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ORIGINAL ARTICLE

Characterization of BMS-911543, a functionally selective small-molecule inhibitor of JAK2

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We report the characterization of BMS-911543, a potent and selective small-molecule inhibitor of the Janus kinase (JAK) family member, JAK2. Functionally, BMS-911543 displayed potent anti-proliferative and pharmacodynamic (PD) effects in cell lines dependent upon JAK2 signaling, and had little activity in cell types dependent upon other pathways, such as JAK1 and JAK3. BMS-911543 also displayed anti-proliferative responses in colony growth assays using primary progenitor cells isolated from patients with JAK2^{V617F}-positive myeloproliferative neoplasms (MPNs). Similar to these *in vitro* observations, BMS-911543 was also highly active in *in vivo* models of JAK2 signaling, with sustained pathway suppression being observed after a single oral dose. At low dose levels active in JAK2-dependent PD models, no effects were observed in an *in vivo* model of immunosuppression monitoring antigen-induced IgG and IgM production. Expression profiling of JAK2^{V617F}-expressing cells treated with diverse JAK2 inhibitors revealed a shared set of transcriptional changes underlying pharmacological effects of JAK2 inhibition, including many STAT1-regulated genes and STAT1 itself. Collectively, our results highlight BMS-911543 as a functionally selective JAK2 inhibitor and support the therapeutic rationale for its further characterization in patients with MPN or in other disorders characterized by constitutively active JAK2 signaling.
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Introduction

The Janus kinases (JAK) are a family of non-receptor tyrosine kinases comprised of JAK1, JAK2, JAK3 and TYK2.[1] JAKs integrate cytokine signal transduction cascades in a variety of diverse cell types through the phosphorylation and subsequent activation of the family of signal transducer and activator of transcription (STAT) proteins that in turn regulate a gene expression program underlying survival, proliferation and differentiation.[2,3] Human genetic disorders or mouse knockout studies have collectively implicated essential roles of JAK-STAT signaling in various aspects of hematopoiesis. The importance of these family members in immune cell function is highlighted by observations that genetic inactivation of human JAK3 or its cognate cytokine receptor, γ_c, results in a severe combined immunodeficiency.[4,5] Similarly, JAK1 deficiency is associated with decreased lymphocyte development through defects in type I (IFN-α) and type II (IL-2) cytokine signaling, whereas human TYK2 deficiency is associated with impaired antimicrobial and allergic responses indicating important roles of these family members in the regulation of key aspects of immune cell development and maintenance.[6,7] In contrast, JAK2 has been implicated in erythropoiesis through its association with the selective association with the single chain cytokine receptor, erythropoietin receptor (EPO-R).[8]

Myeloproliferative neoplasms (MPNs) are clonal malignancies characterized by the uncontrolled expansion of multipotent hematopoeitc progenitor cells.[9] The most common *BCR–ABL*-negative MPNs are polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis. Each MPN is associated with clinically distinct features with PV, ET and primary myelofibrosis being associated with increased erythrocytes, platelets or bone marrow fibrosis, respectively, and all MPNs are associated with progression to acute myeloid leukemia.[10,11] Several laboratories identified *JAK2^{V617F}*-activating mutations at high prevalence in MPNs with frequencies of mutation estimated at ~90% in PV and ~50% in primary myelofibrosis and ET.[12–14] In *JAK2^{V617F}*-negative MPN, mutually exclusive activating mutations in the *JAK2* at exon 12 have also been observed.[15,16] Lastly, in ~5–10% of *JAK2^{V617F}*-negative ET and primary myelofibrosis, mutations that result in the ligand-independent activation of the thrombopoietin receptor have been identified which produce constitutive downstream activation of JAK2-STAT signaling.[17,18] Transgenic expression of these mutations into hematopoietic progenitor cells in mice is able to reconstitute many of the features of human MPN including increased red cell mass, splenomegaly and bone marrow fibrosis.[19,20] Collectively, these findings underscore the critical role of aberrant JAK2 signaling in MPNs and highlight JAK2 as an attractive molecular target for the therapeutic intervention in MPN and other malignancies associated with aberrant JAK2-STAT signaling.

Because of these features, several small-molecule JAK inhibitors are being optimized pre-clinically or are being tested in clinical development for patients afflicted with MPN.[21] The compounds in development inhibit JAK2 tyrosine kinase activity by competing for ATP binding and have varying degrees of cross inhibitory activity to other JAK family members, such as JAK1 and JAK3, as well as other kinases in the kinome.[22] A consequence of non-selectivity to JAK1 or JAK3 is thought to be a potential for immunosuppression given the role of these kinases in immune cell function[23] and as such, compounds with such activities may be associated with additional dose-limiting toxicities compared with other JAK2-selective inhibitors. Here, we report the characterization of BMS-911543, a reversible

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pyrrolopyridine ATP-competitive inhibitor of JAK2. The results of our characterization highlight the high degree of functional selectivity of this small-molecule inhibitor toward JAK2 in a variety of pre-clinical models and support the ongoing clinical testing of BMS-91153 in MPN patients.

Materials and methods

Reagents

The chemical characterization of BMS-911543 (*N,N*-dicyclopropyl-4-((1,5-dimethyl-1*H*-pyrazol-3-yl)amino)-6-ethyl-1-methyl-1,6-dihydroimidazo[4,5-d]pyrrolo[2,3b]pyridine-7-carboxamide) will be described elsewhere (AP and MVL, unpublished data). BMS-911543 was prepared in dimethylsulfoxide as a 10 mM stock solution or in 20% citrate/80% PEG400 vehicle for *in vitro* or *in vivo* experiments, respectively. Recombinant enzymes were purchased from Invitrogen (Carlsbad, CA, USA) or produced as described previously.[24] Keyhole limpet hemocyanin (KLH) used for immunization was purchased from Pierce (Rockford, IL, USA). SET2 cells were obtained from DSMZ (Braunschweig, Germany).

Biochemical kinase assays

The inhibitory activity of BMS-911543 in biochemical kinase assays using recombinant enzymes was performed as described previously.[24] Briefly, incubation mixtures included: 1.1 nM JAK2, 1.5 μM peptide substrate (5-FAM-KKKKEEIYFFFG-OH for JAK2) and 30 μM ATP. The reaction mixture was analyzed on a Caliper LabChip 3000 (Caliper LifeSciences, Hopinkton, MA, USA) by electrophoretic separation of the fluorescent substrate and phosphorylated product after 180 min. The inhibitory activity of BMS-911543 against multiple other recombinant enzymes was evaluated using similar methodology in kinase assays or for interaction with over 450 kinases at 1 μM in collaboration with Ambit Biosciences (San Diego, CA, USA) using competition-binding assays, as described previously.[25] For enzyme kinetics, BMS-911543 was tested from 42 pM to 8.33 μM against JAK1, JAK2 or JAK3. All kinase reactions were carried out at room temperature with γ-[^{33}P]-labeled ATP at 3.75–100 μM for 30 min and terminated by the addition of 1% phosphoric acid. Phosphorylated peptide was captured on 96-well phosphocellulose filter plates using a vacuum manifold and quantified using scintillation counting. k_i was determined from global fits using a competitive inhibition model for JAK1 and JAK3: $v = (V_{max} \times [S])/(k_m \times ((1 + [I]/k_i)^n) + [S])$, and a mixed-type inhibition model for JAK2: $v = V_{max} \times [S]/(k_m \times (1 + ([I]/k_i)^n) + [S] \times (1 + ([I]/k_i')^n))$.

Anti-proliferative and apoptosis assays

The anti-proliferative effects of BMS-911543 on tumor cell lines were monitored by [^{3}H] thymidine incorporation. Cells were incubated with stepwise dilutions of compound for 72 h in RPMI media supplemented with 10% fetal bovine serum. On day 4, 0.022 μCi/ml of [^{3}H] thymidine was added to each well and allowed to incubate for 3–4 h. Cells were harvested onto filter plates, washed and processed for incorporated radioactivity on a scintillation counter. In certain instances, Ba/F3-engineered cells were propagated in the presence of recombinant human erythropoietin (EPO, R&D Systems, Minneapolis, MN, USA) or recombinant mouse IL-3 (Invitrogen). SET2 or Ba/F3 cell lines were treated with BMS-911543 as indicated in the results section and processed for the induction of apoptosis via fluorescence-activated cell sorting (FACS) analysis using annexin V surface staining following the manufacturer's instructions (Sigma, St Louis, MO, USA). Percent apoptosis was expressed as the level of annexin V staining observed versus dimethylsulfoxide control under the same treatment conditions.

Human whole-blood assays and MPN-patient colony growth assays

JAK family functional selectivity was monitored in human whole blood *ex vivo* using FACS and cytokine stimulation. Briefly, blood from healthy volunteers was drawn and added to the 96-well plate containing varying concentrations of compound in dose–response format and incubated with a fixed concentration of cytokine for 1 h. Removal of red blood cells by lysis and fixation of the remaining cells was achieved by incubation at 37 °C for 15 min (Fix and Lyse, BD Biosciences, San Jose, CA, USA). Surface antibodies were then incubated with the cells prior to permeabilzation and phospho-STAT antibodies addition. Cytokine-stimulated JAK2 activity was monitored in human platelets selected with CD61 fluorescein isothiocyanate-conjugated antibody (BD Biosciences), with thrombopoietin (TPO) stimulation (Peprotech, Rocky Hill, NJ, USA) and pSTAT5 detection (pY695 Alexa647 conjugated, BD Biosciences). Samples were analyzed on a FACS Canto II using DIVA 6.1.1 software (BD Biosciences). Assays for JAK1, JAK3 and TYK2 functional selectivity responses were monitored in a similar format using CD3-selected lymphocytes stimulated with IL-2, IL-4, IL-15 and IFN-α (Peprotech). Inhibitory concentration (IC_{50}) determinations were calculated based on 50% inhibition on the fold-induction between cytokine and unstimulated treatments. The protocol for examining the effects of JAK2 inhibitors on MPN-patient colony growth has been reported previously.[26] Briefly, CD34$^+$ progenitor cells were isolated from MPN patient or healthy volunteer blood using FACS. Freshly isolated CD34$^+$ cells were plated in methylcellulose-based, semisolid media (MethoCult, Stem Cell Technologies, Vancouver, Canada) supplemented with cytokines. Colony growth was measured over the course of 2 weeks and was scored according to standard morphological criteria.

Western blot analysis

Evaluation of BMS-911543 effects on Ba/F3 or SET2 cell lines or tumor xenograft lysates was performed by western blotting. For cell lines, roughly 500 000 cells per ml of media were incubated with compound in dose–response format for 2 h, and subsequently processed for western blotting for p-STAT5 (tyrosine 694, BD Biosciences, 1:400 dilution), total STAT5 protein antibodies (BD Biosciences, 1:400 dilution), p-STAT3 (tyrosine 705, Cell Signaling, Beverly, MA, USA, 1:500 dilution), STAT3 (Cell Signaling, 1:500 dilution), ID1 (R&D Systems, 1:1000 dilution), PIM1 (Cell Signaling, 1:500 dilution), pSTAT1 (tyrosine 701, Cell Signaling, 1:1000 dilution) or STAT1 (AbCam, Cambridge, MA, USA, 1:500 dilution) at 1:400 dilution. SET2 cells were also treated for 24 h with BMS-911543 for the analysis of STAT1 levels. Protein extracts from snap-frozen SET2 tumors were prepared and similarly processed for pSTAT5/STAT5, as described for the cell line analysis.

Gene-expression profiling

Following a 4-h treatment of SET2 cells with JAK2 inhibitors, mRNA was processed for the Affymetrix HT-HG-U133A array and the results were analyzed by the SDRS algorithm as described.[27] See Supplementary Information for additional details.

In Vivo *pharmacodynamic (PD) assays*

BMS-911543 dosing solutions were administered to BALB/c mice by oral gavage at the indicated dose levels. After BMS-911543 administration, triplicate animals per time point were euthanized and blood was harvested via cardiac puncture for preparation of pharmacokinetic (PK) or PD analyses. Platelets were stimulated *ex vivo* with murine TPO (mTPO, Peprotech) and stained for CD61 (anti-CD61 FITC, eBiosciences, San Diego, CA, USA). Samples were then processed for p-STAT5 levels, as described above, using anti-pY695 Alexa647-conjugated STAT5 antibody. SET2 cells were inoculated into female athymic mice and propagated as subcutaneous xenografts. Animals with tumors reaching $\sim$500 mm^3 were administered BMS-911543 or vehicle as described above for the indicated times. Tumors were snap frozen in liquid nitrogen and processed for p-STAT5 for western blot analysis as described above.

Analysis of antigen-induced antibody generation in vivo

Female BALB/c mice (6–8 week-old) were immunized intraperitoneally with 250 μg KLH (Pierce) in phosphate-buffered solution on day 0. Mice in appropriate groups were dosed as indicated with BMS-911543. Blood was collected on day 7 and day 14 post immunization. Serum was separated and analyzed for anti-KLH IgM titers (day 7) and anti-KLH IgG titers (day 14) by enzyme-linked immunosorbent assay. Captured anti-KLH antibodies were detected using horseradish peroxidase-conjugated antibody specific for mouse IgM or IgG (Southern Biotechnology Associates, Birmingham, AL, USA) and the TMB peroxidase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Optical densities of developed plates were quantified in a SpectraMax Plus ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). Serum from vehicle-treated BALB/c mice collected on day 7 (IgM) or on day 14 (IgG) after immunization with KLH was pooled and used as a standard comparator in each respective assay.

Results

In Vitro *characterization of BMS-911543*

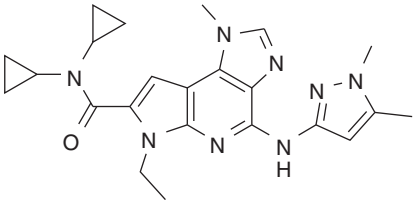
BMS-911543 is a pyrrolopyridine small-molecule inhibitor that was initially characterized using an *in vitro* assay with human recombinant JAK enzyme. BMS-911543 displayed an IC_{50} of 1.1 nM against JAK2 and was approximately 350-, 75- and 65-fold selective to JAK1, JAK3 and TYK2, respectively (Figure 1). Comparison of dissociation constants of BMS-911543 for JAK1, JAK2 and JAK3 extended these findings and displayed a broader selectivity for JAK2 with K_i values of 1114, 0.48 and 357 nM, respectively. BMS-911543 maintained selectivity outside of the JAK family in an additional 26 biochemical kinase assays and against over 450 other kinases using ATP competitive binding assays (see Supplementary Figure 1). Outside the JAK family, the highest inhibitory activity was observed for the Src family member, Lyn (IC_{50} = 300 nM), and the c-FMS receptor tyrosine kinase (IC_{50} = 450 nM), which were both significantly less sensitive than JAK2 (>250-fold). All other kinases examined displayed even less inhibition (500 to >1000-fold less sensitive) to BMS-911543. These results demonstrate the biochemical selectivity of BMS-911543 for JAK2 within the JAK family and across the broader kinome.

Functional selectivity of BMS-911543 on JAK2-dependent cell types

We next examined the effects of BMS-911543 on the cellular inhibition of JAK2-dependent responses. In JAK2^{V617F}-dependent cells such as SET2 or Ba/F3 engineered to express JAK2^{V617F}, BMS-911543 treatment resulted in a dose-dependent anti-proliferative effect with IC_{50} values of 60 and 70 nM, respectively (Figure 2a). Ba/F3 cells expressing a mutated TPO receptor (MPL^{W515L}) that constitutively activates JAK2 signal transduction also displayed an anti-proliferative effect to BMS-911543 but a decreased level (IC_{50} = 260 nM) as compared with JAK2^{V617F}-expressing cells. The anti-proliferative response of BMS-911543 correlated with a similar effect on constitutively active pSTAT5 in Ba/F^{JAK2V617F} cells (IC_{50} = 60 nM) and a dose-dependent induction of apoptosis (Figures 2c and d). Further, the comparison of cytokine-stimulated JAK2^{WT} cells to constitutively activated JAK2^{V617F} cells revealed an increased sensitivity for anti-proliferative and pSTAT5 responses for the ligand-independent pathway (Figures 2a and c). In contrast to these JAK2-dependent cells, cell lines reliant on other JAK family members, including CTLL2 and parental Ba/F cells, stimulated with IL-3 did not show a significant anti-proliferative response to BMS-911543. JAK2-STAT5 signaling has been reported to be constitutively activated in a subset of B-cell lymphomas through the genetic inactivation of the negative pathway regulator SOCS1.[28] In contrast to what was observed for JAK2^{V617F}- or MPL^{W515L}-expressing cell lines, BMS-911543 treatment did not result in a significant anti-proliferative effect in several B-cell lymphoma lines, including KARPAS1106, L1236 or HDLM-2. Lack of anti-proliferative responses were also observed in several solid tumor cell lines of distinct histopathologies (A549, MDA-MB-231, MiaPaCa-2) consistent with these cells dependence upon pathways other than JAK2.

To explore the functional selectivity of BMS-911543 further, we monitored the effects of BMS-911543 on specific cytokine-stimulated pSTATs in a human whole-blood *ex vivo* assay. For JAK2 selective responses, we monitored TPO-stimulated pSTAT5 in human platelets which showed a robust induction of the pathway that was inhibited in a dose-dependent manner by BMS-911543, an observation consistent with this type I cytokine receptor being more reliant on JAK2 for its signal transduction (Figure 2b). We also examined the effects of BMS-911543 on cytokine receptors and cell types that are more dependent upon JAK1 and JAK3, such as IL-2, IL-4 and IL-15,

a



Form. Wt.= 432.5 g/mol

b

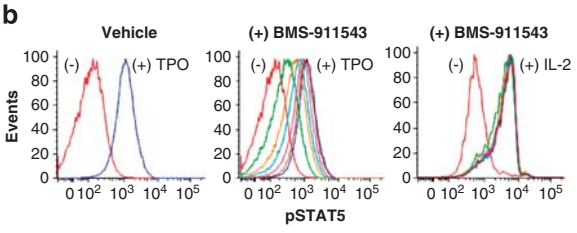
Enzyme	IC_{50} (nM)	K_i (nM)
JAK1	356 -/+ 0.1	1117
JAK2	1 -/+ 0.7	0.48
JAK3	73 -/+ 0.2	357
TYK2	66 -/+ 0.1	n.d.

Figure 1 BMS-911543 is a biochemically selective JAK2 inhibitor. (**a**) Molecular structure and formula weight (Form. Wt.) of BMS-911543. (**b**) IC_{50} of BMS-911543 against recombinant JAK family members using *in vitro* kinase assays (n = 6) or dissociation constant analysis (k_i).

a

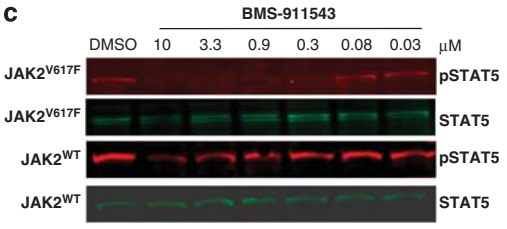
Cell Line	Origin	IC_{50}(M)
SET2	ET (JAK2^{V617F})	0.06
Ba/F3^{JAK2V617F}	Pro-B cell+JAK2^{V617F}	0.07
Ba/F3^{MPLW515L}	Pro-B cell+MPL^{W515L}	0.26
Ba/F3+IL3	Pro-B cell parental	3.5
CTLL2	T cell leukemia	2.9
KARPAS1106	B cell lymphoma	5.2
L1236	B cell lymphoma	1.2
HDLM-2	B cell lymphoma	1.7
A549	Lung cancer	4.0
MDA-MB-231	Breast cancer	4.5
MiaPaCa	Pancreatic cancer	4.7

b



Cytokine	pSTAT	IC_{50} (μM)
TPO	pSTAT5	1.7
IL-4	pSTAT6	> 10
IL-2	pSTAT5	> 10
IFN-α	pSTAT5	3.5
IL-15	pSTAT5	> 10

c



d

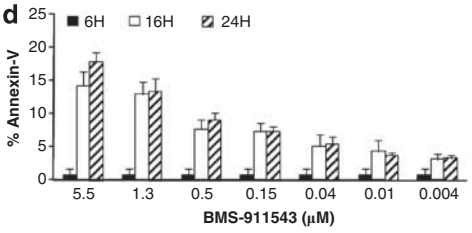


Figure 2 BMS-911543 is a functionally selective inhibitor of JAK2. (**a**) IC_{50} of BMS-911543 in cell proliferation assays using cell lines dependent upon JAK2 (SET2, Ba/F3^{JAK2V617F}, Ba/F3^{MPLW515L}) or other signaling pathways. (**b**) *Ex vivo* human whole-blood assay monitoring wild-type JAK family activation through cytokine stimulation and FACS analysis after treatment with BMS-911543 (lower table). Representative comparison of the dose-dependent effects of BMS-911543 TPO-induced pSTAT5 versus IL-2-stimulated pSTAT5 responses compared with vehicle-treated cells in CD61-gated platelets or CD3-gated lymphocytes is shown in the upper panel. Red traces and blue traces indicate the absence and presence of cytokine treatment, respectively, whereas multiple traces in (+) BMS-911543 represent treatment with different concentrations of inhibitor (10, 3.3, 1.1, 0.4 and 0.1 μM). (**c**) Effects of BMS-911543 on Ba/F3^{JAK2V617F} or Ba/F3^{JAK2WT} pSTAT5 responses in the absence or presence of EPO stimulation, respectively. (**d**) Time course of apoptosis induction in SET2 cells following JAK2 inhibition. SET2 cells were treated with the indicated concentrations of BMS-911543 for 6, 16 or 24 h and processed for FACS analysis using FITC-annexinV to measure apoptotic cells. Percent apoptosis was expressed as the level of annexin V staining observed versus dimethylsulfoxide (DMSO) control under the same treatment conditions. All values are representative of at least two independent experiments.

and found that the inhibitor did not show as significant effects compared with TPO. In contrast, a pyrrolopyrimidine pan-JAK inhibitor (see Supplementary Table 1) displayed potent responses across all cytokine end points with more pronounced effects observed for JAK1 and JAK3 end points than for JAK2 (data not shown). Consistent with the biochemical selectively of BMS-911543, these cellular responses collectively demonstrate the functional selectivity of the inhibitor for JAK2.

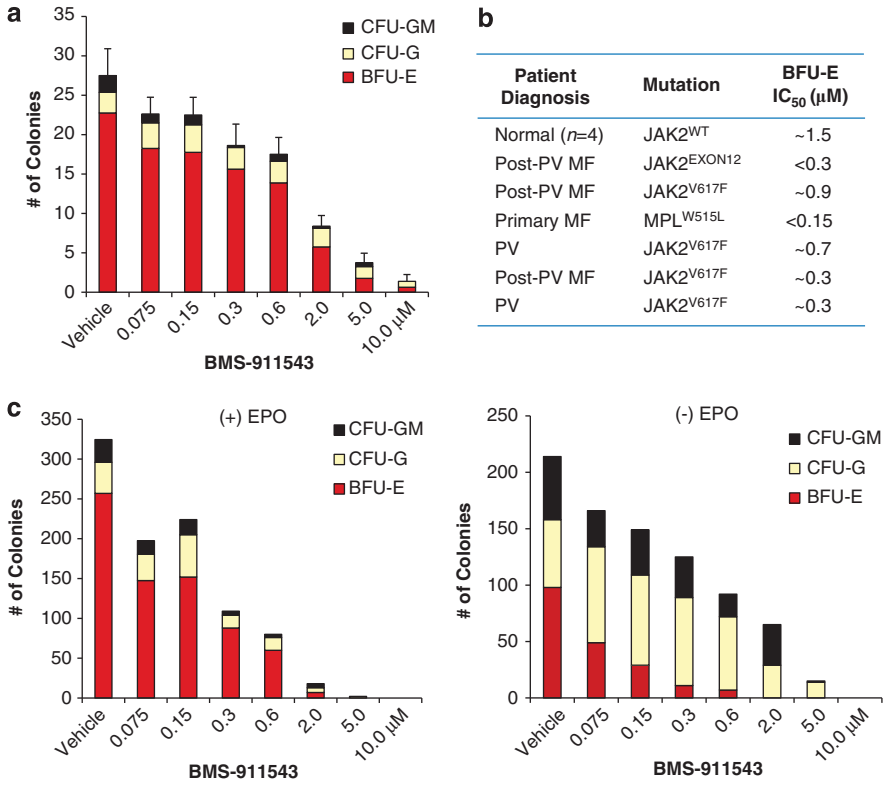
BMS-911543 suppresses the growth of MPN patient-derived cells

To further evaluate the sensitivity of BMS-911543 in a JAK2-mediated disease context, we performed colony growth assays in cytokine-supported media using primary hematopoetic progenitor cells isolated from MPN patients. The assay was first performed using $CD34^+$ progenitors isolated from normal healthy volunteers in the presence of varying concentrations of BMS-911543 to determine the effects on cytokine-stimulated JAK2^{WT} function. BMS-911543 inhibited EPO-mediated burst forming unit-erythroid (BFU-E) colony growth with an IC_{50} of ~1.5 μM (Figure 3a), a response consistent with the TPO-stimulated human whole-blood assay which monitors wild-type JAK2 function (see Figure 2). The inhibitor was next evaluated in the colonogenic growth assays using cells isolated from six different MPN patients that expressed JAK2^{V617F}, JAK2^{EXON12} or MPL^{W515L} mutations. In the presence of EPO, BFU-E IC_{50s} for BMS-911543 in MPN-patient cells ranged from <0.150 to ~0.9 μM (Figure 3b) indicating an increased sensitivity to JAK2 inhibition compared with normal healthy volunteers.

A hallmark of the JAK2-mutated MPN progenitor cells is their ability to undergo EPO-independent erythroid colony formation. To further define the sensitivity on the afflicted progenitor cells, we compared the effects of BMS-911543 on EPO-dependent and EPO-independent BFU-E potential in cells from MPN patients harboring JAK2^{V617F} mutations. In the presence of EPO, BMS-911543 displayed a BFU-E IC_{50} of ~0.3 μM compared with an IC_{50} of 0.075 μM in EPO-independent erythroid colony formation in a representative JAK2^{V617F}-positive MPN patient sample (Figure 3c). These results establish proof of concept for BMS-911543 in MPN patient primary cells *ex vivo* and demonstrate the differential sensitivity of the JAK2^{V617F} pathway compared with the cytokine-stimulated JAK2^{WT} pathway.

In vivo *pSTAT5 inhibition by JAK2 inhibition*

To extend the findings of differential pathway sensitivity to BMS-911543, we next examined the effects of the compound *in vivo*. For the analysis of the JAK2^{WT} response *in vivo*, we utilized a PD–PK model where blood was harvested from animals after compound administration at different time points and dose levels. Platelets were then isolated, treated with TPO *ex vivo* to induce the pSTAT5, and the effects of BMS-911543 at a single



Patient Diagnosis	Mutation	BFU-E IC_{50} (μM)
Normal (*n*=4)	JAK2^{WT}	~1.5
Post-PV MF	JAK2^{EXON12}	<0.3
Post-PV MF	JAK2^{V617F}	~0.9
Primary MF	MPL^{W515L}	<0.15
PV	JAK2^{V617F}	~0.7
Post-PV MF	JAK2^{V617F}	~0.3
PV	JAK2^{V617F}	~0.3

Figure 3 Effects of BMS-911543 on cytokine-dependent and -independent hematopoietic colony growth of MPN patients with activating JAK2 pathway mutations. (**a**) Cytokine-supported colony growth of erythroid cells (BFU-E, red bars), granulocytes (CFU-G, yellow bars) or granulocyte-macrophage progenitors (CFU-GM) from normal healthy volunteers in the presence of varying concentrations of BMS-911543. Error bars indicate the s.d. of mean values from four independent specimens measured in duplicate. (**b**) IC_{50} of BMS-911543 on BFU-E colony growth of cells derived from MPN patients harboring different JAK2 or MPL mutations. Each value represents each MPN patient's BFU-E IC_{50} measured in duplicate. (**c**) Comparison of the effects of BMS-911543 on hematopoietic colony growth from a JAK2^{V617F}-positive MPN patient in the absence or presence of EPO.

oral dose of 5, 10 and 30 mg/kg were compared with vehicle-treated animals. At 30 mg/kg, BMS-911543 fully suppressed (100% inhibition) pSTAT5 induction at all time points (1–18 h post dose) demonstrating the durable effects on the pathway after a single dose (Figure 4a). At the intermediate 10 mg/kg dose, ~75% reduction was observed up to 18 h. The lowest dose level tested in the study, 5 mg/kg, revealed a roughly 50% reduction in TPO-stimulated pSTAT5 by ~8 h. These dose-dependent pSTAT5 reductions correlated with the serum exposures of BMS-911543, with $AUC_{(0-8h)}$ μM h values of 22.9, 41.2 and 109.3 observed, respectively, for 5, 10 and 30 mg/kg dose levels. These results indicate durable and potent reductions of cytokine-activated JAK2^{WT}-pSTAT5 by BMS-911543 after a single oral dose.

We next examined the effects of BMS-911543 *in vivo* on the JAK2^{V617F} pathway. To this end, we developed SET2 cells to grow as a subcutaneous xenograft in athymic mice, and examined the effects of JAK2 inhibition on the constitutively pSTAT5 end point. We examined BMS-911543 at a lower dose range in this model with 1 mg/kg being the lowest dose tested. At the highest dose tested, 10 mg/kg, BMS-911543 showed 90–100% inhibition of pSTAT5 up to 7 h post dose. The 5-mg/kg dose level also showed pronounced activity, with up to 90% pSTAT5 inhibition observed at 7 h post compound administration. The 2- and 1-mg/kg dose levels also showed activity, with the 2-mg/kg dose showing ⩾50% pSTAT5 inhibition, whereas the 1-mg/kg dose showed an activity of 30–40% inhibition. In this model, 2 mg/kg of BMS-911543 was defined as the minimum dose level to achieve 50% inhibition of the pSTAT5 PD end point. Similar levels of activity were also observed for BMS-911543 on pSTAT5 in Ba/F3^{EPORV617F} tumor-bearing mice (data not shown). Collectively, these findings further demonstrate the potent and durable *in vivo* activity of BMS-911543 as well as highlight an increased sensitivity of the JAK2^{V617F} pathway *in vivo*.

Effects of JAK2 inhibition on antigen-induced humoral immune response in vivo

JAK2 selectivity of small-molecule inhibitors is thought to have less immunosuppressive potential than inhibitors containing other JAK family inhibitor activities, particularly JAK1 and JAK3. To evaluate this hypothesis functionally, we administered BMS-911543 in an antigen-induced antibody production model, which is used to assess an adaptive humoral immune response (Figure 5). In this model, mice were immunized with KLH, and serum was isolated and analyzed for anti-KLH IgM titers and anti-KLH IgG titers in vehicle- or BMS-911543-treated animals. BMS-911543 had no effect on IgM antibody titers at day 7 at all doses tested, including the highest dose of 30 mg/kg. Reductions in IgG levels by BMS-911543 were observed at 10 (~65% reduction) and 30 mg/kg (~85% reduction). No effects were observed for 3 mg/kg BMS-911543 on IgG antibody levels after 14 days of compound administration. These findings indicate a

therapeutic window for BMS-911543 on JAK2-mediated pathological responses compared with potential effects on immune system function.

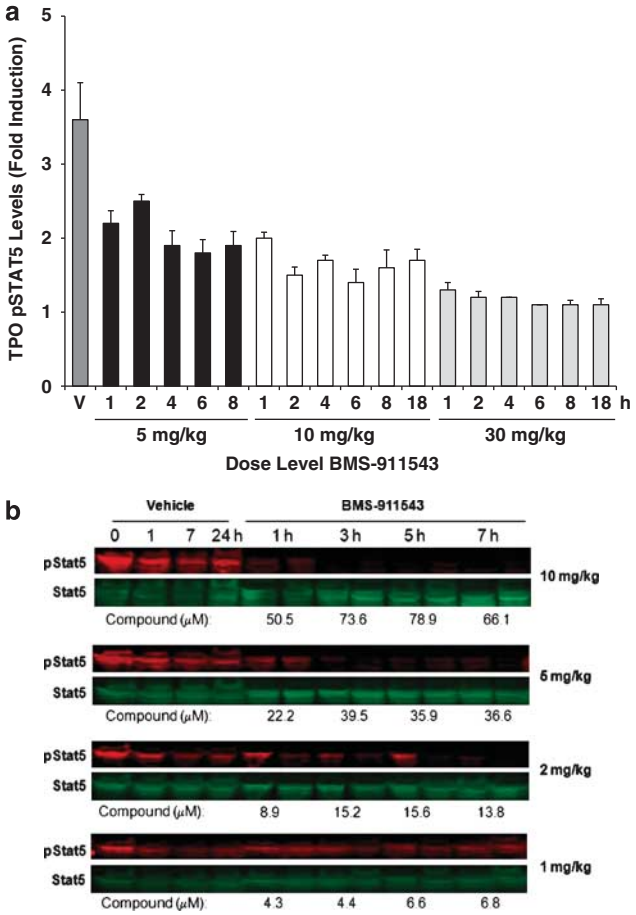


Figure 4 Differential inhibitory sensitivity of JAK2^{V617F} *in vivo*. (**a**) PD effects of BMS-911543 on TPO-induced JAK2^{WT}-pSTAT5 responses in mouse platelets *in vivo*. Mice were orally administered BMS-911543 at 5, 10 or 30 mg/kg, and euthanized at the indicated time points ($n=3$). Platelets were examined *ex vivo* for TPO-STAT5 responses by FACS analysis. Error bars indicated s.d. of mean values obtained from triplicate measure using three mice per time point. (**b**) Effects of BMS-911543 on constitutively active JAK2^{V617F} *in vivo*. SET2 tumor-bearing mice were treated at the indicated dose levels and time course with BMS-911543. SET2 tumor lysates were prepared from BMS-911543-treated animals and examined by western blot analysis for pSTAT5 levels. Compound levels in serum of treated animals for each time point are shown below the blot.

Identification of a JAK2-regulated transcriptional program involving STAT1

JAK2 inhibition in SET2 cells leads to an anti-proliferative effect consistent with the suppression of pSTAT5 but the precise transcriptional program that is affected by pathway inhibition has not been fully elucidated. To investigate the underlying transcriptional changes associated with JAK2 inhibition, we performed gene-expression profiling in SET2 cells. For this analysis, we used a full dose range of three independent JAK2 inhibitors representing distinct chemotypes to identify a shared response signature as well as rule out possible off-target activities (Supplementary Table 1). Analysis of the expression-profiling data identified 25 mRNAs that displayed shared, dose-dependent responses to all three inhibitors in SET2 cells ($P<0.05$, >1.2-fold change; Figure 6a and Supplementary Table 2). The modulation of these shared mRNAs correlated with the anti-proliferative effective concenteration (EC_{50}) of the inhibitors in SET2 cells suggesting these gene products are of biological significance and included genes downstream of cytokine signal transduction, such as *PIM1*, *MYC*, *SOCS2 ID1, PPIF, CISH, IRF1*, *IRF9* and *STAT1*. To confirm these findings, we performed quantitative PCR or western blot analysis with BMS-911543 in dose response on selected gene products. We were particularly interested in STAT1 and STAT1-regulated genes (*PIM1* and *ID1*) as increased STAT1 activity has been implicated recently as a key driver of MPN pathology.[29] Consistent with the previous expression-profiling data from other JAK2 inhibitors, BMS-911543 treatment resulted in a dose-dependent reduction in *STAT1* transcript by quantitative PCR, and the response was correlated with the anti-proliferative IC_{50} of the inhibitor in SET-2 cells (Figure 6b). At the protein level, BMS-911543 treatment also resulted in a dose-dependent reduction in STAT1 levels but required longer compound exposure (24 h) compared with reductions in STAT1 transcript (4 h). PIM1 and ID1 protein levels also were dose dependently decreased in SET2 cells by BMS-911543 (Figure 6c), but with more rapid kinetics than observed for STAT1 suggesting different mechanisms of regulation of the JAK2 core transcriptional program. Interestingly, in addition to lowering STAT1 protein levels, we also observed pSTAT1 reduction following treatment with BMS-911543 that correlated with pSTAT3 reduction suggesting a JAK2-mediated phosphorylation of STAT1 in these cells. Overall, these findings define a JAK2 activation gene-expression profile, which can be used to guide the clinical development of compounds such as BMS-911543 in MPNs and other malignancies associated with aberrant JAK2 signal transduction.

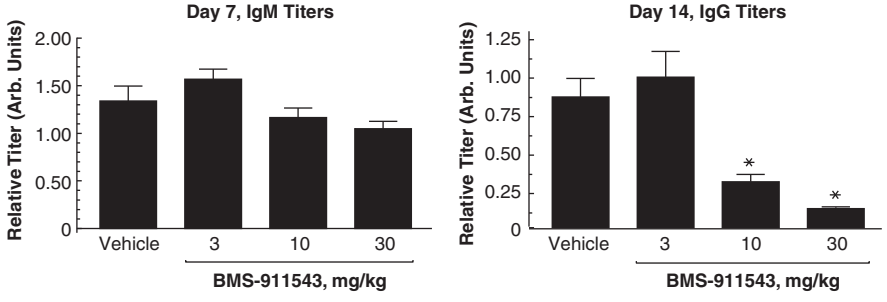


Figure 5 Effects of BMS-911543 in a mouse model of immunosuppression. BALB/c mice were immunized with KLH antigen followed by oral administration of BMS-911543 for 14 days at 3, 10 or 30 mg/kg daily. Anti-KLH IgM levels (left) or anti-KLH IgG levels (right) were monitored at day 7 or 14, respectively. The data are shown as mean ± s.e.m. of $n=10$ animals per treatment group. Asterisks denote $P<0.05$ versus vehicle-treated levels (Analysis of variance).

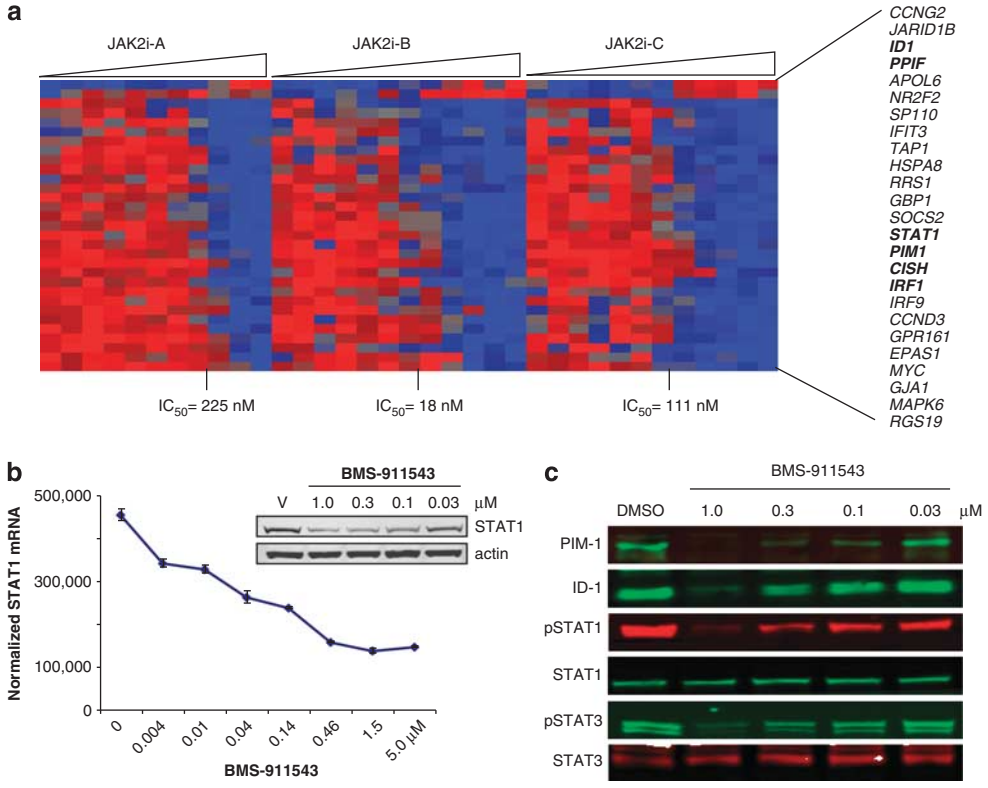


Figure 6 Regulation of STAT1 as part of a JAK2-mediated transcriptional program. (**a**) Gene-expression profiling experiments were performed in SET2 cells treated with three independent chemical classes of JAK2 inhibitors (JAK2i-A, JAK2i-B and JAK2i-C) at 0.06 nM–10 μM. The 25 gene-expression changes shared amongst all the three inhibitors are shown. Across each transcript row the signal intensity data has been linear-scaled from lowest value (blue) to highest value (red). The SET2 anti-proliferative IC_{50} response of each inhibitor is shown beneath the heatmap. (**b**) Effects of BMS-911543 on STAT1 mRNA levels (graph) and protein levels (inset). SET2 cells were treated in triplicate with BMS-911543 for analysis of STAT1 mRNA levels (4-h treatment) or protein levels (24-h treatment) by quantitative PCR or western blot analysis, and were normalized to GAPDH transcript or actin protein levels, respectively. (**c**) Protein extracts from SET2 cells treated with BMS-911543 for 2 h were examined for the indicated proteins by western blot analysis.

Discussion

Constitutive JAK2 signaling is a key feature underlying the pathogenesis of Ph− MPN. This notion is supported by numerous studies which have documented gain-of-function pathway mutations in MPN patients that occur directly in JAK2,[13–16] by ligand-independent activation of MPL/TPOR[17,18] or through the genetic inactivation of negative pathway regulators.[30] Transgenic reconstitution of these genetic lesions in mouse bone marrow progenitors recapitulated much of the pathology of human MPN,[19] and pharmacological administration of JAK family inhibitors has been shown to reverse MPN disease features in these animal models.[20,31] Importantly, clinical testing of JAK inhibitors in MPN patients has demonstrated symptomatic relief of the disease, including reductions in spleen size and decreased disease-associated constitutional symptoms.[22] However, it is important to note that these inhibitors currently in clinical testing are generally not selective for JAK2, with several being equipotent to JAK1 and JAK3, which could manifest in deleterious effects on immune cell function.[23]

Our studies establish the small molecule, BMS-911543, as a highly selective inhibitor of JAK2. Dose-dependent anti-proliferative activity of BMS-911543 was only observed in cells harboring activated JAK2 pathway mutations, and this growth-inhibitory activity correlated with suppression of constitutive pSTAT5 in these cell types. We also observed inhibition of TPO-stimulated JAK2^{WT}-pSTAT5 activity in human platelets *ex vivo* but not upon IL-2- or IL-4-stimulated JAK1/JAK3-pSTAT responses in human T cells. Moreover, at a dose level where BMS-911543 demonstrated *in vivo* activity on JAK2^{V617F} end points, compound administration to antigen-challenged mice had no effect on antibody generation. In contrast, consistent with published findings,[23] we found a pan-JAK inhibitor to potently suppress both antigen-induced IgG and IgM generation (unpublished findings), indicating less immunosuppressive potential for inhibitors with increased JAK2 selectivity. Interestingly, at higher doses of BMS-911543, decreased IgG, but not IgM, levels were observed. This observation may suggest a role of JAK2^{WT} in regulating the IgG to IgM class switch in mice. In this regard, it is important to note that IL-5, which signals in part by activating JAK2, has been shown to promote μ-γ1 switch recombination and IgG1 secretion.[32] Collectively, these cellular and *in vivo* findings are consistent with the selective inhibitory profile of BMS-911543 for JAK2.

JAK2 pathway mutations are thought to confer an 'oncogene-addicted' phenotype.[33,34] Several approaches we pursued established a differential inhibitory sensitivity for cytokine-independent compared with cytokine-dependent pathway activation. Comparison of JAK2^{V617F} versus cytokine-stimulated JAK2^{WT} end points revealed an increased sensitivity to BMS-911543 treatment for cells with constitutive pathway activity, such as in TPO-stimulated pSTAT5 in human platelets compared

with SET2 or Ba/F3$^{EPOR/JAK2V617F}$ cells. Additionally, we observed similar effects of JAK2 inhibitor treatment on the ability of MPN-patient progenitor cells to undergo erythroid colony growth in the presence or absence of exogenous EPO. We were able to extend these findings to our *in vivo* PD models where we observed increased anti-pSTAT5 pathway suppression at a lowered dose level in SET2 tumor tissue compared with cytokine-stimulated platelets harvested from treated animals. These findings support that JAK2 pathway alterations resulting in constitutive signaling impart an increased cellular dependence upon JAK2, and support the concept that pathway inhibition will have a differential sensitivity on JAK2^{V617F} to JAK2^{WT} responses.

Aberrant activation of STAT3 or STAT5 has been reported in a variety of human cancers and expression of constitutively active STAT3 is sufficient to promote malignant transformation.[35] Many investigators have utilized JAK inhibitors to examine the role of JAK family members as molecular targets underlying malignancies particularly associated with STAT3 activation. Studies of this type have ascribed a role for JAK2 dependency in cancer subtypes of breast, prostate, multiple myeloma and B-cell lymphomas.[36–39] We did not observe inhibitory effects of BMS-911543 in various solid tumor or lymphoma cell lines, including the B-cell lymphoma cell line, KARPAS1106, which is reported to have constitutive JAK2 signaling through genetic inactivation of the pathway negative regulator, *SOCS1*.[28] Unlike MPNs, our results suggest that persistent STAT3/5 activation does not confer JAK2 dependence in these other cancers, and that the lack of selectivity of the inhibitors utilized towards other JAK family members or kinases outside the family may underlie the observed effects. Nonetheless, STAT3 activation is a consistent feature in many solid tumors, and it is interesting to note the recent observation documenting the dependence of breast cancer stem-like cells on the JAK2-STAT3 pathway and the association of pathway activation in conjunction with CD44$^+$CD24$^-$ stem cell-surface marker expression with disease relapse.[40] These findings suggest there may be distinct solid tumor subpopulations where pathway inhibition is most relevant as well as highlight the need to incorporate additional parameters to identify the appropriate context to use JAK2 inhibitors alone or in combination with other agents.

To define the underlying cellular responses to JAK2 inhibition we identified a gene-expression signature that was shared amongst distinct JAK2 inhibitors and was associated with cellular growth inhibition. A unifying feature of this transcriptional profile was a core set of regulated genes involved in cytokine-receptor signaling including CISH, PIM1 and SOCS2, previously shown to be regulated by JAK2 pathway inhibition.[29,41] In addition to these changes, we also unexpectedly found STAT1 protein and transcript levels as well as its phosphorylation to be downregulated by JAK2 inhibition. Importantly, the regulation of STAT1 and the associated gene products was correlated highly with JAK2 on-target pharmacology and was shared across multiple inhibitors. Interestingly, pSTAT1 has been observed in a subpopulation of cells in extramedullary hematopoietic tissue from a JAK2^{V617F}-positive PV patient, and the activation of STAT1 has recently been shown to promote an ET versus a PV phenotype, which indicates the potential of STAT1 as an additional modifying factor of MPN disease progression.[41,42] These findings further highlight an interconnection between STAT1 and JAK2^{V617F}-STAT3/5 signal transduction, and support the concept that these proteins should be examined in concert to guide the use of JAK2 inhibitors like BMS-911543 in MPN and other malignancies.

Conflict of Interest

AVP, TMM, HW, DY, BP, XH, RV, YZ, SUR, GLT, LL, MMG, PRM, HS, JH, SLE, YB, EF, TLT KWM, EM, CM, FYL, AW and MVL are employees of Bristol-Myers Squibb, which generated BMS-911543 for clinical trials. All other authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)