Efficacy of the JAK2 inhibitor INCB16562 in a murine model of MPLW515L-induced thrombocytosis and myelofibrosis

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Abstract

The discovery of JAK2 and MPL mutations in patients with myeloproliferative neoplasms (MPN) patients provided important insight into the genetic basis of these disorders and led to the development of JAK2 kinase inhibitors for MPN therapy. Although recent studies have shown that JAK2 kinase inhibitors demonstrate efficacy in a JAK2V617F murine bone marrow transplant model, the effects of JAK2 inhibitors on MPLW515L-mediated myeloproliferation have not been investigated. In this report we describe the in vitro and in vivo effects of INCB16562, a small-molecule JAK2 inhibitor. INCB16562 inhibited proliferation and signaling in cell lines transformed by JAK2 and MPL mutations. In comparison to vehicle treatment, INCB16562 treatment improved survival, normalized white blood cell counts and platelet counts, and markedly reduced extramedullary hematopoiesis and bone marrow fibrosis. We observed inhibition of STAT3 and STAT5 phosphorylation in vivo consistent with potent inhibition of JAK-STAT signaling. These data suggest JAK2 inhibitor therapy may be of value in the treatment of JAK2V617F-negative MPN. However, we did not observe a decrease in the size of the malignant clone in the bone marrow of treated mice at the end of therapy, which suggests that JAK2 inhibitor therapy, by itself, was not curative in this MPN model.
Introduction

Polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF) are classified as BCR-ABL negative myeloproliferative neoplasms (MPNs) typified by clonal proliferation of one or more myeloid lineages. There are approximately 130-150,000 MPN patients in the United States, which makes these disorders among the most common hematopoietic malignancies. MPN patients are at high risk for several disease-related complications including bleeding, thrombosis, splenomegaly, progressive bone marrow failure and transformation to AML. Current therapies for PV and ET are largely empiric, and include anti-platelet therapy, phlebotomy, hydroxyurea, anagrelide and IFN-γ. For patients with PMF or with post-PV/ET myelofibrosis; treatment options are limited, with the notable exception of allogeneic stem cell transplantation for the subset of patients where age and/or comorbidities do not exclude transplantation as a therapeutic option. There is therefore a need for novel therapies for patients with these disorders.

Although previous studies had demonstrated the clonal stem cell origin of these disorders, the genetic basis of these disorders was not known until several groups reported the identification of a recurrent somatic mutation in JAK2 (JAK2V617F) in ~90-95% of PV patients and in ~50-60% of ET and PMF patients. Expression of JAK2V617F in vitro transforms hematopoietic cells to cytokine independent growth and leads to constitutive activation of downstream signaling pathways. In addition, expression of JAK2V617F in vivo using the murine bone marrow transplant (BMT) assay results in a short latency, fully penetrant MPN notable for marked polycythemia, hepatosplenomegaly, and variable progression to myelofibrosis. These data demonstrate the importance of JAK2V617F to the pathogenesis of JAK2V617F-positive MPN.

Although the discovery of JAK2V617F mutations in almost all patients with PV and approximately half of those with ET and PMF provided important insight into the molecular basis of these MPN, the etiology of JAK2V617F-negative MPN remained unknown. Investigators subsequently identified somatic activating mutations in exon 12 of JAK2 in JAK2V617F-negative PV patients; however alternate JAK2 mutations were not identified in JAK2V617F-negative ET and PMF. Based on the observation that the JAK2V617F kinase requires expression of a type I homodimeric cytokine receptor...
(EPOR, MPL, GCSFR) to efficiently transform hematopoietic cells,$^{15}$ investigators sequenced these cytokine receptors in MPN patients and identified somatic mutations at codon 515 of the thrombopoietin receptor (\textit{MPLW515L}) in ET and PMF.$^{21}$ Subsequent to the initial identification of the \textit{MPLW515L} allele, additional somatic mutations at codon 515 (\textit{MPLW515K, MPLW515A})$^{22,23}$ and at codon 505 (\textit{MPLS505N})$^{24}$ have been identified in ET/PMF patients. Analysis of large patient cohorts suggests that somatic \textit{MPL} mutations are present in approximately 3\% of ET and 8\% of PMF patients.$^{24,25}$ Expression of \textit{MPLW515L} transforms murine and human hematopoietic cell lines to cytokine independent growth, and results in constitutive activation of several downstream molecules including STAT3, STAT5, ERK and PI3K/Akt pathways.$^{21}$ More importantly, overexpression of \textit{MPLW515L} in the murine BMT assay results in development of an acute myeloproliferative neoplasm characterized by features of human ET and PMF, including marked thrombocytosis, leukocytosis, and the rapid development of extramedullary hematopoiesis and reticulin fibrosis in all mice expressing this mutant allele.$^{21}$

Based on the identification of activating JAK2 and MPL mutations in these MPN, many groups have initiated efforts aimed at developing small molecule inhibitors of JAK2 signaling for the treatment of MPN.$^{26}$ These compounds inhibit growth and signaling in cell lines transformed by JAK2V617F and \textit{MPLW515L},$^{27}$ in primary MPN patient samples,$^{28}$ and have demonstrated efficacy in a murine BMT model of JAK2V617F-induced PV.$^{29}$ Based on these data, different JAK2 inhibitors have entered early stage clinical trials for patients with PMF and post-PV/ET PMF,$^{30}$ and at this early stage it is difficult to ascertain whether JAK2 inhibition will lead to significant hematological and molecular responses in the different MPN, and if responses will differ based on mutational context. Given that previous \textit{in vivo} studies have focused on the effects of JAK2 inhibition in a JAK2V617F-dependent model of PV, we sought to ascertain whether JAK2 inhibition would improve thrombocytosis, myelofibrosis, and survival in a \textit{MPLW515L}-dependent model of ET/PMF.
Materials and Methods

Reagents: INCB16562 was synthesized by Incyte Corporation (Wilmington, DE, USA). 1 mM stock solutions were prepared and stored in DMSO and diluted in RPMI-1640 with 10% FBS just prior to use. Antibodies used for western blotting included phosphorylated and total JAK2, STAT3, STAT5 and MAPK (Cell Signaling, Beverly, MA, USA), and actin (Santa Cruz Biotechnology, Inc., CA, USA). Luminex assay kits (mouse cytokine 32-plex) were utilized to quantify plasma cytokine levels (Millipore Corp., MA, USA). The hMPL wild type plasmid was generously provided by K. Kaushansky, and cloned into the MSCV-IRE-EGFP retroviral vector. The MPLW515L mutation was generated using site-directed mutagenesis (Quickchange-XL, Stratagene, La Jolla, CA, USA) and confirmed by full-length DNA sequencing. The MSCV-mJAK2-GFP, MSCV-mJAK2V617F-GFP, and MSCV-mJAK2K539L-GFP plasmids have been previously described.16,20

Cell Lines: 293T cells were grown in Dulbecco’s modified Eagle’s medium with 10% FBS. Transient co-transfection of 293T cells and generation of retroviral supernatant were performed using Fugene (Roche, Nutley, NJ, USA) according to manufacturer's guidelines. Ba/F3 cells were maintained in RPMI-1640 with 10% FBS and 1 ng/ml murine IL3 (R&D Systems, Inc; MN, USA). Ba/F3 cells expressing the murine EPOR (BaF3-EPOR)31 were grown in RPMI medium with 10% FBS and 1 unit of EPO per milliliter. Ba/F3 cells were transduced with viral supernatant containing MSCV-hMPLW515L-GFP, while Ba/F3-EPOR cells were transduced with retroviral supernatant containing MSCV-mJAK2-GFP or MSCV-mJAK2V617F-GFP and MSCV-mJAK2K539L-GFP vectors, respectively, sorted for GFP positivity by flow cytometry or by growth in the absence of cytokines. The human leukemic cell lines THP-1, NOMO-1, and HEL were cultured in RPMI-1640 with 10% FBS, while SET-2 cells were maintained in RPMI-1640 with 20% FBS. UKE-1 cells were cultured in RPMI-1640 supplemented with 10% horse serum and 1 μM hydrocortisone.

In Vitro Inhibitor Assays and Western Blot Analysis: 10,000 viable cells were plated in 96 well microtiter plates in 200 μl of RPMI media with different concentrations of INCB16562 in triplicate. 48 hour proliferation was assessed using the Cell viability Luminescent Assay Kit (CellTiter-Glo®, Promega, Cat. No. G7571). Results were
normalized to growth of cells in media containing an equivalent volume of DMSO. The concentration at which 50% inhibition in proliferation occurred was determined using Graph Pad Prism 5.0 software. For Western blot analysis of signaling pathways, cell lines were exposed to different concentrations of INCB16562 or to DMSO for 4 hours. Cells were then collected and lysed in lysis buffer and separated by electrophoresis as described previously. Nitrocellulose membrane was blocked in TBST/5% milk and incubated with antibodies as described above. Immunoprecipitation experiments were carried out as described previously.

**Murine model and Analysis of Mice:** The MPL wild type and W515L murine BMT assay was performed as described previously. Briefly, bone marrow cells from 5FU-treated male donors were harvested and transduced with viral supernatants containing either MSCV-hMPLW515L-GFP or MSCV-hMPLWT-GFP, and 750,000 bone marrow cells of each type were injected into the tail veins of lethally irradiated female BALB/c mice. Non-lethal bleeds were performed 11 days after transplantation to assess disease severity in all mice. MPLW515L mice were then randomized to receive once daily treatment with INCB16562 (20 mg/kg, 60 mg/kg, 120 mg/kg) or with vehicle by oral gavage beginning 12 days after transplantation. We also observed 5 MPLW515L mice not treated with either vehicle or drug in order to study the course of the disease. MPLWT mice were randomized to receive either vehicle (n=5 mice) or INCB 16562 (60 mg/kg) per day. With the exception of mice sacrificed at specific time points for flow cytometric analysis, all mice were treated for 28 days or until any one of several criteria for sacrifice were met, including severe lethargy, >10% body weight loss, and palpable splenomegaly that extends across the midline in accordance with our Memorial Sloan-Kettering Cancer Center IACUC-approved animal protocol. Animal care was in strict compliance with institutional guidelines established by the Memorial Sloan-Kettering Cancer Center, the Guide for the Care and Use of Laboratory Animals [National Academy of Sciences (1996)], and the Association for Assessment and Accreditation of Laboratory Animal Care International. Differential blood counts were assessed by mandibular bleeds before the trial, after 14 days of therapy/vehicle, and at study endpoints. Cytokine levels in mouse serum were determined using the Luminex multiplex bead-based assay system and the Millipore Mouse Cytokine 32-plex kit on a Luminex 200 following manufacturer’s protocols. Data analysis included with the Luminex 200 was used to conduct a 5-parameter logistic fitting method to determine cytokine
concentrations. Bone marrow and spleen samples were subjected to Gordon and Sweeds stain for reticulin fibers (ammoniacal silver procedure). For histopathology, tissues were fixed in formalin and then embedded in paraffin for analysis as previously described. Immunohistochemistry for pSTAT3 was performed as previously described. We performed blinded immunohistochemical analysis of slides from 5 different mice in each group and counted the number of cells with positive-nuclear staining for pSTAT3 at 20X magnification in cohorts of 5 mice treated with vehicle, 60mg/kg, or 120mg/kg respectively.

**Flow Cytometry:** For surface flow cytometry of mouse BM and spleen, cells were washed in PBS + 1% BSA and stained with monoclonal antibodies in PBS + 1% BSA for 20 minutes on ice. Antibodies used were CD41a (PE), CD11b (PECy7), Gr1 (APCCy7), CD71 (PE), and ter119 (PECy7) (all from BD Pharmingen, CA, USA). Cells were gated for viability after incubation with 7AAD (7-amino-actinomycin) and GFP positivity, and more than 100,000 events were analyzed from this subset for marker expression using a FACSCalibur. For phosho-flow analysis, freshly isolated BM from hMPLW515L-transplanted animals was harvested and exposed ex-vivo to either DMSO or INCB16562 (1μM) at 37°C for 2 hours. Following this, cells were stimulated with rh-GCSF (375ng/mL, R&D Systems), rh-TPO (1250ng/mL, R&D Systems) or vehicle for 10 minutes. Cells were then fixed immediately with 2% paraformaldehyde (BD Pharmingen) and permeabilized with 90% ice-cold methanol. Briefly, cells were incubated with CD11b (APCCy7) and CD61 (PE) in combination with anti-phospho-STAT3Y705 (Ax647), anti-phospho-STAT3S727 (Ax647), or anti-phospho-STAT5Y694 (Ax647) (BD Pharmingen). For progenitor sorts, bone marrow cells and splenocytes were stained with the following antibodies for 20 minutes on ice: CD34 (PE), FcγR (Pacific Blue), CD117 (APCH7), Sca1 (PECy7), CD127 (biotin), CD4 (biotin), CD5 (biotin), CD8a (biotin), CD19 (biotin) (all from BD Pharmingen). Cells were then washed and restained with streptavidin (PerCP) (BD Pharmingen) and propidium iodide. Following a final wash, cells were analyzed by flow cytometry on FACSaria to enumerate the common myeloid progenitor (CMPs), granulocyte-macrophage progenitor (GMPs), and megakaryocyte-erythroid progenitor populations (MEPs) as previously described. The gates for defining various subsets were set in the following manner using (1) unstained controls, (2) “fluorescence-minus one” (FMO) controls for experiments when >2 surface markers were used simultaneously, and by (3) the use of gating on discrete cell
populations, when present, and then application of this exact gate to other groups stained with the same fluorophore. Also, all FACS data presented is gated on living cells followed by gating for GFP-positive cells. All data were analyzed using FlowJo software.

**Statistical analyses:** Pooled data was displayed as mean ± SD. Statistical significance between two groups was assessed using the non-parametric exact one-tailed (Wilcoxon-Mann-Whitney) test.

## Results

### Effects of INCB16562 on Proliferation of Hematopoietic Cell Lines

INCB16562 is an orally bioavailable, selective small molecule inhibitor of JAK2 with a cell-free IC\(_{50}\) of approximately 0.3 nM. In enzyme assays, INCB16562 shows maximal inhibitory activity against JAK2, followed by JAK1 (2.2 nM) and then JAK3 (10nM). Activity of the compound was evaluated in a variety of Ba/F3 isogenic cell lines and human leukemic cell lines ([Figure 1 and Supplementary Figure 1](#)). We first evaluated the ability of INCB16562 to inhibit the proliferation of Ba/F3 isogenic cell lines expressing the Tel-JAK1/2/3 fusion proteins. Ba/F3 cells expressing Tel-JAK2 were most sensitive to INCB16562 with an IC\(_{50}\) of 168nM, while Ba/F3 cell lines expressing Tel-JAK1 (IC\(_{50}=2310\)nM) and Tel-JAK3 (IC\(_{50}=2494\)nM) were much less sensitive ([Supplementary Figure 1A](#)). INCB16562 inhibited the proliferation of Ba/F3-EPOR cells expressing JAK2V617F (IC\(_{50}=177\)nM) or the exon 12 mutant JAK2K539L (IC\(_{50}=406\)nM) and of Ba/F3 cells expressing hMPLW515L (IC\(_{50}=600\)nM) but Ba/F3 cells expressing BCR-ABL were much less sensitive with an IC\(_{50}\) of 2840nM ([Figure 1A](#)). Similar results were observed in leukemic cell lines; the JAK2V617F-positive cell lines SET-2 (IC\(_{50}=46\)nM) and UKE-1 (573nM) were sensitive to growth inhibition by INCB16562 whereas the JAK2-wild-type cell lines THP-1 (IC\(_{50}=3460\)nM) and NOMO-1 (IC\(_{50}>3000\)nM) were much less sensitive ([Supplementary Figure 1B](#)).
Effects of INCB16562 on Signal Transduction

We investigated the effects of INCB16562 on signal transduction pathways in sensitive and resistant hematopoietic cell lines. Treatment with INCB16562 markedly reduced phosphorylation of JAK2 in Ba/F3-EPOR-JAK2V617F, Ba/F3-EPOR-JAK2K539L, and Ba/F3-MPLW515L cells (Figure 1B). We also observed dose-dependent inhibition of downstream signaling pathways, including phosphorylation of STAT3, STAT5 and MAP Kinase at doses comparable to those required for growth inhibition (250nM-1000nM). In contrast, signaling in Ba/F3-BCR-ABL cells was not affected, with persistent STAT5 and MAPK phosphorylation seen in the presence of 2000 nM INCB16562 (Figure 1C). We observed similar results in JAK2V617F-positive and JAK2V617F-negative human leukemia cell lines, with potent inhibition of downstream signaling pathways in JAK2V617F-positive SET-2 and UKE-1 cells but not in JAK2V617F-negative NOMO-1 cells (Supplementary Figure 1C).

INCB16562 Improves Survival in the MPLW515L Model of ET/PMF

We have previously shown that expression of MPLW515L using the murine BMT assay induces a rapid and lethal myeloproliferative disease in mice that recapitulates many features of human ET and PMF, including marked thrombocytosis, myelofibrosis and extramedullary hematopoiesis.21 Given the in vitro efficacy of INCB16562 in JAK2 dependent cell lines, we assessed the efficacy of INCB16562 in the MPLW515L retroviral model of ET/PMF (Supplementary Figure 2). We assessed engraftment and disease severity by measuring blood counts in all mice 11 days after tail vein injection; at this time point all mice had evidence of myeloproliferation with leukocytosis (110.7 +64.8 K/μL) and thrombocytosis (1,595 +556.4 K/μL). Animals were then randomized to begin treatment with vehicle or with 20 mg/kg/day, 60 mg/kg/day or 120 mg/kg/day of INCB16562 administered by oral gavage beginning on day 12. All mice receiving vehicle died within 21 days of treatment initiation, consistent with previous transplantation experiments with MPLW515L. The survival, body weight, liver/spleen weight, and blood counts of mice treated with or without vehicle were not significantly
different (Supplementary Figure 3) arguing against an adverse effect of vehicle on disease severity.

Although all mice receiving 20 mg/kg/day succumbed soon thereafter, all mice randomized to receive 60 mg/day or 120 mg/kg/day mice were alive on day 28 of drug therapy (p < 0.0001; Figure 2A). We noted a rapid decline in the weight of mice treated with vehicle (or untreated W515L mice) whereas mice receiving 60 mg/kg or 120 mg/kg INCB16562 regained weight lost after transplantation and maintained their weight throughout the rest of the trial. This difference in weights was statistically significant starting 17 days of treatment with 120mg/kg/day (p=0.007) and after 18 days of treatment with 60mg/kg/day (p=0.004) (Figure 2B). This observation is consistent with the improvement in body weight and constitutional symptoms of PMF patients that have received JAK2 inhibitors in early clinical trials.35

INCB16562 Improves Thrombocytosis, Leukocytosis, and Extramedullary Hematopoeisis in MPLW515L Model

In order to assess the effects of INCB16562 on MPLW515L-induced thrombocytosis and leukocytosis, we performed peripheral blood analyses of all mice on the day that they were randomized to receive treatment (Day 0), 14 days after treatment initiation (Day 14), and at end of treatment (Day 28). Mice receiving INCB16562 had a dose-dependent reduction in leukocytosis and thrombocytosis over time (Figure 2C). Specifically mice receiving INCB16562 60 mg/kg day had a 50% reduction in WBC counts compared to their counts before therapy, and their platelet counts were unchanged whereas platelet counts in vehicle treated mice increased by more than 100% by day 14 of the trial. Mice receiving INCB16562 120 mg/kg day had a 98% reduction in WBC counts compared to before therapy, and their platelet counts were normalized by day 14 of drug therapy. By day 28, platelet counts in mice treated with 60 mg/kg or 120 mg/kg INCB16562 increased compared with levels at 14 days following treatment but still remained within normal limits (Figure 2C). In contrast, drug treatment of mice transplanted with MPLWT cells did not result in any difference in weights (Supplementary Figure 4A), blood counts (Supplementary Figure 4B) or in liver/spleen weights (Supplementary Figure 4C) at time of sacrifice. These data demonstrate that expression of MPLW515L,
but not MPLWT, results in marked leukocytosis and thrombocytosis that is reversed by JAK2 inhibitor therapy.

Planned sacrifice of a subset of mice 12 days after treatment initiation allowed us to assess the effects of INCB16562 on extramedullary hematopoiesis and on histology in target organs. We observed a dose dependent decrease in spleen and liver weights of W515L mice receiving 60 mg/kg or 120 mg/kg INCB16562 compared to vehicle treated mice (p<0.001; Figure 2D). Histological evaluation of bone marrow revealed a decrease in overall cellularity compared with vehicle-treated mice (Figure 3A). In addition to the reduction in spleen size (Figure 2D), INCB16562 therapy was associated with partial normalization of splenic histology and periarteriolar lymphatic sheaths whereas vehicle-treated mice showed neutrophilic infiltration of the spleen with complete effacement of normal splenic architecture (Figure 3B). There was a dose dependent decrease in the number of megakaryocytes in treated mice versus vehicle (p<0.01, Figure 3F). We also noted marked infiltration of neutrophils in the livers of vehicle-treated mice which greatly distorted the normal hepatic architecture; in contrast hepatic histology was preserved in mice receiving 60mg/kg or 120mg/kg INCB16562 (Figure 3C). Histopathologic analysis of lung tissue revealed neutrophilic and megakaryocytic extramedullary infiltrates in the lungs of vehicle control, but not INCB16562 treated mice (data not shown). Mice transplanted with MPLWT-transduced cells, regardless of their assigned treatment (vehicle or INCB16562) had bone marrow, spleen, and liver histopathology similar to that seen in INCB16562 treated MPLW515L mice (Figures 3A through 3D; first panel).

Most importantly, reticulin-staining of bone marrow and spleen from mice treated with 60 mg/kg or 120 mg/kg INCB16562 showed a marked reduction in fibrosis compared to vehicle-treated mice (Figure 3D and 3E). We also assessed bone marrow fibrosis in MPLW515L mice scarified just prior to treatment initiation (day 11) and found that all MPLW515L mice had at least moderate bone marrow and spleen fibrosis at this early time point. These data suggest that fibrosis in hMPLW515L mice is reduced, and not merely prevented, by INCB16562 treatment (Figure 3D and 3E, Supplementary Figure 5). A minimum of 6 reticulin slides (each from bone marrow and spleen) taken from at least 3 different animals per treatment group was reviewed.
In addition to improving body weight and reducing WBC and platelet counts, treatment with INCB16562 produced profound decrements (≥2 log₂) in inflammatory cytokines and angiogenic growth factors, many of which have been associated with the hypercatabolic state and constitutional symptoms observed in MPN patients including IL-6, TNFα, interleukin 1β (IL-1β) and VEGF (Figure 4).36

**Flow Cytometric Analyses of INCB16562 Treated Mice versus Vehicle Control MPLWT and MPLW515L Mice**

Consistent with histopathologic analyses, we noted a decrease in myeloid cell burden in the spleens and bone marrows of INCB16562 treated MPLW515L mice by surface flow cytometry (Supplementary Figure 6). We noted a reduction in the proportion of Gr1/Mac-1 double positive population in the spleen, but not bone marrow, of drug treated mice (Supplementary Figure 6A), and noted a decrease in the proportion of CD41 positive megakaryocytes in the bone marrow and spleen of drug-treated MPLW515L mice (Supplementary Figure 6C). We also observed a decrease in the proportion of early erythroid precursors (CD71 single positive and CD71/ter-119 double-positive cells) in mice treated with 60mg/kg and 120mg/kg of INCB16562 compared to vehicle treated mice (Supplementary Figure 6B). No differences were seen in the proportion of B cell populations in mice treated with 60mg/kg and 120mg/kg of INCB16562 compared to vehicle treated mice (data not shown). MPLWT mice treated with 60 mg/kg/day INCB16562 did not show any significant differences in the granulocyte, neutrophil or erythroid populations between vehicle treated or drug treated samples either in the bone marrow or spleen (Supplementary Figures 7A, 7B and 7C).

To further determine whether the decrease in myelopoiesis in bone marrow and spleens of treated MPLW515L mice could be attributed to a decrease at the level of the myeloid precursors, we performed multiparameter flow cytometric analysis of stem and progenitor populations on bone marrow and spleen from MPLW515L mice treated with 60mg/kg of INCB16562 compared to vehicle treated MPLW515L mice. First, in comparison to control Balb/C mice and mice transduced with MPLWT bone marrow cells, we noted a marked increase in the proportion of megakaryocyte-erythroid precursor cells (MEPs) in vehicle-treated MPLW515L mice such that they accounted for the majority of progenitor cells in the spleens of diseased mice (Supplementary Figure 8A).
In contrast, mice treated with 60mg/kg of INCB16562 demonstrated a distribution of cells within the GMP, CMP, and MEP gates in the spleen consistent with normalization of the size of the MEP compartment (Supplementary Figure 8A). We consistently observed a significant decrease in the size of the GFP positive spleen MEP compartment with INCB16562 treatment of MPLW515L mice: the average proportion of GFP positive spleen MEPs in 3 independent experiments was 0.0117±0.009 in drug-treated mice compared with 0.155±0.035 in vehicle-treated mice (p=0.04, Mann Whitney U test; Supplementary Figure 8B). In contrast to the marked effects seen in the spleen MEP population, the absolute number of GFP positive MEP cells in bone marrow was not statistically significant after 12 days of INCB16562 treatment at 60mg/kg/day (Supplementary Figure 8B). We also assessed the percentage of GFP positive cells within the MEP population in bone marrow and spleen of vehicle and treated mice. The average percentage of GFP-positive MEP cells without treatment was 68.0% with vehicle treatment compared with 31.3% with INCB16562 (p=0.043, Mann-Whitney U Test). This suggests that although the effects of INCB16562 on the bone marrow MEP population are modest in comparison to the spleen MEP population, there is a relative decrease in the relative proportion of mutant MEPs in bone marrow and spleen relative to WT-MEPs. We did not observe significant differences in the bone marrow or splenic MEP population of MPLWT vehicle treated or MPLWT INCB16562 treated mice (Supplementary Figure 9), and stem/progenitor populations of MPLWT transplanted mice 12 days after transplantation, regardless of vehicle/drug treatment, were similar to control BALB/c mice.

**Inhibition of JAK-STAT Signaling with INCB16562 Treatment In Vivo**

In order to investigate the effects of INCB16562 on signal transduction *in vivo*, we performed Western blotting and phospho-protein specific flow cytometry on spleen and bone marrow cells from mice treated with INCB16562 or with vehicle. Western blot analysis demonstrated marked reduction of STAT3 and STAT5 phosphorylation in splenocytes from mice treated with 60 mg/kg or 120 mg/kg INCB16562 compared to mice treated with vehicle or with 20 mg/kg INCB16562 (Figure 5A). We observed a near-complete or complete inhibition of STAT3 and STAT5 phosphorylation *in vivo* in 3/4 60 mg/kg and in all 3 120 mg/kg treated mice; in contrast we observed persistent, marked STAT3 and STAT5 phosphorylation in all vehicle treated mice and in mice
treated with 20 mg/kg INCB16562. Of note pMAPK levels did not differ between vehicle treated and drug treated mice consistent with persistent activation of the MAPK pathway in vivo in the setting of JAK2 inhibition. We then asked whether INCB16562 treatment of mouse hematopoietic cells inhibited JAK2-mediated phosphorylation of STAT3 and STAT5 in specific myeloid cell subsets by phospho-flow cytometry. Two hours of ex-vivo INCB16562-treatment resulted in inhibition of phosphorylation of STAT3 and STAT5 in response to stimulation by rhGCSF and rhTPO in CD11b and CD61-positive cells (Figure 5B). These results were representative of 3 separate experiments, and we consistently observed a greater effect of INCB16562 on inhibition of STAT3 phosphorylation compared to STAT5 phosphorylation in purified megakaryocyte and neutrophil populations. In addition, immunohistochemical staining for pSTAT3Y705 in mice spleen revealed a significant decrease in pSTAT3Y705 with INCB16562 therapy compared with vehicle-treated mice (Figure 5C). Specifically, the average number of pSTAT3 stained positive nuclei was 14.8 ±5.2 in the vehicle treated group compared with 4.2 ± 1.5 and 3.8 ±1.9 in the 60 mg/kg and 120 mg/kg INCB 16562 treated groups respectively (p<0.05, Mann Whitney U test).

28 Days of Therapy with INCB16562 Does Not Eradicate MPLW515L-expressing Cells

Once daily therapy with INCB16562 improved survival, thrombocytosis, leukocytosis, extramedullary hematopoiesis, and myelofibrosis in MPLW515L mutant mice. We then asked if INCB16562 therapy resulted in a reduction in the relative size of the mutant clone by assessing the GFP percentage of different hematopoietic subsets in drug treated mice. We did not note a decrease in the proportion of GFP positive peripheral neutrophils in drug treated mice over time (Supplementary Figure 10B). Treatment with INCB16562 did not result in a significant decrease in the percentage of GFP-positive bone marrow lineage-negative, Sca-1 positive, c-Kit positive (LSK) cells. (p=0.9). There was a non-significant reduction in the proportion of GFP-positive LSK cells in the spleen with INCB16562 treatment (p=0.06, Mann-Whitney U test) (Supplementary Figure 10A). We have also observed 3 mice after discontinuation of INCB16562 (60 mg/kg) treatment after 28 days of treatment and found that these mice survived an average of 20 days after discontinuation of INCB16562 treatment. These data suggest that once daily
therapy with INCB16562 for 28 days at efficacious doses does not eradicate the mutant clone in this model of ET/PMF.

Discussion

Mutations in the JAK-STAT signaling pathway are the most common somatic genetic events in PV, ET and PMF, and MPL mutations are the most frequent class of disease alleles encountered in MPN patients after JAK2V617F. This has led to the development of small molecule inhibitors of JAK2 for the treatment of PV, ET, and PMF, and several of these agents are in late preclinical studies or in early phase clinical trials. The agent with the most extensive clinical experience to date is INCB18424, a selective JAK1/2 inhibitor that is currently in Phase I/II clinical trials for patients with PMF or with post-PV or ET MF. The majority of patients treated to date have experienced rapid reductions and marked improvements in constitutional symptoms; however it remains to be seen whether INCB18424 therapy will allow more than the occasional patient to become transfusion independent and whether there will be reductions in JAK2V617F allele burden over time. Based on preclinical data in JAK2V617F-positive patient samples and preclinical models, several other JAK kinase inhibitors are currently being evaluated in Phase I/II studies for MPN patients. However, there remain several important questions regarding the use of JAK kinase inhibitors in MPN patients: (A) Do the existing JAK2 inhibitors completely abrogate JAK2 kinase activity and downstream signaling at clinically achievable doses? (B) Do different JAK inhibitors have similar efficacy/side effects, or will differences in selectivity or pharmacokinetics/pharmacodynamics alter activity of different compounds? (C) Will JAK inhibitors demonstrate efficacy regardless of JAK2/MPL mutational status? and (D) Will these agents offer more significant benefit to patients with PV and ET compared to patients with PMF or with post-PV or ET MF?

In this report, we evaluate the effects of INCB16562, a selective, small molecule JAK2 kinase inhibitor, on a mouse model of MPLW515L-induced thrombocytosis and myelofibrosis. Both INCB16562 and INCB18424, the clinical compound used in PMF patient trials, inhibit JAK2 most potently, followed by JAK1 (INCB16562 is 5-fold less
potent against JAK1 compared to JAK2 in enzyme based assays versus 2-fold for INCB18424), TYK2 (14-fold less potent versus 7-fold for INCB18424), and then JAK3 (30-fold less potent versus 150-fold for INCB18424). We believe therefore that the observations made regarding the effects of JAK2 inhibition with INCB16562 in our in vitro and in vivo studies are relevant to clinical experiences with INCB18424 (35 and Li, J, et al, manuscript submitted) and with other JAK2 inhibitors (TG101348, CEP701).28,29 We demonstrate that treatment once daily with doses of INCB16562 that inhibit JAK-STAT signaling in vivo, markedly improve survival in the MPLW515L murine model. Moreover, we found that INCB16562 therapy resulted in dose-dependent improvement in leukocytosis and thrombocytosis with normalization of white blood cell counts and platelet counts in all mice treated with 60 mg/kg and 120 mg/kg once daily. We also observed marked improvements in extramedullary hematopoiesis including reductions in liver and spleen size and marked improvement in liver and spleen architecture. Treatment with INCB16562 also resulted in normalization of multiple inflammatory cytokines and angiogenic factors elevated in MPLW515L-mutant control mice and in MPN patients. Mice receiving INCB16562 gained weight consistent with what has been observed in the initial clinical trials with INCB18424 in PMF patients. We also noted a significant effect of INCB16562 therapy on the proliferation of specific myeloid progenitor populations including normalization of the size of the MEP compartment in spleens of INBC16562 treated mice. Most importantly, INCB16562 therapy led to significant, dose-dependent reduction of reticulin fibrosis in bone marrow and spleen of all mice treated with doses of INCB16562 that inhibited signaling in vivo.

We were able to demonstrate that INCB16562 specifically abrogated JAK-STAT signaling induced by constitutively activated JAK2 and MPL alleles. In vitro studies demonstrated that INCB16562 inhibited proliferation, JAK2 phosphorylation, and downstream signaling pathways in cell lines transformed by JAK2/MPL mutations, but not in hematopoietic cell lines without activating JAK2/MPL alleles. Moreover we were able to demonstrate potent, dose-dependent inhibition of JAK-STAT signaling in vivo, including potent inhibition of STAT3 and STAT5 phosphorylation in primary tissues from MPLW515L mice treated with INCB16562. The observation that INCB16562 potently inhibits JAK2 activity and downstream signaling in vitro and in vivo provides important insights into the effects of potent JAK2 inhibition in a model of MPN based on a human disease allele. Moreover, we did not see significant benefit on blood counts,
histopathology, and survival until we used INCB16562 doses sufficient to inhibit JAK-STAT signaling in vivo, suggesting more attention should be paid to the degree of target inhibition achieved with JAK2 inhibitors in the clinic, potentially using flow-cytometric analysis of phosphorylated signaling intermediates to assess pathway inhibition in specific myeloid cell subsets.

Although INCB16562 improved survival and reduces myeloproliferation in the MPLW515L murine BMT model, we did not observe a reduction in the proportion of GFP positive cells in peripheral blood or in the LSK population with 28 days of treatment. Furthermore, all previously treated mice developed fatal thrombocytosis/leukocytosis within 2-3 weeks after cessation of therapy. These data demonstrate INCB16562 does not fully eradicate the MPN clone in our murine model despite in vivo evidence of pathway inhibition and improvements in clinical signs of disease. This may in part reflect the acute nature of the myeloproliferation induced by MPLW515L in the retroviral bone marrow transplant assay, in comparison to the chronic myeloproliferation seen in human ET/PMF. However, our experience with INCB16562 in vivo parallels the observations seen thus far in the initial clinical trials of JAK2 inhibitors where there has been improvement in hematologic parameters and organomegaly with JAK2 inhibition but minimal decrease in allele burden with JAK2 inhibitor therapy. This may reflect the relatively short duration of therapy, insufficient pathway inhibition, insensitivity of the stem cell compartment to targeted therapies, or the possible emergence of resistant/persistent clones over time due to JAK2 dependent or independent mechanisms. The preclinical and clinical experience with these compounds suggest that treatment with JAK2 inhibitors alone may not be truly curative for these MPN, and preclinical studies combining JAK2 inhibitors with other existing and novel therapeutic approaches are warranted. Of note, we did not observe in vivo inhibition of MAPK signaling with INCB16562 therapy, and future studies into the basis for persistent in vivo MAPK activation in the setting of JAK2 inhibition and into the potential efficacy of combined JAK2/MAPK inhibition are warranted in MPN models and in MPN patient samples. Taken together, our data show that JAK2 inhibitor therapy offers significant efficacy and limited toxicity in a MPL-mutant model of ET/PMF, and suggest that JAK2 inhibitors may offer significant clinical benefit to JAK2V617F-negative ET/PMF patients, particularly those with MPL mutations.
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Author Contributions

PK, OA, JSF, and RLL designed the study. PK, OA, SM, JP, NK, JRG, TCB, ES, PJH, AG, APC, and MR performed the experiments. PK, OA, JP, CH, JSF, MLH, and RLL analyzed data. KV and JFB provided critical reagents. PK, OA, and RLL wrote the manuscript.

Conflict of Interest Disclosure

KV, PJH, TCB, MR, APC, and JSF are employed by the Incyte Corporation. All other authors declare no conflict of interest.
References:


Figure Legends:

Figure 1: Viability curves (A), immunoprecipitation analysis (B), and Western blots (C) revealing viability and down-modulation of signaling intermediates downstream of JAK2 with various concentrations of INCB16562 in Ba/F3 isogenic cell lines bearing mutant MPN alleles. (A) Cells bearing mutations which result in constitutive activation of the JAK-STAT signaling pathway (JAK2V617F, JAK2K539L, and MPLW515L) have a lower IC50 compared with Ba/F3 cells bearing BCR-Abl. (B) Immunoprecipitation analysis demonstrated decrease in JAK2 phosphorylation in Ba/F3 isogenic cell lines with INCB16562 treatment. 20 million cells were incubated with either DMSO or INCB16562 for 4 hours, followed by immunoprecipitation as described previously13. (C) Western blots revealing a dose-dependent downmodulation of signaling intermediates in the JAK-STAT pathway following treatment with INCB16562.

Figure 2: Phenotypic characteristics of hMPLW515L mice treated with vehicle versus INCB16562 at varying doses over time. (A) Treatment with INCB16562 resulted in significant increase in survival at 60 mg/kg and 120 mg/kg doses compared with vehicle as shown by Kaplan-Meier survival curve (p<0.0001, log-rank test). (B) Mice treated with INCB16562 regained weight lost after tail-vein injections compared with vehicle-treated mice which continued to lose weight. This difference in weights were statistically significant starting 17 days of treatment with 120mg/kg/day (p=0.007, Mann-Whitney U test) and after 18 days of treatment with 60mg/kg/day (p=0.004, Mann-Whitney U test). (C) Measurement of hematologic parameters at various time points in INCB16562 treatment reveal significant improvement in WBC and platelet count with 60mg/kg and 120mg/kg of INCB16562 at day 14 post treatment initiation. (D) Treatment with INCB16562 resulted in decreased hepatosplenomegaly as measured in organ weight at 12 days of treatment. Asterisk (*) indicates p-value <0.05 compared with vehicle-treated mice.

Figure 3: H+E staining of bone marrow (A), spleen (B), and liver (C) and reticulin (D and E) of hMPLWT mice and hMPLW515L mice treated with vehicle or INCB16562 for 12 days. H+E stains demonstrate reduced cellularity in the (A) bone marrow and (B) reduced myeloproliferation in the spleen of INCB16562-treated W515L mice. Enumeration of megakaryocytes per 20X field in 5 fields from the spleen of 2-3 mice sacrificed on day 12 after beginning treatment reveals significant dose-dependent decrease in number of megakaryocytes with 60 and 120mg/kg of INCB16562 (F). Liver histology reveals greatly reduced extramedullary hematopoiesis in the liver with INCB16562 treatment at either 60mg/kg or 120mg/kg of W515L mice (C). Reticulin staining of marrow (D) and spleen (E) reveals a decrease in myelofibrosis with 12 days of INCB16562 treatment. Asterisk (*) indicates p<0.05 compared with vehicle-treated mice. hMPLWT mice on the other hand, show normal bone marrow (A, leftmost panel) and splenic (B, left most panel) architecture with minimal fibrosis (D and E, left most panel), and a normal hepatic structure (D, left most panel).

Figure 4: Effect of INCB16562 treatment on serum cytokine levels in hMPLW515L mice. Displayed are log2 fold changes in serum cytokine levels in vehicle-treated
hMPLW515L mice after 9 days of treatment compared with levels in normal BALB/c mice as well as serum cytokine levels in hMPLW515L in mice treated with 9 days of 60 or 120mg/kg INCB16562 relative to vehicle-treated mice. Treatment with INCB16562 led to a >2 log_2 fold decrease in inflammatory cytokines such as IL-1β, IL-9, IL-6, and TNF-α.

**Figure 5: Inhibition of JAK-STAT signaling with INCB16562 treatment of primary cells from hMPLW515L mice.** (A) Western blotting of JAK-STAT signaling intermediates in hMPLW515L splenocytes following 12 days of INCB16562 treatment reveals abrogation of phosphorylation of STAT3, STAT5, and MAPK with INCB16562 treatment compared with vehicle. (B) Phospho-protein specific flow cytometry of CD11b+ and CD61+ myeloid cells in hMPLW1515L bone marrow cells treated *ex-vivo* with INCB16562 at 1μM or with vehicle for 2 hours. Treatment with INCB16562 greatly decreased phosphorylation of STAT3 and STAT5 in response to 10 minute *ex-vivo* stimulation with rhGCSF (375ng/mL) and rhTPO (1250ng/mL). (C) Immunohistochemical staining (40x) of pSTAT3Y705 in mouse spleen after 12 days of treatment with vehicle or INCB16552 at 60 or 120mg/kg reveals a decrease in pSTAT3 in treated versus control mice tissue.
Figure 1

A

EPORV617F with INCB16562

IC50 - 177 nM

LOG [INCB 16562]

-2.5 -2.0 -1.5 -1.0 -0.5 0.5

W515L with INCB16562

IC50 - 600 nM

LOG [INCB 16562]

-2.5 -2.0 -1.5 -1.0 -0.5 0.5

EPORK539L with INCB16562

IC50 - 406 nM

LOG [INCB 16562]

-2.5 -2.0 -1.5 -1.0 -0.5 0.5

BCR-abl with INCB16562

IC50 - 2840 nM

LOG [INCB 16562]

-2.5 -2.0 -1.5 -1.0 -0.5 0.5

B

MPLW515L  JAK2V617F  JAK2 Exon 12

0 1000 0 1000 0 1000 nM INCB16562

IP-JAK2

WB-pJAK2

IP-JAK2

WB-JAK2

C

JAK2V617F  JAK2 Exon 12

MPLW515L  BCR-ABL

0 100 250 500 1000 2000 0 100 250 500 750 1000 2000 nM INCB16562

pSTAT3

STAT3

pSTAT5

STAT5

pMAPK

MAPK
Figure 4
Efficacy of the JAK2 inhibitor INCB16562 in a murine model of MPLW515L-induced thrombocytosis and myelofibrosis

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