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An inhibitor of Janus kinase 2 prevents polycythemia in mice

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ABSTRACT

Polycythemia vera (PV) is a myeloproliferative disorder characterized by increased red cell mass and splenomegaly in the absence of secondary causes [Tefferi A., Spivak J.L., Polycythemia vera: scientific advances and current practice. Semin Hematol 2005;42(4):206–20.]. Recently, several laboratories have discovered that the vast majority of patients with PV carry a single, activating mutation (V617F) in the pseudokinase domain of Janus kinase 2 (Jak2) [Zhao R, Xing S, Li Z, Fu X, Li Q, Krantz SB, et al., Identification of an acquired JAK2 mutation in polycythemia vera. J Biol Chem 2005;280(24):22788–92; James C, Ugo V, Le Couédic JP, Staerk J, Delhommeau F, Lacout C, et al., A unique clonal JAK2 mutation leading to constitutive signalling causes polycythemia vera. Nature 2005;434(7037):1144–8; Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, et al., A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 2005;352(17):1779–90; Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, et al., Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005;7(4):387–97.]. This discovery has spurred interest in developing therapies for PV via inhibition of Jak2. We induced polycythemia in mice by administering high dose recombinant erythropoietin (Epo) and determined that administration recapitulates almost all of the major and minor diagnostic features of human PV. We then tested a selective, small molecule inhibitor of Jak2 (Jak2i) and showed that this treatment prevents polycythemia. This prevention of polycythemia was accompanied by lower hematocrits, reduced spleen sizes and reductions in Stat5 phosphorylation (pStat5). Surprisingly, Epo rapidly (<1 h) induces mobilization of activated erythroid precursors into the blood, thus allowing drug–response relationships to guide discovery. We conclude that inhibition of Jak2 prevents polycythemia in mice, and furthermore present this model as an efficient tool for the discovery of drugs that effectively treat human PV.

1. Introduction

A human disease characterized by increased red blood cell mass, plethora and susceptibility to vascular thromboses has been recognized for over a century (reviewed in Ref. [1]). Later, this condition was termed polycythemia vera (PV), and is now grouped with other Philadelphia chromosome-negative myeloproliferative disorders (MPDs) including myeloid metaplasia with myelofibrosis (MMM) and essential thrombocythemia (ET). Patients with PV are at increased risk of vascular thromboses and hemorrhage [6], although periodic phlebotomy along with antiplatelet treatment (aspirin) have lowered this risk and improved survival [7,8]. Nonetheless, despite achieving normal hematocrits after phlebotomy, patients with PV continue to experience debilitating morbidities and reduced lifespan [9–12]. In addition, a small fraction progress to develop terminal myelofibrosis or acute myeloid leukemias [13]. Thus, further efforts to develop effective treatments for PV are warranted.

A series of investigations into a potential molecular basis of PV have been underway for a number of years (reviewed in Ref. [14]), based on our growing knowledge of the erythropoietin signaling pathway. Erythropoietin (Epo) binds to a single type 1 erythropoietin receptor (EpoR), and induces a series of signaling events that result in homodimerization of EpoR, autophosphorylation of Jak2, transphosphorylation of EpoR, and activation/docking of signaling molecules including signal transducers and activators of

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transcription (STATs) [15,16]. As evidenced by the fatal anemias consequent to gene disruption, the erythropoietin-EpoR-Jak2-Stat5 pathway plays a critical role in the development and proliferation of erythropoietic lineages [17–21]. Recently, a much clearer understanding of the molecular basis of PV has emerged. After years of study revealing no consistent pathologic mutations in the Epo or EpoR loci, in 2005 several independent labs discovered that nearly all patients with PV harbor a single mutation (V617F) in the JH2 pseudokinase domain of Jak2. This mutation is associated with an increased level of Jak2 phosphorylation and constitutive activation (phosphorylation) of Stat5 [2–5]. Furthermore, transplantation of V617F-transduced bone marrow cells into lethally irradiated mice results in a polycythemic state that closely resembles human PV [22–25]. However, mouse bone marrow transplant models, while crucial to demonstrating the origins of PV, are sufficiently time and labor intensive to limit their broad usage in developing therapeutics to further understand and treat the disease.

Informed by these previous findings, we reasoned that Epo-driven polycythemia in the mouse might represent a highly relevant model for human PV for several reasons. First, expansion of the erythroid lineage is the major pathological abnormality in PV [20] and is also a primary result of Epo administration. Second, EpoR is required for V617F mutant signaling *in vitro* [26]. Third, selective small molecule inhibitors of Jak2 have been synthesized that inhibit both wild type and mutant kinase, thus arguing that Epo-WtJak2 and Epo-V617FJak2 driven polycythemias would be inhibited equivalently by such Jak2 equipotent drugs.

We show below that Epo administration indeed phenocopies almost all salient features of human PV and describe a newly synthesized, selective, orally tolerated Jak2i that is significantly improved over prior small molecule inhibitors of the kinase [27]. Using this molecule to treat polycythemic mice, we demonstrate further that inhibition of Epo-activated Jak2-Stat5 signaling is correlated with prevention of the polycythemic state *in vivo*. Our findings strongly support the hypothesis that Jak2 is required for Epo-driven polycythemia. These findings further suggest that Epo-stimulated mouse modeling of human PV can play a significant role in development of targeted molecular therapy for this disabling disease.

2. Materials and methods

2.1. Reagents

2.1.1. P1 compound synthesis and measurement

P1 was synthesized internally, US Patent #6,852,727. P1 was qualified by comparing peak area ratio of P1 and internal standard in samples to a standard curve in a LC/MS method using a positive MS/MS transition from m/z 363.9 to 199.6

2.2. In vitro assays

2.2.1. JAK enzymatic assays

Reactions (50 μL) contained 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 0.01% Brij-35, 1 mM EGTA, 2 μM synthetic peptide (all from Sigma, St. Louis, MO), 15 μM MgATP (JAK2 kinases) or 5 μM MgATP (JAK3 kinase). Under these conditions, various concentrations of P1 and either JAK2 JH2JH1 kinase fragment (Invitrogen, Carlsbad, CA), or JAK3 JH1 kinase fragment (Upstate/Millipore, Billerica, MA) were included to a final assay concentration of 25 pM or 250 pM, respectively. To prepare the reaction, all components except JAK2 inhibitor and ATP were mixed as a 1.33× solution and dispensed in 37.5 μL aliquots into a 96-well plate. To this, 2.5 μL of 20× JAK2 inhibitor was added and mixed. Reactions were initiated by the addition of 10 μL of 5× MgATP, and allowed to continue for 40 min at room temperature, then stopped by the addition of 50 μL of Stop/Detect buffer, containing 10 mM EDTA, 25 mM HEPES, 0.1% TRITON X-100 (all from Sigma, St. Louis, MO), 4.7 μM Europium-Py20 (PerkinElmer, Waltham, MA) and 2.07 mg/mL Strepavidin-APC (Prozyme, San Leandro, CA). Samples were incubated 1 h at room temperature. Sample fluorescence was measured with an HTRF protocol (Label 1: Lance 615, Label 2: Lance 665, for both: delay = 50 μs, window time = 100 μs, cycle = 1000 μs, flash energy level = 103).

2.2.2. ViaLight cell proliferation assay

HEL 92.1.7 cells growing in log phase were plated into white walled 96-well tissue culture treated plates (Corning Life Sciences, Lowell, MA) at 2×10^5 cells/mL (100 μL per well) in optimal growth media (see ATCC). Cells were then treated in triplicate with 10× JAK2 inhibitor at varying concentrations for 48 h; wells incubated with an equal volume of DMSO served as controls. To carry out the assay, 50 μL of pre-warmed ViaLight lysis buffer was added to each well, and then incubated at room temperature for 20 min. At that time, 100 μl of room temperature ViaLight substrate was added to each well. After incubation in the dark for 5 min, each plate was then read on a Victor V3 plate reader set for luminescence with a read rate of 1 s per well.

2.2.3. Stat5 phosphorylation

The level of Stat5 phosphorylation was quantified using a pStat5 Beadlyte Assay: 5×10^5 HEL 92.1.7 cells in log phase of growth were dispensed into each well of a pre-wet 96-well filter plate (Millipore, Billerica, MA) in RPMI growth media supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% dialyzed fetal calf serum (all from Invitrogen, Carlsbad, MA). Cells were then treated with 10× JAK2 inhibitor titration series for 1 h at 37 °C under 5% CO_2. Control cell wells contained diluent (DMSO) only. The plate was aspirated by vacuum manifold and rinsed once with 100 μL ice cold Tris buffered saline followed by vacuum aspiration. Cells were then lysed in each well by the addition of 60 μL of ice cold Universal Lysis Buffer (Upstate/Millipore, Billerica, MA) with one Roche mini-protease inhibitor tablet (as described by manufacturer). The plate was vigorously agitated on a plate shaker at +4 °C for 20 min and then placed onto a 96-well low protein binding collection plate (Corning Life Sciences, Lowell, MA) and centrifuged at 1000 × g for 10 min at +4 °C. The cleared lysate was split into duplicate wells in a fresh, pre-wet 96-well filter plate. All remaining steps are as described by Upstate/Chemicon in Phospo-Stat5A/B (Tyr694–699) Beadmates protocol. Plates were analyzed on the BioPlex system (Bio-Rad) set to read bead 35.

2.3. Flow cytometry

2.3.1. Erythroid progenitor measurements

Antibodies were purchased from BD Biosciences, San Jose, CA (CD71- PE) and eBioscience, San Diego, CA (Ter119 APC-Cy7). Spleens were collected and placed in 10 mL of RPMI on ice, mashed through a 0.22 μM strainer, collected and suspended in medium. Cells were centrifuged @ 335 × g for 5 min, re-suspended in 10 mL of BD FACS staining buffer (BSA), counted on a Vi-cell and aliquots of 1.5×10^6 cells were placed into light protected eppendorf tubes, and pelleted @ 718 × g for 4 min. Supernatant was removed and cells were re-suspended in 50 μl of Fc Block (1:100) and stained on ice for 15 min. Cells were then stained in 1 mL Stain Buffer (BSA) on ice for 2–5 min, centrifuged @ 718 × g for 5 min, then supernatant was aspirated and 50 μl of master mix was added (containing appropriate dilution of CD71 and Ter119 antibodies), incubated on ice for 15 min and 1 mL stain buffer was added followed by

centrifugation, suspension in BSA and acquisition on an LSRII machine (BD Biosciences, San Jose, CA).

2.4. In vivo studies

2.4.1. Acute PK/PD studies

C57Bl/6 mice (Charles River Laboratories, Wilmington, MA) aged 4–6 weeks were dosed with either Aranesp™ (Henry Schein, Melville, NY) (10 U/g of body weight) by subcutaneous injection plus vehicle (by oral gavage) or with Aranesp™ plus the Jak2 inhibitor P1 (100 mg/kg body weight by oral gavage) as described in International Patent Application WO 2009/017714. Retro-orbital blood was collected 1, 3 and 8 h after dosing for drug concentration and phosphorylated Stat5 levels. Phosphorylated Stat5 levels were measured by X-MAP technology using Phosphorylated Stat 5A/B Beadmates (Millipore, Billerica, MA) on a BioPlex machine (Bio-Rad, Hercules, CA). Statistics were performed using GraphPad Prism software.

2.4.2. Efficacy studies

C57Bl/6 (Charles River Laboratories) mice, age 4–6 weeks, were given either Aranesp™ alone (10 U/g of body weight) by subcutaneous injection every other day for 7 days or Aranesp™ plus P1 (100 mg/kg by oral gavage, once a day for 7 days). On day 7, mice were euthanized and blood (via cardiac puncture) was collected for determinations of hematocrit, drug concentration and phosphorylated Stat5. Spleens were also isolated, weighed, and analyzed by immunohistochemical staining for Bcl-xL, caspase 3 and TUNEL assay. Hematocrits were determined using a Hemavet 960 (Drew Scientific, Dallas, TX). Phosphorylated STAT5 levels were measured by X-MAP technology as described above.

2.4.3. Phospho-epitope staining for flow cytometry

Mouse bone marrow and spleen were homogenized directly in phosphate buffered saline (500 μL), filtered through 70 μm-pore-size mesh. Cells were fixed by mixing one volume of single cell suspension with 20 volumes of pre-warmed BD™ Phosflow Lyse/Fix Buffer (BD Pharmingen, San Jose, CA). Cells were incubated at 37 °C for 10 min, pelleted by centrifugation (300 × *g*) for 5 min. Cells were washed once with PBS, pelleted by centrifugation (300 × *g*) for 5 min and permeabilized by adding 1 mL (for 1–10 × 10^6 cells) of BD™ Phosflow Perm Buffer III (BD Pharmingen, San Jose, CA) for 30 min on ice. Cells were then washed twice with BD Pharmingen™ Stain Buffer (BD Pharmingen, San Jose, CA) and pelleted at 300 × *g* for 5 min. BD Pharmingen Stain Buffer was used to re-suspend the cells. Cells were incubated with 0.06 μg of BD Pharmingen FcBlock™ antibody for 15 min on ice. Cells were then incubated for 30 min at room temperature with a 1:50 dilution of Phosphostat5 antibody (Cell Signaling Technologies, Beverley, MA) followed by two PBS washes. Cells were then incubated with 100 μL of a 1:50 dilution of R-Phycoerythrin-conjuated AffiniPure F(ab′)2 Frangment Donkey Anti-Rabbit IgG (H + L) (Jackson ImmunoResearch, West Grove, PA) in the dark for 30 min at room temperature. Cells were washed twice with PBS and re-suspended in PBS (500 μL) before analysis on a BD FACSCalibur machine (BD Pharmingen, San Jose, CA).

2.5. Histology and immunohistochemistry

Mouse spleen, liver and bone marrow were fixed in 10% formalin. Bone marrow was decalcified post fixation. Standard hematoxylin and eosin staining were performed on spleen, liver and bone marrow. For IHC, slides were incubated in goat serum blocking solution and labeled with antibodies to Ki67 (monoclonal, Neomarkers, Fremont, CA) and Bcl-xL (polyclonal, BD Pharmingen, San Jose, CA).

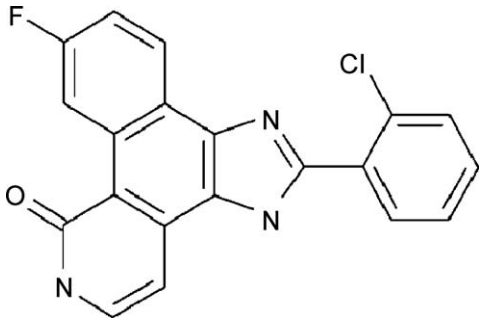

Fig. 1. Structure of P1. P1 is a tetracyclic inhibitor of Jak2 in numerous enzymatic and cellular assays.

3. Results

3.1. Synthesis of pyridone 1 (P1), a selective inhibitor of Jak2

A tricyclic small molecule inhibitor of Jak2, P1, was synthesized (Fig. 1). P1 is a potent inhibitor of Jak2, exhibiting an IC50 (concentration required to inhibit 50% of enzyme activity) of 1 nM against recombinant Jak2 *in vitro*. P1 was equally potent against a recombinant V617F mutant form of Jak2 in enzymatic assays. The compound P1 inhibited activity of other Jak family proteins (50% inhibitory concentrations) including Jak1, Tyk2, and Jak3 (Table 1). Thus, P1 showed 11-fold selectivity over Jak3 *in vitro* (Table 1). The biochemical characteristics of P1 were similar to a highly structurally related molecule (P6) in that both have relatively low selectivity for Jak2 family members but are highly selective when tested against other kinases [28]. For example, screening P1 against a panel of 175 kinases demonstrates that P1 inhibits only 11(6%) of kinases (PanLabs), and with much lower potency than against Jak2 (Table 1). P1 also potently inhibited JAK2 activity in cells, with biochemical (pStat5) and biological (proliferation of human erythroleukemia HEL cells) IC50 s of 85 and 510 nM, respectively. Of note, HEL cells are driven by the human PV-related Jak2 V617F mutation [29,30]. Thus, the Jak2-selective inhibitor P1 effectively inhibits Jak2 enzymatic and cellular activities in both wild type and mutant (V617F) contexts.

3.2. Development of mouse polycythemia model

While chronic Epo administration can cause significant polycythemia in mice [31], the extent to which erythropoietin

Table 1
P1 selectively inhibits Jak family members (Jak1-3, Tyk2). Recombinant enzymatic data (*) and Upstate enzymatic/radioligand data showing 50% inhibitory concentrations (IC50's) *in vitro* for all enzymes that were affected by P1 of 175 kinases tested.

Enzyme	IC50 (nM)
Jak2*	1
Jak1*	2.1
PKC (rat, nonselective)	3.4
Tyk*2	7
Jak3*	11
PKA (bov, nonselective)	132
Adensoine A_3	165
PDE2	360
PDE5	370
COX2	419
PDE6 (bov)	436
ERK1	487
SHT_{2B}	527
PKC_α	751
Monoamine transporter (rabbit)	881

administration can cause a state mimicking PV in humans has not been completely established. To simulate continuous JAK2 dependent signaling and induce polycythemia as closely approximating human PV as possible, we used a long acting derivative of human Epo, Aranesp™, that has been shown to increase red cell mass and hematocrit even after once weekly administration in mice [32].

We first determined an optimal, simple dosing regimen of Epo that results in a robust polycythemic phenotype as defined by three criteria: increased red cell mass (hematocrit), splenomegaly, and trilineage hyperplasia/hypercellularity in the bone marrow. Several regimens of Epo administration, including continuous minipump, daily injection or every other day injection (not shown) resulted in a similar degree of polycythemia in mice after 7 days (Fig. 2, top). Administration of Epo (10 U/g as describe above) every other day for 5–7 days was selected for further studies as a suitable regimen causing both progressive polycythemia and increased spleen size (splenomegaly) in mice (Fig. 2). At this dose, Aranesp increased hematocrit by approximately 1–2 units (%) per day. Increased spleen size (7-fold or more) was accompanied by evidence of extramedullary hematopoiesis in spleen, with effacement of white pulp by red pulp and the appearance of erythroid progenitor cells (Fig. 2, bottom panels). In addition and similar to prior reports, Epo induced Bcl-xL expression in the spleen; this is also a feature of human PV (Fig. 2) [33]. As well, a 2–3-fold increase in erythropoiesis was observed in bone marrow, with effacement of bone marrow fat in response to Epo and the appearance of *de novo* erythropoiesis in liver (Fig. 2, far right panels). Mild neutrophilia and megakaryocytosis (trilineage hyperplasia) were also observed in bone marrow after Epo administration, although peripheral leukocyte and platelet counts were unchanged (data not shown). From these findings, we conclude that many of the biochemical and anatomic characteristics of V617F-driven polycythemia vera are recapitulated in Epo-induced polycythemia in mice.

3.3. Quantitation of EpoR signaling in peripheral blood

To measure activation of the EpoR signaling pathway, we employed a Beadlyte assay for phosphorylated Stat5 (pStat5) protein. Remarkably, we found that Epo treatment increases pStat5 levels more than 10-fold in peripheral blood as early as 15 min after injection, and this effect persists until 24 h after dosing (Fig. 3A). There is no change in total Stat5 in whole blood (inset, Fig. 3A). Noting that normal peripheral blood has a low percentage of Epo-responsive mononuclear cells, we employed flow cytometry to further identify the cells that might respond to Epo. Using this assay, we observed a greater than 30-fold increase in the percentage of erythrocyte progenitor (CD71/Ter119+) cells in peripheral blood (Fig. 3B), while there was no effect on overall white blood cell counts (data not shown).

Interestingly, co-administration of Epo and our JAK2 inhibitor P1 had no effect on enrichment of erythroid progenitors in the periphery of treated mice (Fig. 3B). Variation in the timing of P1 administration also had no effect on the erythroid population increase in blood: the results were unchanged by treatment with P1 1 h prior to, simultaneously with, or 1 h after Epo administration (data not shown). Selective Epo-induced mobilization of erythroid precursors from an "erythroblastic niche" has recently been described [34]. These results confirm that Epo stimulates a pathway of erythropoiesis that can easily be measured in blood.

Group	Hct (%)	Reticulocytes (%)	Spleen (mg)
PBS	46.5 ± 1	3.9 ± 0.1	80
Epo-daily oral	66.3 ± 6	27.6* ± 0.3	561
Epo-Minipump	62.1 ± 2.1	26.8* ± 2	645

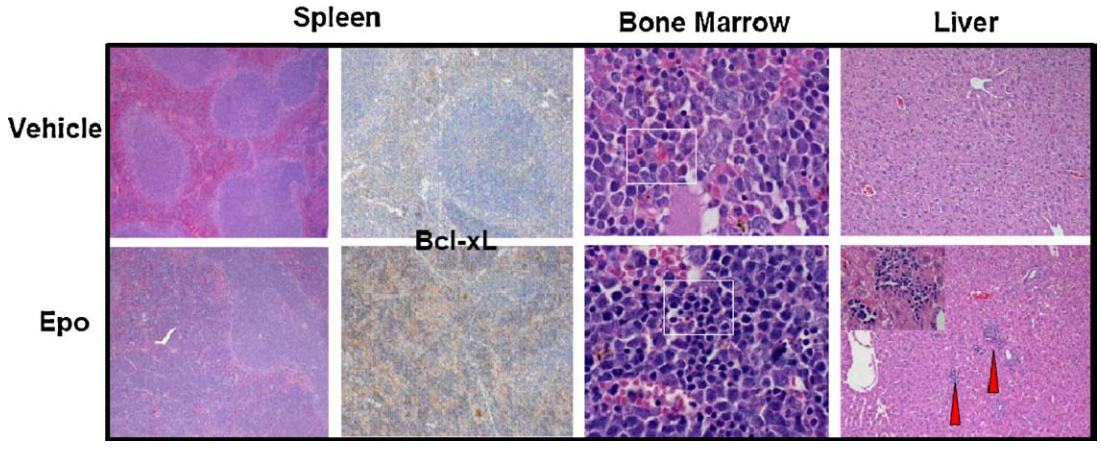

Fig. 2. 7 days of Epo administration results in polycythemia in mice. Table, above, shows effects of 7 days of oral Epo, Epo administered continuously by minipump (equivalent of 5 U/day in PBS) on hematocrit (Hct), reticulocytes (* denotes upper limit of detection for Advia) and spleen size. Bottom panels show H and E histology and immunohistochemistry in vehicle-treated (top panels) and Epo-treated (lower panels) mice. Compared to vehicle-treated mice, Epo-treated mice show effacement of white pulp in spleen (50×), induction of Bcl-xL (100×), erythropoiesis in bone marrow (640×) and liver (100×). Inset in lower right panel shows an erythroblastic island (640×). White-lined boxes in bone marrow slides show window of measurement, indicating 2–3-fold increase in erythropoiesis in Epo-treated mice. Red arrowheads indicate islands of erythropoiesis in liver parenchyma.

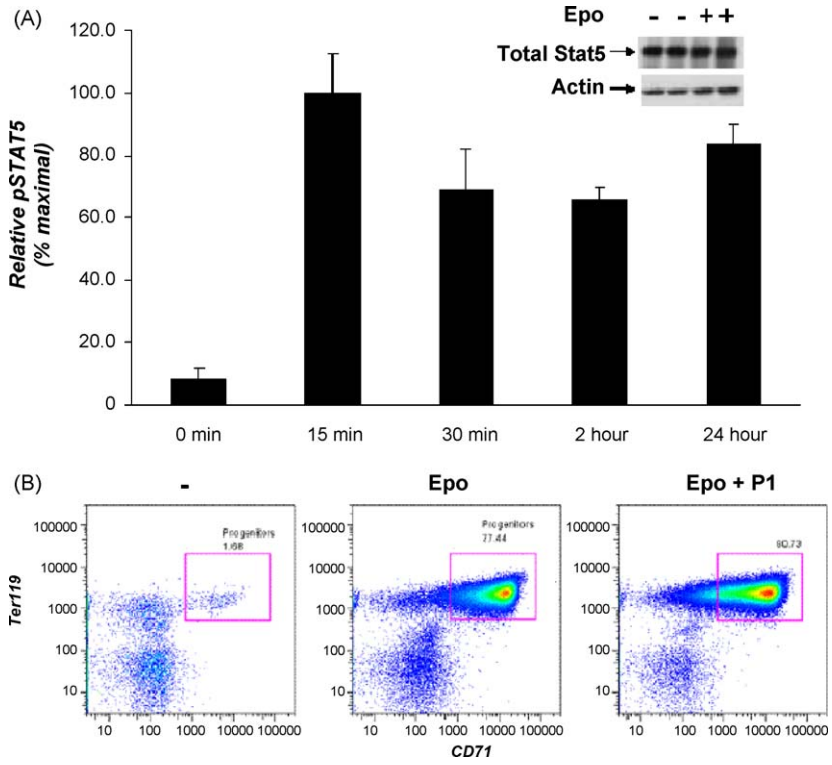

Fig. 3. Administration of Epo (single dose) increases pStat5 in peripheral blood and mobilizes erythroid mononuclear cells (MNCs). (A) pStat5 levels increase in peripheral blood cells and remain elevated in response to a single injection of Epo. Epo administration does not change total Stat5 levels or actin in response to Epo (above inset). (B) Peripheral blood MNCs stained with erythroid markers Ter119 and CD71 are enriched 1 h following single dose Epo administration. Administration of P1 has no effect on erythroid cell % (right panel).

3.4. P1 inhibits Jak2/pStat5 signaling in peripheral blood

To help establish the potential efficacy of pyridine JAK2 inhibitors as therapeutics for PV, we demonstrated effective inhibition of Epo signaling with a small molecule (P1) in mice. As shown in Fig. 4, administration of P1 reduced Epo-induced phosphorylation of Stat5 in blood to baseline (20%) levels. Significant reductions in pStat5 were seen from 1 to 8 h post administration and persisted until approximately 12 h (data not shown) after dosing. Thus, despite a marked increase in the number of stimulated erythroid cells in the peripheral blood in response to Epo, activation of Stat5 (pStat5) was completely prevented by P1. Inhibition of Epo-induced pStat5 by P1 was observed in peripheral blood, spleen and bone marrow (phosphoflow, data not shown) with a 50% inhibitory concentration of approximately 3–5 μM (IC50). The concentration of P1 was similar in blood, spleen and bone marrow, and tissue concentrations were consistently commensurate with blood levels (data not shown). A maximally effective and tolerated oral dosing regimen of 100 mg/kg P1 once daily exceeds the *in vivo* IC50 for pStat5 inhibition for approximately 12 h (data not shown).

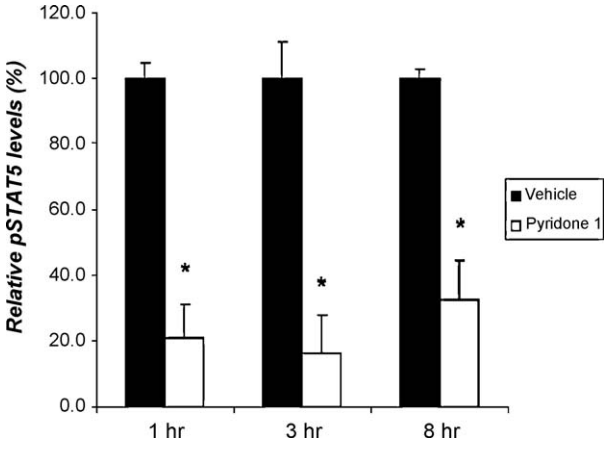

Fig. 4. Administration of P1 inhibits activation of Stat5 in Epo-treated mice ($n = 4$/group). A single 10 U dose of Epo was administered to C57Bl6 mice simultaneously with P1. P1 inhibits phosphorylation and activation of Stat5 in peripheral blood after Epo injection. The effect is sustained for up to 8 h ($^*p < 0.001$).

3.5. P1 inhibits development of Epo-induced polycythemia in mice

We next tested the hypothesis that Jak2 is required for polycythemia. As shown above, the compound P1 potently inhibits the Epo signaling pathway *in vitro* and *in vivo*, and therefore offers a suitable tool to test this hypothesis. We tested this hypothesis by using the Jak2i P1 to prevent the development of Epo-induced polycythemia in mice.

Polycythemia is induced in mice following as few as 5 days of Epo treatment, and this experimental design was used as a simplified assay for prevention of polycythemia in mice. Mice receiving Epo every other day were also dosed either vehicle or P1 (100 mg/kg). On days in which Epo was administered, vehicle or P1 was administered simultaneously. Mice treated with vehicle alone in addition to Epo show a 9-point increase in hematocrit relative to untreated mice (Fig. 5A). Using hematocrit as a surrogate of red cell mass (RCM), this increase corresponds to 20–25% increase in total RCM in response to Epo treatment. In contrast, P1-treated mice showed only a 4-point increase in Hct, indicating that P1 prevented approximately 60% Epo-induced polycythemia. Similar effects on spleen size, which is a surrogate for Epo-induced erythropoiesis, were also observed in response to P1 (Fig. 5B). Effects of P1 on the Epo pathway were assessed both during (data not shown) and at the end of the study (day 7, 3 h post dose). As shown in Fig. 5C, P1 significantly reduced pStat5 levels in peripheral blood compared to

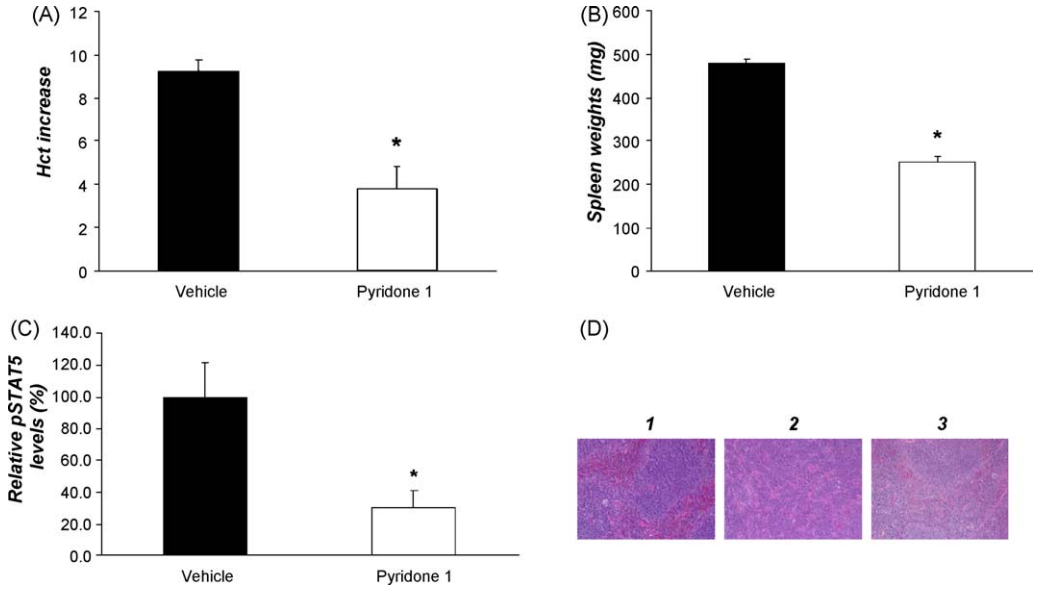

Fig. 5. P1 inhibits activation of Stat5 and development of PV and splenomegaly in Epo-treated mice after 7 days (n = 9–10/group). (A) P1 prevents rise in hematocrit (Hct) in response to Epo; (B) P1 prevents increase in spleen size in response to Epo; (C) P1 inhibits activation and phosphorylation of Stat5 levels compared to vehicle; (D) H and E stains showing normal splenic red and white pulp architecture (1) is disrupted in response to Epo (2) but is restored in response to Epo/P1 administration (3) (*$p < 0.001$).

vehicle-treated mice. Similarly, we observed concomitant and dose-dependent reductions in pStat5 levels in spleen and bone marrow in all treatment groups (Fig. 6A) by phospho flow, X-MAP technology and western blot (data not shown). Therefore, P1 prevented the development of PV in an Epo-driven mouse model, using hematocrit, spleen size and pStat5 as primary endpoints. The effectiveness of P1 in preventing Epo-induced polycythemia by P1 was, moreover, proportional to the estimated exposure to P1. Histological analysis failed to show significant apoptosis in spleen or bone marrow in response to P1 after 7 days (data not shown), but in contrast reduced proliferation was observed (Fig. 6).

These findings indicate that an Epo-driven mouse model of PV offers a rapid, phenotypically robust system for testing the impact of inhibitors of Jak2 on PV. Further, they demonstrate that synthesis of a potent Jak2i (P1) blocks EpoR pathway signaling activity (induced by either wild type or mutant (V617F) Jak2

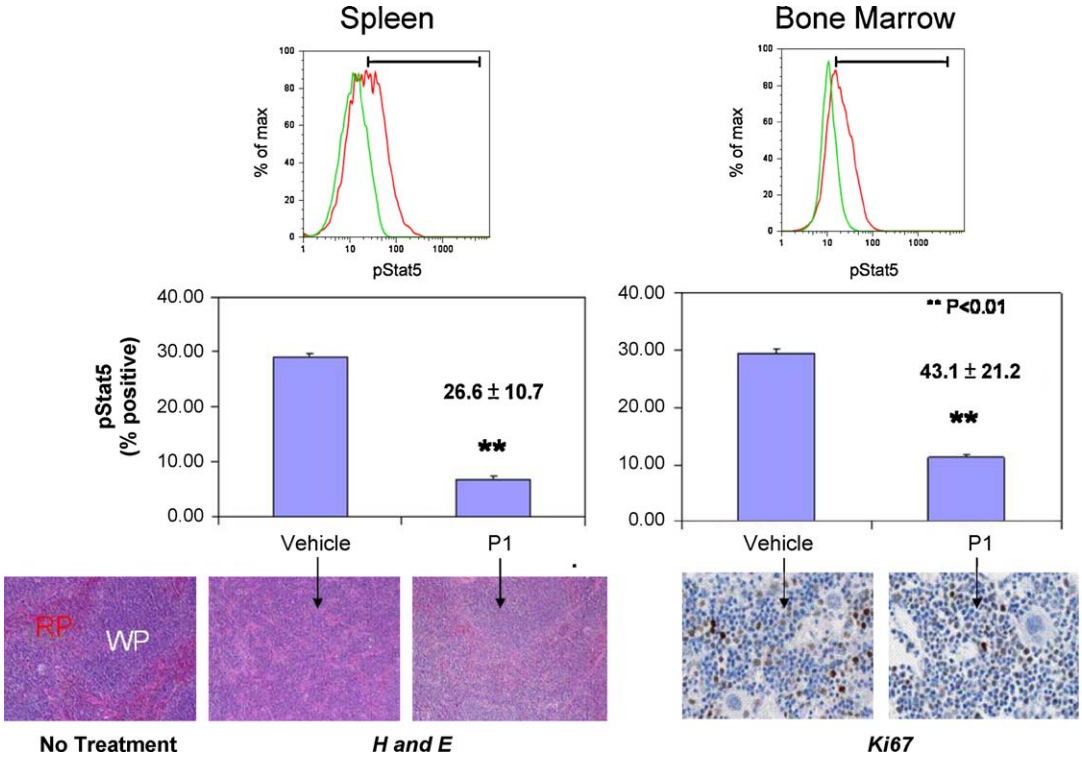

Fig. 6. Efficacy of P1 correlates with decreased levels of pStat5, restoration of normal spleen architecture and decreased proliferation in spleen and bone marrow. Top panels: M2-gated (pStat5 positive) splenocytes and total bone marrow cells in P1-treated mice (green line) compared with vehicle (red line), as indicated by the significant shift to the left. P1 concentration in blood ± S.D. indicated above bars; bottom: (left panels) H and E staining of spleen shows expansion of red pulp (RP), effacement of white pulp (WP) in response to 7 days of Epo treatment with restoration after 7 days of P1; (right) cellular proliferation in bone marrow (Ki67) is reduced in response to 7 days of P1 treatment.

molecules) and prevents polycythemia in a mouse model. Finally, these results strongly suggest that Jak2 activity is required for Epo-induced polycythemia.

4. Discussion

The discoveries that nearly all patients with PV harbor a single nucleotide mutation in Jak2 and that this mutation is sufficient to cause PV in mice have generated great interest in developing inhibitors targeting Jak2. Still, there remains much work to be done before safe and efficacious therapeutics become available. We have now developed an Epo-driven mouse model of human PV that phenocopies many features of the human disease. Of great importance, it is significantly more facile for routine use than the bone marrow transplant systems previously described. In this new setting, we have demonstrated that a selective inhibitor of Jak2 prevents polycythemia in mice and have offered partial proof of the concept that Jak2 inhibition can be effective treatment for PV. Recent reports, using a different, mutant Jak2 (V617F)-expressing mouse model, also support the contention that Jak2 inhibitors may be effective treatment for PV [35,36].

These studies demonstrate that Jak2 activity is required for Epo-driven polycythemia, and suggest that Jak2 inhibitors may be effective treatment for human PV. This observation is noteworthy because while Jak2 has been shown to be required for Epo signaling, several pathways either parallel to or downstream of Jak2 can functionally substitute for Jak2 in erythropoiesis. For example, both Akt and bcr-abl are capable of inducing erythropoiesis in Jak2-deficient fetal liver cells [35,36]. We show here, however, that Epo-induced polycythemia in mice is prevented by a relatively selective Jak2i. While our surrogate marker for Jak2 activation (pStat5) lies downstream of numerous cytokines and receptors [37], measurement of its inhibition correlates closely with drug exposure and biologic effect, arguing that we are measuring disruption of Epo-Jak2 signaling. In addition, pStat5 activity as readout for Epo or Jak2 activity has broad precedent in the literature [2–5]. In fact, constitutively active STAT5 is sufficient to induce Epo-independent cell growth in human erythroid progenitors, thus demonstrating that STAT5 activity lies directly downstream of Epo signaling [38]. Finally, the small molecule inhibitor P1 inhibits both wild type and mutant (V617F) Jak2, and can be tested *in vivo* in JAK2V617F-driven polycythemia as recently shown for other molecules [23,39].

Due to the inhibitory activity of P1 against other Jak family members, however, it is reasonable to consider whether these off-target effects of P1 could contribute to prevention of polycythemia [40]. Numerous lines of evidence support the hypothesis that erythropoiesis requires Jak2 specifically, and that inhibition of other Jak family members would have no impact on polycythemia. For example, gene disruption experiments show that Jak2 deficiency results in early embryonic lethality with complete failure of erythropoiesis, and support that hypothesis that Jak2 has a required, non-redundant role in erythropoiesis [20]. Mice with homozygous deletion of Jak1, Jak3 and Tyk2, however, have normal erythropoiesis but abnormalities in immune function [41–45]. Furthermore, selective Jak3 inhibitors show expected immunomodulatory, rather than anemia-inducing effects in mice and in primates, arguing that the effects of P1 are most likely a consequence of disruption of erythrocytosis via the Epo-Jak2-Stat5 pathway [46]. Theoretically, inhibition of other Jak family members could lead to immune defects, and this possibility would need to be rigorously tested in the appropriate clinical setting.

The limitations of an Epo-stimulated model of PV need to be considered, noting that the effects of Epo administration in mice results in many but not all of the clinical features of human PV. Similar to the human disease, mice administered high dose Epo exhibit elevated hematocrit, splenomegaly and induction of Bcl-xL, but lack V617F allele presence and granulocytosis. In addition, Epo-driven polycythemia cannot strictly be considered polycythemia "vera" or "true" PV because Epo is the known cause of the disease, while primary human erythrocytoses (PV or familial erythrocytosis) occur in the absence of circulating erythropoietin [47]. The kinetics of Epo-stimulated PV in this model are rapid (days), and therefore differ both from V617F-driven PV in mice (approximately 4 weeks) and human PV (months to years) [22,23,25]. In addition, while EpoR is required both for Epo signaling and Epo-independent growth of the V617F mutation in cells, it remains unclear how Jak2 interacts with other type 1 cytokine receptors, particularly during myeloid development. The V617F mutation, in addition to its association with mild granulocytosis in PV, is found less frequently in human myeloid malignancies and hematologic diseases of varied clinical phenotypes [48]. These observations suggest that the context within which the V617F mutation signals plays a major role in disease progression. By contrast, Epo administration presumably directly affects only EpoR expressing cells and lineages that are affected downstream of Epo stimulation. Finally, the structure of PV-inducing Jak2 proteins (wild type, V617F, exon 12, TEL-JAK2) are likely to differ, thereby affecting downstream targets of cytokine signaling, as has been recently demonstrated [49–52]. As a consequence, the therapeutic mechanisms (antiproliferative versus apoptotic) and potential of Jak2 inhibitors may differ between Epo- and mutant Jak2-driven disease. Thus, while a high-dose Epo-driven model of PV recapitulates the major clinical feature (elevated hematocrit) and significant aspects of disease pathophysiology, we appreciate that the effects of inhibiting mutant Jak2 will need to be further investigated using more cumbersome models or in the clinic itself.

Currently available treatments for patients with PV extend their lifespan, although achieving normalized hematocrits fails to significantly ameliorate morbidities such as fatigue, pruritis and symptoms of iron deficiency. Successful development of inhibitors of Jak2 may both treat these symptoms and also function as tools to aid our further understanding of the pathogenesis of PV. Our results are consistent with the hypothesis that Jak2 is required for polycythemia, and offer a facile tool for further discovery. In this way, results from higher-throughput studies in the Epo-driven mouse model of PV can be confirmed in more limited numbers of other mice whose hemopoietic cells over express Jak2-V617F, for example. Such an approach promises to rapidly advance our efforts to develop treatments and potentially a cure for human PV.

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