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Limited efficacy of BMS-911543 in a murine model of Janus kinase 2 V617F myeloproliferative neoplasm

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Abstract

Activation of JAK2, frequently as a result of the JAK2^{V617F} mutation, is a characteristic feature of the classical myeloproliferative neoplasms (MPN) polycythemia vera, essential thrombocythemia and myelofibrosis and is thought to be responsible for the constitutional symptoms associated with these diseases. BMS-911543 is a JAK2 selective inhibitor that induces apoptosis in JAK2-dependent cell lines and inhibits the growth of CD34^{+} progenitor cells from patients with JAK2^{V617F} - positive MPN. To explore the clinical potential of this inhibitor, we tested BMS-911543 in a murine retroviral transduction – transplantation model of JAK2^{V617F} MPN. Treatment was initiated at two dose levels (3 mg/kg and 10 mg/kg) when the hematocrit exceeded 70%. Following the first week, white blood cell counts were reduced to normal in the high dose group and were maintained well below the vehicle-treated mice throughout the study. However, BMS-911543 had no effect on red blood cell parameters. After 42 days of treatment, the proportion of JAK2^{V617F} - positive cells in hematopoietic tissues was identical or slightly increased compared to controls. Plasma concentrations of IL-6, IL-15, and TNFα were elevated in MPN mice and reduced in the high dose treatment group, while other cytokines were unchanged. Inhibitor activity after dosing was confirmed in a cell culture assay using the plasma of dosed mice and pSTAT5 flow cytometry. Collectively, these results show that BMS-911543 has limited activity in this murine model of JAK2^{V617F} – driven MPN and suggest that targeting JAK2 alone may be insufficient to achieve effective disease control.

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Authorship: ADP designed experiments and implemented the study, performed analyzes, and prepared the manuscript. AME assisted with experiments and provided manuscript critiques. AVS assisted with experiments. MSZ provided assistance with the manuscript. JEM designed and performed flow cytometry. JTP provided guidance regarding the model and manuscript critiques. TO provided project direction and manuscript critiques. MWD is the principal investigator, provided project direction, technical guidance, manuscript critiques, and takes primary responsibility for this manuscript.

No potential conflicts of interest were disclosed by the other authors.

Keywords

Polycythemia vera; Myelofibrosis; Janus kinase; pSTAT5

Introduction

An activating mutation in the JAK2 gene ($JAK2^{V617F}$) is common in patients with myeloproliferative neoplasms (MPN), including over 90% of patients with polycythemia vera (PV) and 30–50% of patients with primary myelofibrosis (PMF) or essential thrombocythemia (ET) (1–6). Ruxolitinib, the only JAK inhibitor thus far approved for clinical use in PMF, is equipotent against JAK1 and JAK2 (Table 1), but is less active against the remaining JAK family members TYK2 and JAK3 (7–12). While ruxolitinib effectively controls MF symptoms, it has no significant impact on disease burden, raising the question whether more potent and specific inhibitors of JAK2 may target the MPN clone more effectively. BMS-911543 is a highly selective inhibitor of JAK2. In kinase assays, BMS-911543 is 356-fold more potent against JAK2 compared to JAK1, 73-fold more potent against JAK3 and 66-fold more potent against TYK2 (12). BMS-911543 was shown to selectively inhibit the proliferation of JAK2-dependent cell lines and to reduce colony growth by $V617F^{+}$ patient samples at submicromolar concentrations (12). BMS-911543 is bioavailable in mice and has been shown to inhibit pSTAT5 activity in xenografts using the $JAK2^{V617F}$ heterozygous SET2 cell line or BaF3 cells co-expressing $JAK2^{V617F}$ and EpoR, with IC_{50} values achieved at ~2 mg/kg and up to 90% suppression of pSTAT5 at 7 hours post dosing (12). As effects on tumor growth were not reported and xenografts are imperfect models of human MPN, we decided to test BMS-911543 in a retroviral transduction – transplantation model of MPN. This model closely resembles human PV and progresses to secondary MF (13).

Methods

Induction of MPN, study design and drug administration

A PV-like myeloproliferative neoplasm (MPN) was induced in female Balb/c mice as previously described (11, 13). Once the average hematocrit of the cohort exceeded 70%, four mice were euthanized and examined to confirm MPN through the presence of splenomegaly and GFP^{+} cells in the spleen and bone marrow. Mice were randomly assigned to three treatment groups; vehicle control, 3 mg/kg (low dose, LD), and 10 mg/kg (high dose, HD)). The 3 and 10 mg/kg doses were chosen based on the near complete suppression of STAT5 phosphorylation observed following 2, 5, and 10 mg/kg dosing. (12). BMS-911543{(N,N-dicyclopropyl-4-((1,5-dimethyl-1H-pyrazol-3-yl)amino)-6-ethyl-1-methyl-1,6-dihydroimidazo[4,5-d]pyrrolo[2,3b]pyridine-7-carboxamide)} was prepared in a solution of 20% citrate/80% PEG400 with brief sonication and aliquots were stored at −20°C. Mouse weight was recorded weekly and drug dilutions were made according to the average weight of each group. Details of the compound, including structure and IC_{50} values, are provided elsewhere (12). Pharmacodynamic studies were performed on three mice per group (nine total) four hours after administration of the first dose of BMS-911543. Mice were dosed by oral gavage (100 μl/dose) once daily for 42 days, at which point one healthy

control, six vehicle-treated mice and seven mice per treatment group were harvested for examination. Two mice from each drug treatment group were observed for an additional 32 days after discontinuation of drug. Complete blood counts were performed once or twice weekly on a Heska HemaTrue (Loveland, CO) and GFP percentage (as a proxy for disease burden) was determined weekly on a Guava 6HT flow cytometer (Millipore, Billerica, MA). A schematic of the study design is presented in Figure 1. Mouse procedures were carried out according to guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Utah.

Histology and reticulocyte counts

Six vehicle-treated, seven low dose, and seven high dose mice were evaluated. Livers, lungs, and spleens were dissected and placed in freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate buffered saline for 24 hours then moved to 70% ethanol. Bones were placed in 4% paraformaldehyde in phosphate buffered saline overnight, moved to Formical-2000 (Decal Chemical Corp., Tallman, NY) for 7–9 days, then placed in 70% ethanol. Tissues were embedded in paraffin, sectioned at 5 micrometers, and stained with H&E. Separate bone marrow sections were also stained with Chandler's Reticulin Stain Kit (American MasterTech, Lodi, CA). For reticulin scoring, three images per mouse were captured at 20x and blindly scored according to the 0–3 semi-quantitative method described by Thiele et al. (14). Reticulocyte counts were performed on 10 μl whole blood after 10 minutes incubation with 8 μl 0.5% new methylene blue solution (Ricca Chemical Company, Arlington, TX). Two hundred red blood cells were scored.

Flow cytometry and antibodies

For pSTAT analysis, blood, spleen, and bone marrow cells were harvested 4 hours after treatment with vehicle, 3 mg/kg or 10 mg/kg BMS-911543 (N=3 for each group), fixed in 2% paraformaldehyde at 37°C for 10 minutes, stored on ice for one minute, permeabilized with 90% cold methanol for 30 minutes on ice and stored at −20°C until analyzed. Cells were washed twice in PBS supplemented with 0.5% bovine serum albumin (PBS/BSA) and then resuspended in 100 μl PBS/BSA at room temperature. Following incubation for 10 minutes, antibody was added at 1:50 for one hour. After two washes in PBS/BSA, the cells were analyzed on a BD FACS Canto. Alexa Fluor 647 conjugated antibodies directed against $pSTAT1^{Y701}$, $pSTAT3^{Y705}$ and $pSTAT5^{Y694}$ were purchased from Cell Signaling Technology (Danvers, MA; catalog numbers 8009S, 4324S, 9365S, respectively). Data was analyzed for each pSTAT as well as separately for GFP^+ and GFP^- cells. Ba/F3 cells were cytokine starved overnight then simultaneously stimulated with IL-3 (50 ng/ml) and IL-6 (100 ng/ml) for 5 minutes to serve as species-specific controls. HEL cells served as an additional control. Lineage antibodies were purchased from BD Biosciences (San Jose, CA; Ter119-V450, CD19-PE-Cy7, cKit-PerCP-Cy5.5, CD34-Alexa Fluor 647, CD19-PE-Cy7, CD41-PE, CD3-V450, CD71-PE) and eBiosciences (San Diego, CA; Gr1-PerCP-Cy5.5, B220-APC, Fc block). Aqua Live/Dead (Life Technologies, Carlsbad, CA) was included at 1:100 per sample and only live (negative) cells were analyzed. For detection of lineage markers, freshly isolated cells were incubated in a 96 well plate in PBS/BSA and 0.5 μg Fc block for 10 minutes, antibody for 30 minutes, washed twice with PBS, fixed with 2%

paraformaldehyde and analyzed on a BD FACS Canto the following day. Compensation was set with OneComp eBeads from eBiosciences.

Cytokine profiling and statistics

Cytokine profiling was performed with the Milliplex Mouse Cytokine/Chemokine kit by Millipore and analyzed with a MAGPIX instrument (Luminex Corporation, Austin, TX). Cytokine quantifications more than threefold above or below the data set median were not used for analysis. All data sets in this publication were compared in Microsoft Excel with a student's t-test using two-tailed distribution and two-sample equal variance. Significance was assigned to p values ≤ 0.05.

Indirect plasma drug activity assay

Given that BMS-911543 was minimally effective at altering the natural course of murine MPN, we sought to determine whether the drug was bioavailable. We dosed four mice at 30 mg/kg and harvested plasma 90 minutes later. Plasma was centrifuged twice at 21,000 g for 10 minutes to remove all cellular debris, incubated with 1.8 mg/mL of Proteinase K (Qiagen, Netherlands) at 37°C for 1 hour to digest proteins (including cytokines), and centrifuged through an Amicon Ultra-0.5 centrifugal filter unit with Ultracel-3 membrane (3 kDa nominal molecular weight cut off, Millipore) to ensure removal all cytokines. Ba/F3 cells (1×10^5/well, 96 well plate, 100 μL) were IL-3 starved overnight, incubated for 30 minutes with 50 μL of either vehicle plasma (n=3) or with plasma from mice dosed with BMS-911543 at 30 mg/kg (n=4). This dose was chosen to accommodate the need to dilute the plasma 1:3 in cell culture medium (100 μL plasma plus 50 μL plasma) while maintaining a sufficiently high concentration to allow for a clear effect to be observed. Following the 30 minute incubation period, murine IL-3 (50 ng/mL, Peprotech, Rocky Hill, NJ) was added to each well, incubated for 5 minutes at 37°C, then cells were prepared for flow cytometry following standard fixation/permeabilization steps and anti-pSTAT5 (Y694)-Alexa Fluor 647 (Cell Signaling Technology). Data was collected on a BD FACSCanto and analyzed with FlowJo X (Treestar, Ashland, OR).

Results & Discussion

Induction of MPN

$JAK2^{V617F}$-positive MPN was induced in lethally irradiated Balb/c mice by transplantation of bone marrow cells retrovirally transduced with MIG-$JAK2^{V617F}$, as described (Figure 1) (13). An average hematocrit over 70% was predefined as the trigger for the initiation of treatment. Twenty-one days after transplant, the hematocrit was 74±6%, red blood cells $10.7\pm0.8\times10^{12}$/L, hemoglobin 195±11.6 g/L and white blood cells $31.0\pm27.9\times10^9$/L, with 27.7±3.0% granulocytes and 68.4±5.8% lymphocytes. FACS revealed 34.2±12.2% GFP^+ cells in the peripheral blood. The following day, four mice were sacrificed and subjected to autopsy and histopathology. Spleen weights were increased to 304.5±46.9 mg (healthy control=76.8 mg), bone marrow was hypercellular and splenic architecture was effaced. GFP^+ cells in bone marrow and spleen were 26.1±7.6% and 16.7±2.9%, respectively. Together, these parameters established a diagnosis of MPN. On the following day, BMS-911543 treatment was initiated at two dose levels [low dose (LD) = 3 mg/kg and high

dose (HD) = 10 mg/kg, once daily by oral gavage] based on the pharmacokinetic data and maintained for 42 days (except in mice harvested for pharmacodynamic studies). Vehicle control mice received carrier only.

BMS-911543 suppresses leukocytosis but not erythrocytosis in JAK2^{V617F}-induced MPN

Four hours after the first treatment, three mice per group were euthanized for pSTAT analysis. Spleen, bone marrow, and blood were harvested and cells were analyzed by flow cytometry to evaluate pSTAT1^{Y701}, pSTAT3^{Y705}, and pSTAT5^{Y694}. pSTAT5^{Y694} was reduced in the peripheral blood of treated mice compared to controls, both in GFP^{+} and GFP^{-} cells, although the difference did not quite reach statistical significance ($p<0.10$ for each, Supplemental Figure 1). In contrast, pSTAT5^{Y694} was identical in the marrow and spleen. pSTAT1^{Y701} and pSTAT3^{Y705} in treated mice were generally comparable to controls (Supplemental Figures 1–3). The mice were followed by daily inspection and 1–2 weekly complete blood counts (CBC). After an initial decrease in all groups (Figure 2), the white blood cells (WBC) in the vehicle treated group steadily increased, reaching 40×10^9/L on day 25 of treatment. In contrast, the WBC in the LD and, more so in the HD treatment groups, were reduced, although differences became smaller toward the end of the study. Relative proportions of granulocytes decreased over the treatment period. Erythrocytosis, hemoglobin and hematocrit gradually decreased into the normal range, but were not different between treatment groups and controls (Figure 2). Reticulocyte counts were also identical between treated mice and controls (Supplemental Figure 4), and platelet counts remained in the normal range, as previously described with this model (13). Thus, BMS-911543 suppressed the leukocytosis that is associated with JAK2^{V617F} MPN, without affecting red cell parameters. Following the 42-day treatment period, two randomly selected high dose and two low dose mice were observed for an additional 32 days after discontinuation of BMS-911543. All mice showed a further increase in WBC that was maintained for the 32-day observation period (Supplemental Figure 5). Red cell parameters remained stable, probably reflecting the fact that the disease had entered the ‘spent phase’ and transformed to myelofibrosis. At autopsy, spleens were enlarged but their weight was reduced (~184 mg; data not shown) compared to the spleens harvested at day 1 (~300 mg) or day 42 (~230 mg) (Supplemental Figure 4). Five healthy mice were dosed with 10 mg/kg BMS-911543 and five with vehicle in a similar, separate experiment and no changes in spleen mass, spleen or bone marrow histology (Supplemental Figure 6), or blood parameters (Supplemental Figure 7) were observed. Gr1^{+} cells were reduced from 11.6 to 8.6% ($p=0.026$) in the BMS-911543 group while B and T cell populations were not altered (data not shown, compare to Figure 4), suggestive of mild splenic and/or myeloid toxicity.

BMS-911543 treatment does not significantly alter MPN histology

On treatment day 42, spleens, livers and femurs were harvested from seven mice in each of the two treatment groups and six vehicle controls. Spleen weight was similar in all groups (Supplemental Figure 4). Histology showed trilineage hematopoiesis with prominent megakaryocytes (Figure 3). Spleens from the vehicle and LD (not shown) groups exhibited complete effacement of splenic architecture, while the HD group showed some restoration of germinal centers (Figure 3). Liver histology revealed megakaryocytes and extramedullary hematopoiesis, especially near portal veins, without significant differences between groups.

Bone marrows were uniformly hypocellular and reticulin staining showed severe fibrosis in each group, with average scores near 2.5 on the 0–3 scale (Supplemental Figure 4) (14). We observed a higher percentage of total GFP^+ cells ($JAK2^{V617F}$ – positive) in the spleen (85.1±0.3% vs. 75.6±8.3%; p=0.014) and bone marrow (67.1±6.7% vs. 57.2±7.3%; p=0.026) of mice treated with HD BMS-911543 compared to the vehicle control group (Supplemental Figure 4).

BMS-911543 partially restores lineage composition of spleen but not marrow or blood

Spleen, marrow, and peripheral blood were harvested after 42 days of treatment and analyzed by flow cytometry for lineage markers. A dose dependent reduction of granulocytes ($Gr1^+$) and partial restoration of B ($B220^+$, $CD19^+$) and T ($CD3^+$) cell distributions was observed in the spleen (Figure 4), but not in the blood or bone marrow (Supplemental Figure 8). No significant differences were observed in other lineage markers, except a small reduction of peripheral blood B cells in the HD group (1.3±0.4 vs. 0.8±0.3%, $p<0.05$). To evaluate the impact of BMS-911543 treatment on MPN burden we measured GFP expression as well as immunophenotypically defined subsets of blood, bone marrow, and spleen cells. BMS-911543 had little effect on the percentage of GFP^+ cells in each cellular subset or even led to a mild increase (e.g. GFP^+ bone marrow granulocytes were increased to 20.3±4.8% in the HD group compared to 15.0±3.2% in the vehicle group, p=0.0377 (Supplemental Figure 9). These results suggest that BMS-911543 fails to provide JAK2 wild-type cells with a competitive advantage over MPN cells throughout all cell compartments analyzed.

BMS-911543 partially normalizes serum cytokine concentrations

JAK family inhibitors like TG101209 or momelotinib (formerly Cytopia387) have been shown to partially normalize cytokine concentrations in mice with $JAK2^{V617F}$-induced MPN (13, 15). We therefore measured serum cytokine profiles after 42 days of treatment with BMS-911543. TNFα, IL-6, and IL-15 concentrations were significantly reduced in the HD group compared to the vehicle control, with TNFα and IL-15 reaching the normal range (Figure 5). G-CSF and KC concentrations were reduced in MPN mice compared to normal mice, with further reduction upon treatment with BMS-911543 (Supplemental Figure 10). IL-10, IL-17, and VEGF concentrations were also elevated in MPN mice but not reduced by treatment (Supplemental Figure 10).

BMS-911543 is present and active in the plasma of treated mice

The limited efficacy of this inhibitor mandated verification of the presence of BMS-911543 in the plasma of mice following oral dosing. While this has been reported elsewhere (12), we sought to confirm bioavailability in our system. To this end we developed a functional, indirect plasma drug activity assay, using plasma from mice treated with BMS-911543 in a cell culture assay with phosphorylation of STAT5 (Y694) as the end point. At a dose of BMS-911543 equivalent to 10 mg/kg we observed a 48% reduction of pSTAT5 MFI compared to controls (p=0.002; Supplemental Figure 11), confirming the presence of active drug.

Our data show that BMS-911543 has limited efficacy in our murine model of $JAK2^{V617F}$ -induced MPN. Although we did not perform a direct comparison, the results are clearly inferior to those of momelotinib tested in the same disease model (11). As momelotinib is equipotent against JAK1 and JAK2, but BMS-911543 is highly selective for JAK2, it is conceivable that JAK1 inhibitory activity is required for more profound effects in this mouse model of MPN. Direct in vivo validation of this notion would require treating mice with drug concentrations that are equipotent toward JAK2, which would be challenging due to differences in their pharmacokinetic properties. Similar to CYT387 (11), treatment was initiated when the hematocrit of the cohort exceeded 70%. Given that this is a late model of MPN, more profound effects may have been observed with earlier treatment initiation. The limited activity of BMS-911543 against the MPN phenotype is reflected by PD studies. BMS-911543 reduced $pSTAT5^{Y694}$ only in the peripheral white blood cells, but not the bone marrow or spleen cells, even at high doses (10 mg/kg). This is in contrast to its strong effects in cell line xenografts, where a 90% suppression of $pSTAT5^{Y694}$ was reported after a single dose of 10 mg/kg (12), suggesting that $pSTAT5^{Y694}$ in these key disease sites is not, or is not exclusively, under the control of JAK2, and that the physiological microenvironmental context is critical for PD assessment. Similarly, BMS-911543 did not reduce $pSTAT3^{Y705}$, suggesting that JAK1 inhibition may be required to suppress this key regulator of inflammatory cytokine responses. A phase 1/2 study of BMS-911543 is ongoing and it will be interesting to see whether limited activity of the compound in murine $JAK2^{V617F}$-induced MPN model is reflected clinically (16)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A highly selective JAK2 inhibitor was tested in a murine model of JAK2^{V617F+} MPN
- Despite high preclinical potential, BMS-911543 is minimally effective in murine MPN
- JAK2 inhibition is not sufficient to control the symptoms of murine MPN
- Selective inhibition of JAK2 may be detrimental to healthy cells and/or advantageous to malignant cells

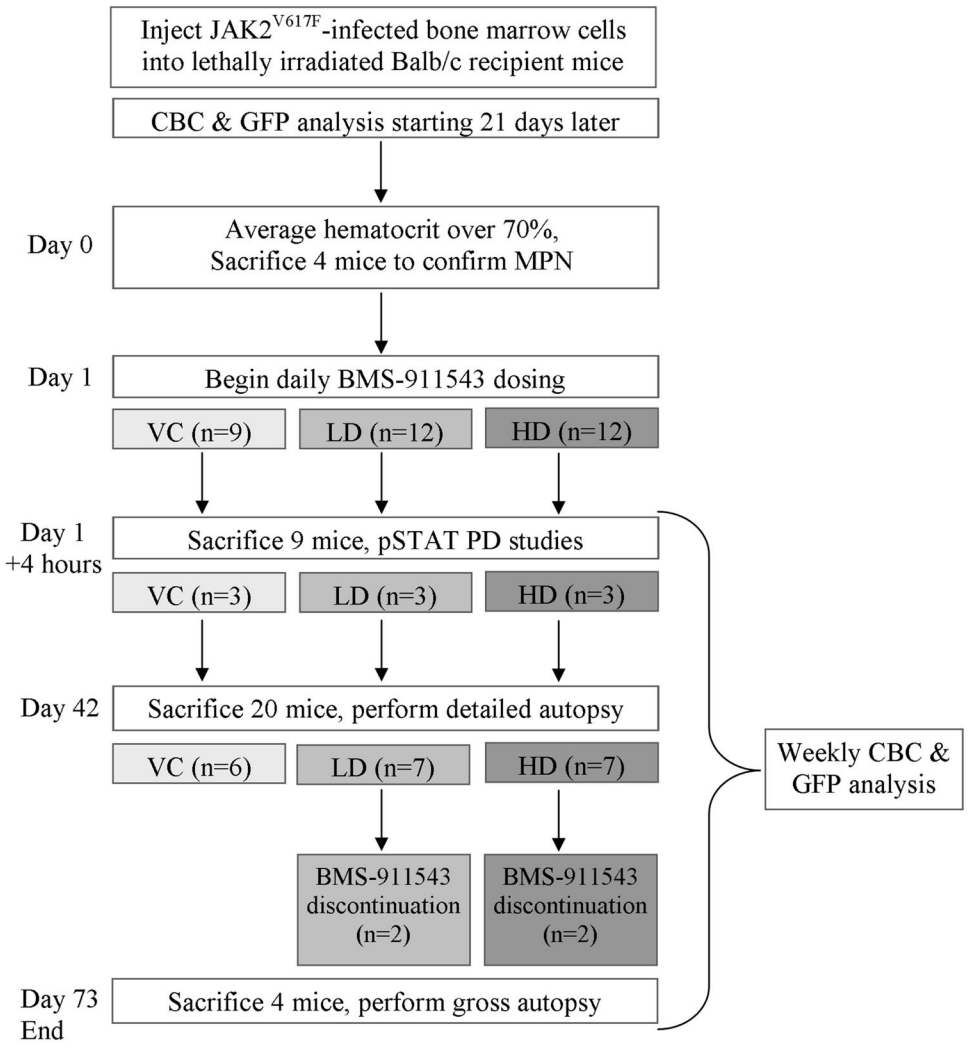

Figure 1. A schematic of the research plan

Donor mice received 5FU and bone marrow cells were harvested five days later. These cells were infected with MIG-JAK2^{V617F} and injected into lethally irradiated recipient mice. Dosing with BMS-911543 began when the average hematocrit exceeded 70% and continued for 42 days. VC - vehicle control, LD - low dose (3 mg/kg), HD - high dose (10 mg/kg), PD - pharmacodynamics, CBC - complete blood count, GFP - green fluorescent protein

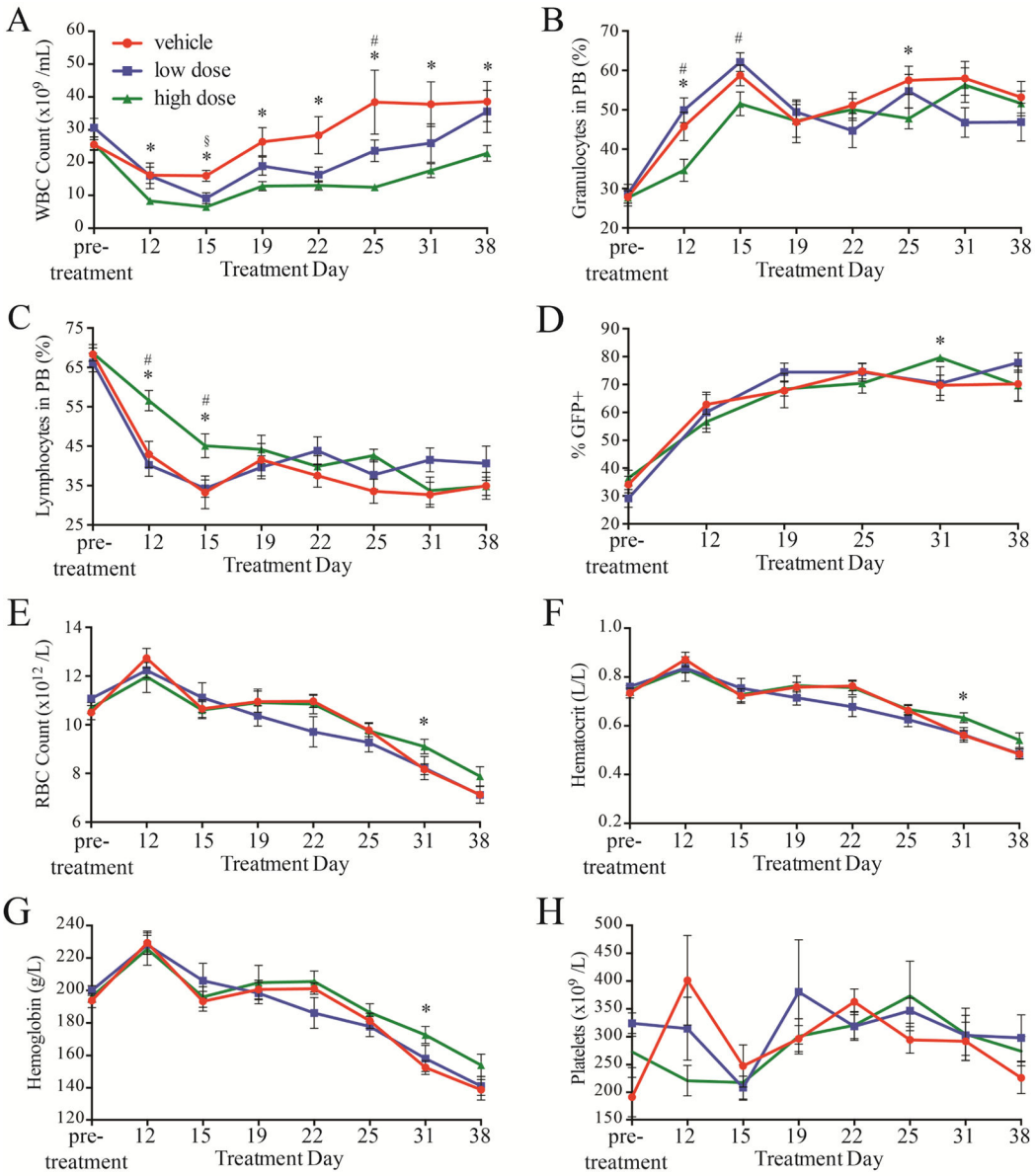

Figure 2. BMS-911543 lowered and maintained white blood cell counts in murine MPN

(A) The high dose group maintained white cell counts that were nearly normal and well below the vehicle-treated group for the treatment period. (B) Granulocytes, as a percentage of white blood cells, were reduced and (C) relative lymphocyte counts were elevated by high dose BMS-911543 early in the treatment period, but all groups became indistinguishable after 15 days of treatment. (D) The percentage of peripheral white cells positive for GFP increased steadily in all groups throughout the treatment period and were slightly higher in the HD group at day 31. (E, F, G) Red blood cells, hematocrit, and hemoglobin decreased steadily throughout the treatment period. (H) Platelet counts did not vary between groups. Data from ten vehicle, ten low dose, and nine high dose mice are shown. * $p \leq 0.05$ high dose vs. vehicle; § $p \leq 0.05$ low dose vs. vehicle; # $p \leq 0.05$ low dose vs. high dose. Error bars represent standard error of the mean.

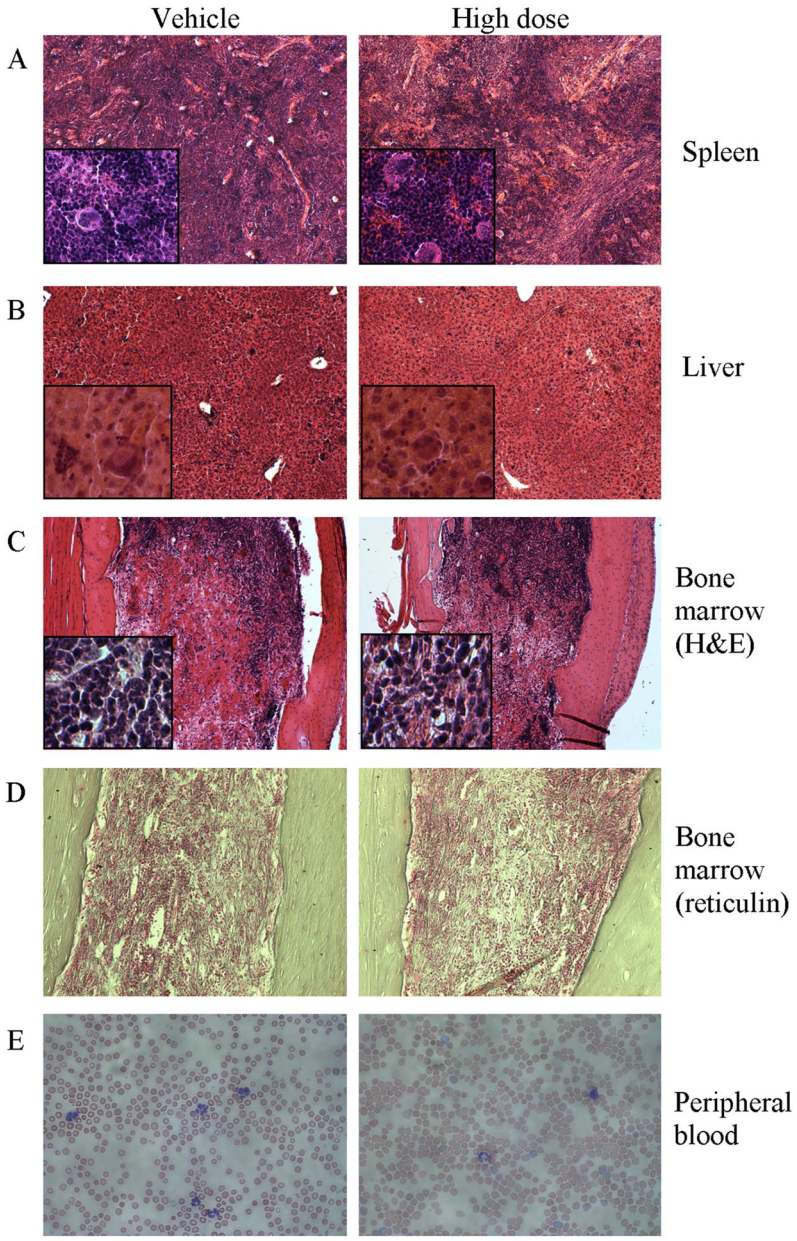

Figure 3. Histopathology after 42 days of treatment with BMS-911543

Representative histological sections are shown for (A) spleen, (B) liver, (C) bone marrow stained with H&E, (D) bone marrow stained with Chandler's reticulin stain, each at Day 42, and (E) peripheral blood (Wright's stain) at Day 35. A–C are 10x with 40x insets, D is 20x, and E is 40x.

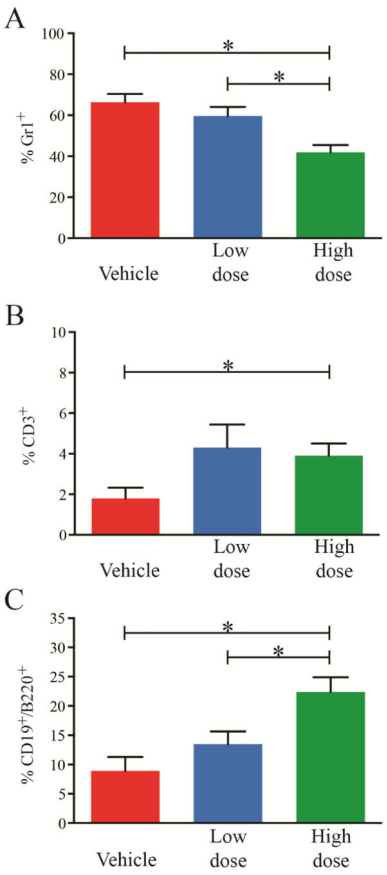

Figure 4. Effect of BMS-911543 on hematopoietic cell lineages in MPN

Following 42 days of treatment, high dose BMS-911543 reduced the granulocytes (A, $Gr1^+$) and increased the T ($CD3^+$) and B ($CD19^+/B220^+$) cells (B and C, respectively) in the spleen, but not the blood or bone marrow (not shown). Flow cytometry data from six vehicle, seven low dose, and seven high dose mice are shown. Error bars represent standard error of the mean. *$p \leq 0.05$

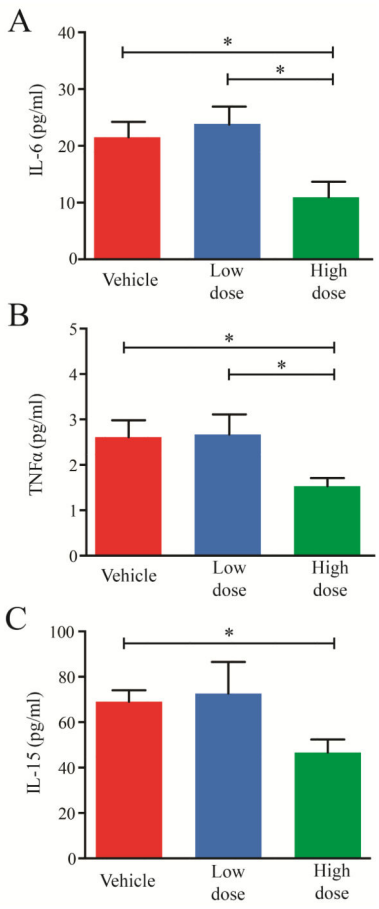

Figure 5. Effect of BMS-911543 on cytokine concentrations in MPN

Serum was evaluated after the 42 day treatment period and IL-6 (A), TNFα (B), and IL-15 (C) were elevated compared to normal mice and reduced in the high dose group compared to the vehicle-treated group. Data from six vehicle, seven low dose, and seven high dose mice are shown. Error bars represent standard error of the mean. *p≤0.05

Table 1

Summary of JAK family inhibitors.

Inhibitor name	Company	JAK1	JAK2	JAK3	TYK2	Citation
BMS-911543	Bristol-Myers Squibb	356	1	73	66	Purandare et al., 2012
ruxolitinib (INCB018424)	Novartis	3.3	2.8	428	19	Quintas-Cardama et al., 2010
TG101209	Sanofi-Aventis	ND	6	169	ND	Pardanani et al., 2007
tofacitinib (CP-960,550)	Pfizer	112	20	1	ND	Changelian et al., 2003
pacritinib (SB1518)	S*BIO	1280	23	520	50	William et al., 2011
momelotinib (CYT387)	Gilead	11	18	155	17	Tyner et al., 2010