano H and Nakatani T: Expression of lipoxygenase in human prostate cancer and growth reduction by its inhibitors. Int J Oncol 24: 821-827, 2004.
34. Hyde CA and Missailidis S: Inhibition of arachidonic acid metabolism and its implication on cell proliferation and tumour-angiogenesis. Int Immunopharmacol 9: 701-715, 2009.
35. Ding XZ, Iversen P, Cluck MW, Knezetic JA and Adrian TE: Lipoxygenase inhibitors abolish proliferation of human pancreatic cancer cells. Biochem Biophys Res Commun 261: 218-223, 1999.
36. Wen Z, Liu H, Li M, Li B, Gao W, Shao Q, Fan B, Zhao F, Wang Q, Xie Q, *et al*: Increased metabolites of 5-lipoxygenase from hypoxic ovarian cancer cells promote tumor-associated macrophage infiltration. Oncogene 34: 1241-1252, 2015.
37. Hassan S and Carraway RE: Involvement of arachidonic acid metabolism and EGF receptor in neurotensin-induced prostate cancer PC3 cell growth. Regul Pept 133: 105-114, 2006.
38. Karlage KL, Mogalian E, Jensen A and Myrdal PB: Inhalation of an ethanol-based zileuton formulation provides a reduction of pulmonary adenomas in the A/J mouse model. AAPS PharmSciTech 11: 168-173, 2010.
39. Romano M, Catalano A, Nutini M, D'Urbano E, Crescenzi C, Claria J, Libner R, Davi G and Procopio A: 5-Lipoxygenase regulates malignant mesothelial cell survival: Involvement of vascular endothelial growth factor. FASEB J 15: 2326-2336, 2001.
40. Ye YN, Wu WK, Shin VY, Bruce IC, Wong BC and Cho CH: Dual inhibition of 5-LOX and COX-2 suppresses colon cancer formation promoted by cigarette smoke. Carcinogenesis 26: 827-834, 2005.
41. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, *et al*: Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell 139: 891-906, 2009.
42. Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, Le QT and Giaccia AJ: Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell 15: 35-44, 2009.
43. Kirschmann DA, Seftor EA, Fong SF, Nieva DR, Sullivan CM, Edwards EM, Sommer P, Csiszar K and Hendrix MJ: A molecular role for lysyl oxidase in breast cancer invasion. Cancer Res 62: 4478-4483, 2002.
44. Graham SM, Vass JK, Holyoake TL and Graham GJ: Transcriptional analysis of quiescent and proliferating CD34+ human hemopoietic cells from normal and chronic myeloid leukemia sources. Stem Cells 25: 3111-3120, 2007.
45. Roos J, Oancea C, Heinssmann M, Khan D, Held H, Kahnt AS, Capelo R, La Buscato E, Proschak E, Puccetti E, *et al*: 5-Lipoxygenase is a candidate target for therapeutic management of stem cell-like cells in acute myeloid leukemia. Cancer Res 74: 5244-5255, 2014.
46. Chen Y, Hu Y, Zhang H, Peng C and Li S: Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. Nat Genet 41: 783-792, 2009.
47. Ruiz-Herguido C, Guiu J, D'Altri T, Inglés-Esteve J, Dzierzak E, Espinosa L and Bigas A: Hematopoietic stem cell development requires transient Wnt/β-catenin activity. J Exp Med 209: 1457-1468, 2012.
48. Hu Y, Chen Y, Douglas L and Li S: Beta-catenin is essential for survival of leukemic stem cells insensitive to kinase inhibition in mice with BCR-ABL-induced chronic myeloid leukemia. Leukemia 23: 109-116, 2009.
49. Zhao C, Blum J, Chen A, Kwon HY, Jung SH, Cook JM, Lagoo A and Reya T: Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell 12: 528-541, 2007.
50. Heidel FH, Bullinger L, Feng Z, Wang Z, Neff TA, Stein L, Kalaitzidis D, Lane SW and Armstrong SA: Genetic and pharmacologic inhibition of beta-catenin targets imatinib-resistant leukemia stem cells in CML. Cell Stem Cell 10: 412-424, 2012.
51. Lucas CM, Harris RJ, Giannoudis A, Mcdonald E and Clark RE: Low leukotriene B4 receptor 1 leads to ALOX5 downregulation at diagnosis of chronic myeloid leukemia. Haematologica 99: 1710-1715, 2014.
52. Dolinska M, Piccini A, Wong WM, Gelali E, Johansson AS, Klang J, Xiao P, Yektaei-Karin E, Strömberg UO, Mustjoki S, *et al*: Leukotriene signaling via ALOX5 and cysteinyl leukotriene receptor 1 is dispensable for in vitro growth of $CD34^+$ $CD38^-$ stem and progenitor cells in chronic myeloid leukemia. Biochem Biophys Res Commun 490: 378-384, 2017.