Efficacy of TG101348, a Selective JAK2 Inhibitor, in Treatment of a Murine Model of JAK2V617F-Induced Polycythemia Vera

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DOI 10.1016/j.ccr.2008.02.009

SUMMARY

We report that TG101348, a selective small-molecule inhibitor of JAK2 with an in vitro IC_{50} of ~3 nM, shows therapeutic efficacy in a murine model of myeloproliferative disease induced by the *JAK2V617F* mutation. In treated animals, there was a statistically significant reduction in hematocrit and leukocyte count, a dose-dependent reduction/elimination of extramedullary hematopoiesis, and, at least in some instances, evidence for attenuation of myelofibrosis. There were no apparent toxicities and no effect on T cell number. In vivo responses were correlated with surrogate endpoints, including reduction/elimination of *JAK2V617F* disease burden assessed by quantitative genomic PCR, suppression of endogenous erythroid colony formation, and in vivo inhibition of JAK-STAT signal transduction as assessed by flow cytometric measurement of phosphorylated Stat5.

INTRODUCTION

The prevalence of myeloproliferative diseases (MPD) polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF) in the United States has been estimated at 80,000–100,000 cases, with ~2–5 new cases per 100,000 per year. These MPD collectively have a higher prevalence than BCR-ABL-positive chronic myelogenous leukemia (CML). In addition to thrombotic events and bleeding complications, these patients are at risk for progression to acute myelogenous leukemia, and PV and ET patients are at risk for progression to myelofibrosis. Current therapies are similar to those used to treat BCR-

ABL-positive chronic myeloid leukemia prior to the development of the imatinib and include hydroxyurea, and interferon-alpha (Kiladjian et al., 2006; Tefferi et al., 2006). In addition, phlebotomy may be used to induce iron deficiency to control polycythemia in patients with PV, and anagrelide may have benefit in selected MPD patients with thrombocytosis (Harrison et al., 2005). However, there are currently no molecularly targeted therapies available for treatment of this large cohort of patients that would be analogous to use of imatinib for treatment of CML (Druker et al., 2001a).

The *JAK2V617F* mutation is observed in ~99% of PV patients and approximately half of the patients with myelofibrosis and

SIGNIFICANCE

The *JAK2V617F* mutation is present in the majority of cases of myeloproliferative disease (MPD), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), and is an attractive candidate for molecularly targeted therapy. However, the potential toxicities of JAK2 inhibition in vivo and identification of appropriate surrogate endpoints for response are challenges that may limit clinical usefulness in treatment of these relatively indolent diseases. We tested the activity of a clinical lead compound, TG101348, in a murine model of *JAK2V617F*-induced myeloproliferative disease. We observed that TG101348 is safe and effective in treatment of MPD in this model system and identify surrogate endpoints for response that may be of value in clinical trials in humans.

essential thrombocythemia, respectively (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). Expression of JAK2V617F confers factor-independent growth to various hematopoietic cell lines, including Ba/F3, 32D, and FDCP (James et al., 2005; Levine et al., 2005), and does so most efficiently in the presence of Type I cytokine receptors that lack a common chain, such as the erythropoietin, thrombopoietin, and G-CSF receptors, respectively (Lu et al., 2005). In each of these cellular contexts and in human HEL cells that also harbor the *JAK2V617F* allele, apoptotic cell death is induced by small molecule inhibitors of JAK2 (Levine et al., 2005). Thus, hematopoietic cells transformed by JAK2V617F exhibit oncogene dependence that has been observed in cells transformed by BCR-ABL. These findings suggest that JAK2V617F may be a valid target for therapeutic intervention in MPD associated with this mutation. Of note, in vitro data suggest that the rare PV patients with exon 12 activating mutations in *JAK2* (Scott et al., 2007) and the ~5% of PMF and ET patients that have the MPLW515L/K-activating mutation might also be targeted with JAK2 inhibitors (Pikman et al., 2006).

Here, we report efficacy of a small-molecule JAK2 inhibitor, TG101348, with suitable characteristics for in vivo use in humans, in a murine model of disease (Wernig et al., 2006). Furthermore, we utilize this model to develop and validate surrogate endpoints for response in treated animals that include assessment of genomic burden of disease using quantitative PCR, abrogation of erythropoietin-independent colony formation that is a hallmark of the MPD, and inactivation of STAT5 as a critical effector of JAK2V617F as assessed by phosphoflow cytometry. We also observe that the inhibitor has minimal impact on T cell number with dosing regimens that impact the disease phenotype. TG101348 is well tolerated, is an effective therapeutic agent in a murine model of disease, and may be suitable for the initiation of clinical trials in humans with *JAK2V617F* associated MPD.

RESULTS

In Vitro Characteristics of TG101348

TG101348 is a small-molecule, ATP-competitive inhibitor designed and synthesized using structure-based drug design methods to inhibit JAK2, but not other closely related kinases (Figure S1 available online; Table 1; Table S1). TG101348 had a high degree of kinase selectivity for JAK2. For example, TG101348 had an IC$_{50}$ ~300-fold higher for the closely related JAK3 and was a less potent inhibitor of the JAK1 and TYK2 family members. The activity of TG101348 was evaluated in a variety of cell-based assays. TG101348 inhibited proliferation of a human erythroblast leukemia (HEL) cell line that harbors the *JAK2V617F* mutation, as well as a murine pro-B cell line expressing human JAK2V617F (Ba/F3 JAK2V617F), with an IC$_{50}$ value of approximately 300 nM for either line (Figure 1A; Table S2). Proliferation of parental Ba/F3 cells was inhibited to a comparable level, with an IC$_{50}$ value of ~420 nM, consistent with the essential role of IL-3-dependent signaling in the parental cell line (Figure S2A). Exposure of these cells to TG101348 reduced STAT5 phosphorylation at concentrations that parallel the concentrations required to inhibit cell proliferation (Figure 1B). In accordance with the above results and the premise that these

Table 1. TG101348 Is JAK Family Selective and Relatively Specific

Kinase Selectivity Profile of TG101348			
Primary Target		JAK Family Kinases	
Kinase	Enzyme IC$_{50}$ (nM)	Kinase	Fold Selectivity[a]
JAK2	3	JAK2	1
JAK2 V617F	3	JAK3	334
Flt3	15	JAK1	35
Ret	48	Tyk2	135

No other tested kinase with IC$_{50}$ < 50 nM.
[a] Fold selectivity over JAK2.

cells require JAK2 activity for both proliferation and survival, TG101348 induced apoptosis in both HEL and Ba/F3 JAK2V617F cells in a dose-dependent manner (Figure 1C). In contrast, TG101348 did not show proapoptotic activity in control normal human dermal fibroblasts at concentrations up to 10 μM, and the antiproliferative IC$_{50}$ against fibroblasts was >5,000 nM. (Figure S2B). These data indicate that TG101348 is a potent and highly selective inhibitor of JAK2 kinase in cell-based assays of transformation.

Pharmacokinetic Properties of TG101348 in C57Bl/6 Mice

The pharmacokinetic parameters of TG101348 were evaluated in C57Bl/6 mice following single oral administration of doses ranging from 30 mg/kg to 200 mg/kg. Maximum plasma concentrations (C$_{max}$) of 0.68, 3.58, and 4.28 μM were observed at 3 hr postdose following oral gavage of 30, 100, and 200 mg/kg, respectively (Figure 1D). Following oral administration of TG101348, the total plasma exposure (AUC) increased linearly with respect to dose. At 7 and 24 hr postdose, the mean plasma concentrations were 0.483 and 0.02 μM for a 100 mg/kg dose, indicating that sustained plasma concentrations above the cellular IC$_{50}$ could be achieved with twice daily (bid) administration.

The steady-state plasma concentrations following bid administration showed no appreciable plasma accumulation. Based on the linearity and predictability of the TG101348 oral pharmacokinetics over dose range of 30 to 200 mg/kg, bid doses of 60 and 120 mg/kg were selected for evaluation in the murine model of polycythemia vera.

Efficacy of TG101348 in a Murine Model of *JAK2V617F*-Induced Polycythemia Vera
Study Design

We tested the efficacy of TG101348 in a murine bone marrow transplant assay of established polycythemia vera that recapitulates many of the features of the human disease. In brief, primary hematopoietic cells were transduced with a murine ecotropic retrovirus harboring the mutant *JAK2V617F* allele, and on day 26 after bone marrow transplantation into lethally irradiated syngeneic recipient mice, the development of polycythemia was assessed by differential peripheral blood count. All mice developed erythrocytosis with average hematocrits ≥ 70% prior to initiation of treatment on day 27. The animals were divided into treatment or vehicle control groups (n = ~20 mice/group). Because the
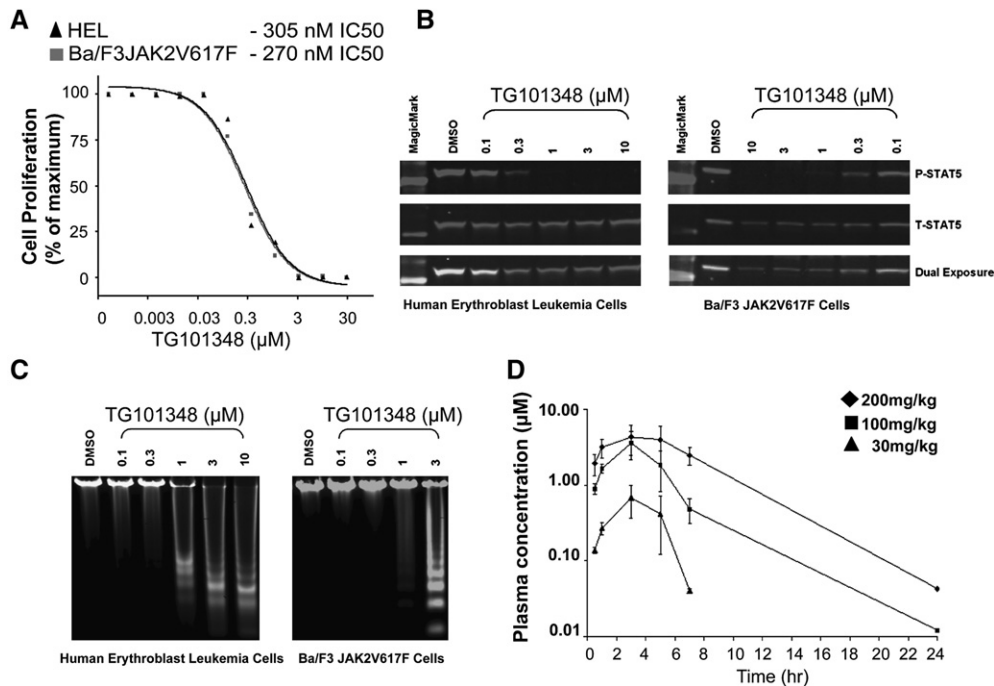
Figure 1. In Vitro Activities and Pharmacokinetics of TG101348
(A) HEL and Ba/F3 JAK2V617F cells were incubated with indicated concentrations of TG101348, and their proliferation was determined.
(B) HEL and Ba/F3 JAK2V617F cells were incubated with 0.1, 0.3, 1, 3, and 10 μM of TG101348, and the phospho-Stat5 status was assessed.
(C) TG101348 eliminated malignant cells by induction of apoptosis as shown by increased DNA fragmentation in a dose-dependent manner.
(D) C57Bl/6 mice were dosed with 30 mg/kg, 100 mg/kg, and 200 mg/kg of TG101348, and serum concentrations of the drug were assessed at 30 min, 1 hr, 3 hr, 5 hr, 7 hr, and 24 hr posttreatment. The error bars represent the mean ± SD.

half-life of murine erythrocytes is on the order of 40–50 days, treatment trials were employed in animals that were treated for 42 days to assess the effect of treatment on polycythemia, as well as to assess potential for hematologic toxicities including T cell immunosuppression and other toxicities. TG101348 was administered by oral gavage at 60 mg/kg or 120 mg/kg bid for 42 days, whereas the control group received vehicle only. Moribund mice were sacrificed during the trial, and all remaining mice were sacrificed at trial endpoint. Three independent trials were performed where mice were treated with TG101348 or vehicle, involving a total of 56 placebo and 112 drug-treated mice with JAK2V617F-induced polycythemia.

Survival and Response of Treated Animals
During the time course of the study, six animals died in the placebo group, and one animal in the 60 mg/kg drug group at day 18, whereas all animals treated with 120 mg/kg of TG101348 were all alive at study endpoint (Figure 2A). Retro-orbital sampling of peripheral blood demonstrated a mean reduction in hematocrit of ∼5.1% (hct 80.9%) in animals treated with 60 mg/kg ($p < 0.05$) and ∼17.9% (hct 68.1%) in animals treated with 120 mg/kg ($p < 0.0001$) compared to placebo (hct 86%) by study endpoint at day 42. Thus, there was a dose-dependent reduction in polycythemia (Figure 2C). In addition, there was marked dose-dependent reduction in splenomegaly of treated animals compared to vehicle-treated controls (Figures 2B and 2C).

As a correlate of reduction in spleen weights, there was also clear evidence of dose-dependent histopathologic response as assessed by the degree of extramedullary hematopoiesis in

spleens and livers of treated animals (Figures 2Da–2Di). Histopathological analysis of spleens from PV animals treated with vehicle showed effacement of normal splenic architecture and a significant expansion of the red pulp by a predominant population of erythroid elements, mature myeloid cells, and clusters of dysplastic megakaryocytes. In contrast, animals treated with 120 mg/kg showed normal splenic architecture, with small residual pockets of erythroid precursors (Figures 2Da–2Df). In addition, hepatic sections of mice treated with placebo demonstrated extramedullary hematopoiesis consisting of infiltrates of maturing myeloid and erythroid elements that was completely abrogated upon treatment with TG101348 in both the 60 mg/kg and 120 mg/kg treatment arms (Figures 2Dg–2Gi). As expected, based on previous experience with this model, there was 2+ reticulin fibrosis at study endpoint in all placebo-treated animals that were analyzed (n = 3), whereas in three of four animals treated on the 120 mg/kg arm there was no evidence of reticulin fibrosis. These findings indicate that efficacious treatment of the polycythemia-vera like syndrome may result in reversion of myelofibrosis (Figures 2Dj–2Dl).

Efficacy of TG101348 as Assessed by Flow Cytometry and Hematopoietic Colony Formation
Comparative flow cytometric analysis was performed on splenocytes or bone marrow of mice treated with TG101348 or placebo. There was an ∼2-fold decrease in JAK2V617F-positive CD71-single-positive early erythroid precursors ($p < 0.01$) in the bone marrow of animals at the 120 mg/kg dose compared
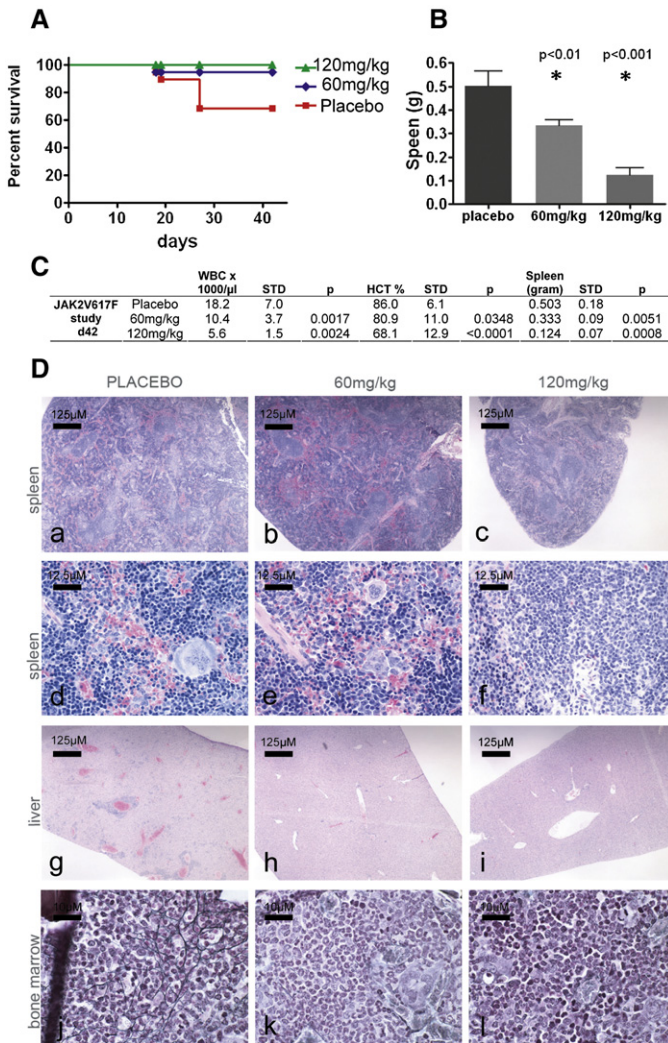
Figure 2. Survival and Response of JAK2V617F BM Transplant Animals Treated with TG101348

(A) Kaplan-Meier survival plot showing death of six JAK2V617F-expressing mice in the placebo (n = 20), of one in the 60 mg/kg group (n = 20), and normal life expectancy for the 120 mg/kg group treated with TG101348 (n = 20).

(B) 1.5-fold reduction in spleen weights in the 60 mg/kg group (p < 0.01), and 4-fold reduction in the 120 mg/kg group (p < 0.001) compared to the placebo-treated arm. The error bars represent the mean ± SD.

(C) Differential blood counts in JAK2V617F-expressing mice treated with placebo, 60 mg/kg, and 120 mg/kg of TG101348.

(D) Histology of JAK2V617F mice treated with placebo, 60 mg/kg, or 120 mg/kg of TG101348 with representative sections of spleen displaying complete effacement of normal splenic architecture ([Da]–[Dc], H&E) with a prominent expansion of red pulp comprised of maturing myeloid elements, erythroid elements, and clusters of megakaryocytes ([Dd]–[Df], H&E stain). Liver sections from JAK2V617F placebo-treated mice displayed evidence of extramedullary hematopoiesis in a perivascular and sinusoidal distribution that was notably absent in 60 mg/kg and 120 mg/kg of TG101348-treated mice ([Dg]–[Di], H&E). Moderately to marked diffusely increased 2+ reticulin fibrosis in JAK2V617F placebo-treated mice (n = 3) decreased to a 1+ reticulin fibrosis or was absent in 75% of JAK2V617F-expressing mice treated with 120 mg/kg (n = 4) ([Dj]–[Dl], reticulin stain).

T cell number. In addition, there was no reduction in cells of the neutrophil/monocyte lineage that express both Gr1 and Mac1 markers. This observation indicates that the drug has less inhibitory effect in wild-type hematopoietic cells than in JAK2V617F-positive cells. (Figure 3B; Figure S3A–S3C).

In the bone marrow, there was a marked dose-dependent effect on the number of BFU-E with a reduction of 40% in the 60 mg/kg group compared with control and complete suppression of BFU-E formation in the 120 mg/kg group. There were minimal effects at either dose level among other colony-forming units (Figure 3C). There were similar findings in the spleen, in which there was abrogation of erythroid colony-forming capacity at the 120 mg/kg dose compared to control (p < 0.01), though there was less of an effect at the 60 mg/kg dose. In addition, in the spleen there was a reduction in CFU-GM and CFU-M colony formation by ~10- or ~3-fold at the 60 mg/kg or 120 mg/kg doses, respectively (p < 0.05) that correlated with an overall reduction of colony-forming activity of ~2-fold compared to vehicle-treated animals (Figure 3D).

Assessment of Surrogate Endpoints of Response

Conventional response criteria include assessment of complete blood counts, histopathology including myelofibrosis, and assessment of immunophenotypic and colony-forming unit responses. To determine which surrogate markers correlated with these responses, we also assayed for disease burden using quantitative genomic PCR for the presence of the integrated JAK2V617F retrovirus, the ability of serum derived from treated animals to inhibit growth of human BFU-E, and reduction of phosphorylated Stat5 as assessed by flow cytometry.

Quantitative PCR for the JAK2V617F retrovirus was performed on DNA derived from peripheral blood, bone marrow, and spleen of treated and control animals at study endpoint. We observed a dose-dependent reduction in genomic disease burden using
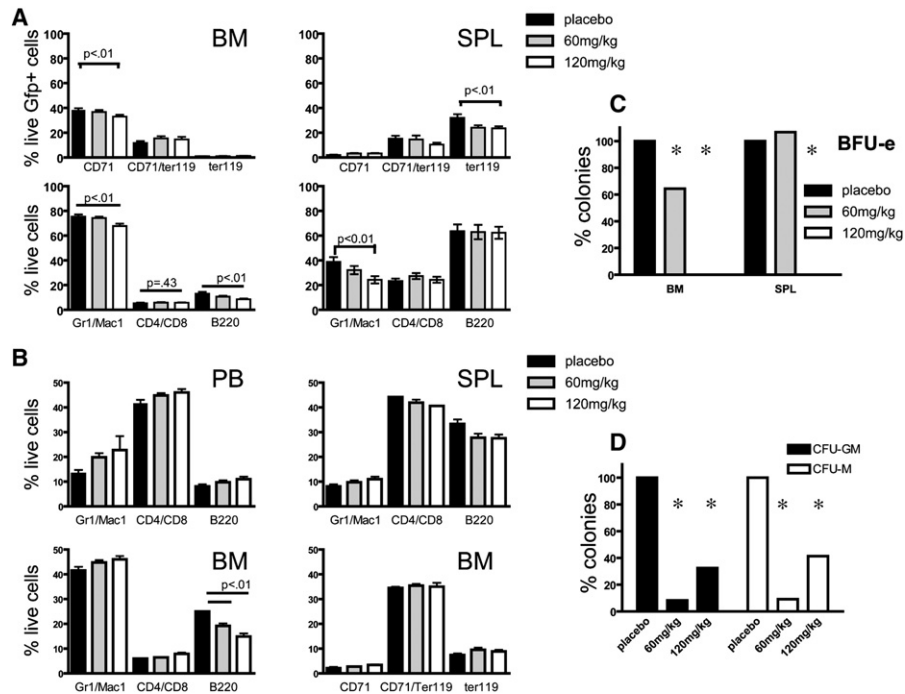
with vehicle. A similar reduction was observed in the number of cells of the neutrophil/monocyte lineage that express both Gr1 and Mac1 markers that delineate these cells in mice in the bone marrow or spleens at this drug dose. There were modest effects observed on mature B cells when compared with control (Figure 3A).

The potential effect of the drug on lymphopoiesis was difficult to assess in the context of a fulminant myeloproliferative disease after lethal irradiation for bone marrow transplantation. To better understand the effects of TG101348, a cohort of three groups of wild-type nonirradiated animals (n = 7) was treated concurrently for 42 days by oral gavage with vehicle, 60 mg/kg or 120 mg/kg of drug, respectively. Differential blood counts demonstrated a slight reduction of total WBC and hematocrits for wild-type animals in the 100 mg/kg treatment arm but within the normal range (Table S3). With flow cytometric analysis we detected no effect on T cell number in either the bone marrow or spleen of animals treated with TG101348. However, we observed a statistically significant reduction, albeit modest, in B cell number in the bone marrow of JAK2V617F and the bone marrow and spleen of wild-type animals, but not in other compartments. Thus, 42 days of treatment with TG101348 had no appreciable effect on

Figure 3. Assessment of Efficacy by Flow Cytometric Analysis and Hematopoietic Colony-Forming Capacity in Response to Treatment with TG101348

(A) The bar graphs in the top row display staining of the average of the erythroid compartment of live JAK2V617F-expressing cells and demonstrate a 2.3-fold decrease in the CD71 single-positive (p < 0.01) immature erythroid precursor population in bone marrow of animals treated with 120 mg/kg of drug and a 2.5-fold decrease in ter119 single-positive more mature erythroid precursors (p < 0.01) in spleen. The bar graphs in the bottom row represent percentages of neutrophil/monocyte cells expressing Gr1 and Mac1 treated with 120 mg/kg (p < 0.01).

(B) The bar graphs represent the hematopoietic compartment of PB, BM, and spleen of wild-type nonirradiated mice. Notably, no differences among the placebo and both drug-treated groups for neutrophil/monocyte cells expressing Gr1 and Mac1 markers, T-lymphocytes, erythroid precursors, and B-lymphocytes in spleens were detected, although a modest reduction of B-lymphocytes for both drug-treated groups was observed in BM. The error bars in (A) and (B) represent the mean ± SD.

(C) Total numbers of BFU-E were decreased by 40% in the bone marrow of the 60 mg/kg group (p < 0.01) and absent in the bone marrow and spleens of the 120 mg/kg group (p < 0.01) after 42 days of treatment with TG101348 compared to placebo.

(D) Total numbers of CFU-GM and CFU-M of splenocytes were reduced at least 2.5-fold (p < 0.05).

this metric, with ablation of hematopoietic cells containing the JAK2V617F retrovirus at the 120 mg/kg dose (PB p < 0.001, BM and Spl p < 0.01), with a more limited response at the 60 mg/kg dose level (Spl p < 0.05) (Figure 4A and Figure S4A). These data indicate that TG101348 effectively reduces disease burden in treated animals, and suggests that quantitative assessment of *JAK2V617F* allele burden may be of value in monitoring response to therapy.

JAK2V617F-positive MPD is also associated with the pathognomonic finding of erythropoietin independent erythroid colony formation (EEC) that offers an opportunity to assess functional eradication of this population by JAK2 inhibitors. In the presence of erythropoietin in vitro, we observed an ~5-fold or ~8.5-fold reduction of BFU-E derived from patients with PV at 300 or 600 nM TG101348, respectively. Of note, in the absence of erythropoietin, TG101348 showed increased potency, with nearly complete inhibition of EEC formation at a concentration of 300 nM (Figure 4B). We next assessed inhibition of human EEC from PV bone marrow by treatment with serum derived from drug-treated mice. Serum was collected 2 hr after a dose of 60 mg/kg or 120 mg/kg, respectively, and added to methylcellulose cultures of bone marrow cells from human PV patients with *JAK2V617F*.

We observed abrogation of erythroid colonies scored at 7 days at each dose level of TG101348 (Figure 4C).

We next assessed the potential value of measuring activation status of signal transduction targets of JAK2V617F such as STAT5 in monitoring response to therapy. We first characterized the effect of TG101348 on signal transduction pathways activated by JAK2V617F in vivo using flow cytometry with phospho-specific antibodies to STAT5, Erk or S6 kinase (S6K), an effector of the PI3K/AKT pathway. Flow cytometry was used to isolate disease-relevant hematopoietic progenitors derived from animals by gating on GFP+CD71+ immature erythroblasts, or GFP+Mac1+Gr1+ myeloid cells that harbored the JAK2V617F retrovirus. We assessed the activation state of signaling intermediates in cells starved of growth factors for 4 hr and then stimulated with Interleukin 3 (IL3) and erythropoietin (EPO). JAK2V617F expressing myeloid cells had comparable basal levels of activation of Stat5, Erk, or S6K compared to wild-type cells, but showed a marked relative increase in activation state of these proteins after stimulation with IL3 and EPO (Figure 5A). In contrast JAK2V617F expressing erythroid cells had activated basal signaling levels, but upon cytokine stimulation (IL-3 and EPO), the degree of phosphorylation further
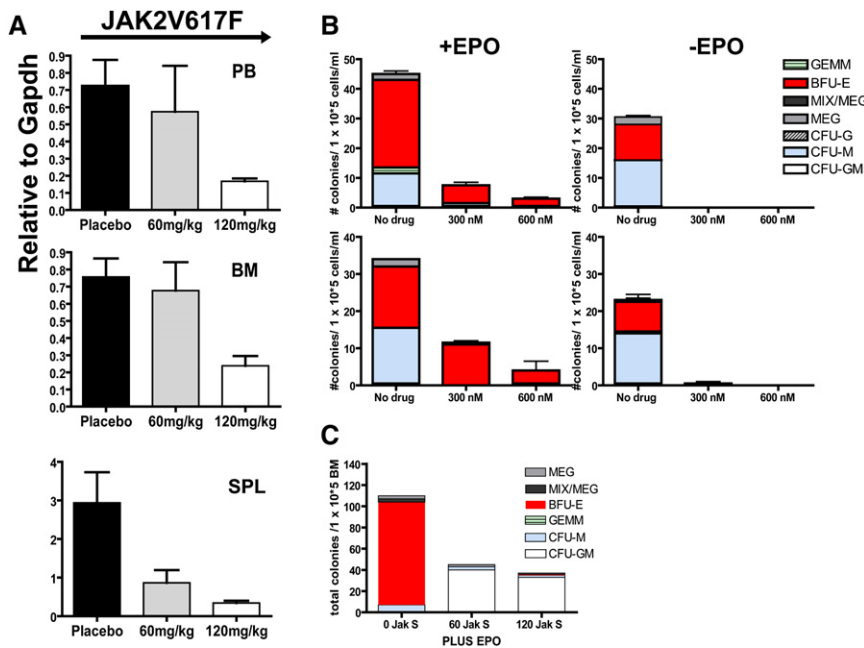
Figure 4. Evaluation of Surrogate Endpoints of Response after TG101348 Treatment

(A) Quantitative PCR for JAK2V617F retrovirus on DNA from peripheral blood, bone marrow, and spleen of treated and control animals at study endpoint.

(B) TG101348 inhibits EEC formation of bone marrow (upper panel) and peripheral blood (lower panel) of PV patients with and without erythropoietin in a dose-dependent manner.

(C) Inhibition of erythroid colony formation of PV bone marrow with mouse serum collected 2 hr after a dose of 60 mg/kg or 120 mg/kg. The error bars in (A)–(C) represent the mean ± SD.

increased. In vitro treatment of stimulated JAK2V617F cells, myeloid as well as erythroid with TG101348 resulted in a marked decrease in activation of Stat5 as assessed by staining with phosphospecific antibody. These data indicate that TG101348 is capable of inhibiting phosphorylation of STAT5 and other effectors of the JAK-STAT signaling pathway in relevant primary hematopoietic cells expressing JAK2V617F derived from animals.

We then tested whether flow cytometry could be used to measure pharmacodynamic response to therapy in treated animals in vivo. Cells were harvested from animals 2 hours after treatment with TG101348, sorted for the markers described above, and immediately stained for phosphoStat5. We observed a marked reduction in phosphorylated Stat5 and phosphorylated S6K in GFP+CD71+ immature erythroblasts, as well as GFP+Mac1+Gr1+ myeloid cells derived from TG101348 treated versus vehicle control animals (Figure 5B; Figure S4B).

We also assayed activation state of Stat5 in BFU-E derived from bone marrow of patients with polycythemia vera. After growth of BFU-E for 7 days in methylcellulose cultures, individual colonies were isolated and treated with TG101348 at a concentration of 600 nM on glass slides. Immunofluorescence confocal microscopy demonstrated a >90% reduction in phosphoStat5 in treated cells versus controls (Figure 5C).

Taken together, these findings indicate that one or more experimental approaches may be of value in evaluating surrogate endpoints for response in clinical trials of JAK2 inhibitors in MPD, including (1) assessment of disease burden using quantitative genomic PCR for the *JAK2V617F* disease allele, (2) functional assessment of the EEC activity in treated patients, and (3) flow cytometry using antibodies that delineate activation state of signal transduction effectors of JAK2V617F.

DISCUSSION

JAK2V617F is an attractive therapeutic target in MPD, and there is a substantial patient population that may benefit from improved therapy that is considerably larger than for related diseases such as CML. Although there is evidence for genetic heterogeneity among PV, ET, and PMF(Levine et al., 2006) and the acquisition of *JAK2V617F* may be a secondary event in at least some cases of sporadic and heritable MPD, this single allele is associated with the majority of cases. *JAK2V617F* or *JAK2* exon 12 mutations appear to be present in all cases of polycythemia vera, and *JAK2V617F* is present in ~50% of cases of ET or PMF, respectively (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Scott et al., 2007). Exon 12 mutations appear to occur more frequently in individuals whose clinical phenotype is characterized by polycythemia with a trend toward normal leukocyte and platelet counts. This observation in humans and data from the mouse model suggest that the exon 12 mutations confer a relative predilection for erythroid cell-fate determination compared with the exon 14 mutation. Although a solved structure is not available, predicted structures place the exon 12 and exon 14 mutations as physically proximate. *JAK2V617F* and related alleles each confer factor-independent growth to hematopoietic cell lines, and murine models of disease recapitulate many of the features of human MPD (Wernig et al., 2006; Lacout et al., 2006; Zaleskas et al., 2006). In these settings, JAK2 inhibition induces apoptotic cell death, indicating that, like other oncogenic tyrosine kinases, JAK2V617F confers dependence on JAK signaling to the resident hematopoietic progenitor cell. Thus, a safe and efficacious small-molecule inhibitor of JAK2 might be expected to have therapeutic benefit in these cases, analogous to that observed in stable phase BCR-ABL-positive CML patients treated with imatinib. Furthermore, cellular cytotoxicity assays with novel alleles discovered in the remainder of MPD cases, such as MPLW515L/K that is associated with ~2%–5% of ET or PMF, are also sensitive to inhibition with JAK2 inhibitors (Pikman et al., 2006).

Our data indicate that the JAK2 selective inhibitor TG101348 has potential for efficacious treatment of JAK2V617F-associated MPD. We utilized a murine model in which disease was fully established prior to initiation of therapy and demonstrated dose-dependent responses to therapy. We designed these studies to accommodate the long half-life of red cells in mice (40–50 days) by treating animals for 42 days with TG101348. Although
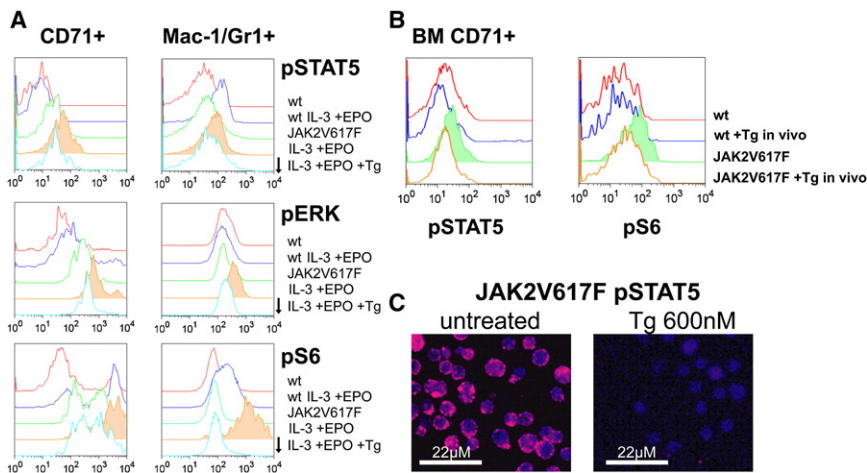
Figure 5. Assessment of JAK2V617F Signal Transduction Pathways in Response to Therapy with TG101348

(A) Bone marrow of wild-type (WT) or JAK2V617F were harvested then starved for 4 hr. Cells were incubated with indicated inhibitors for 15 min and then stimulated with IL-3 and EPO for 10 min before fixation and staining. Cells were then analyzed by flow cytometry and gated for CD71 or Mac-1/Gr-1 double-positive cells. (A) and (B) are representative of 3–5 independent experiments.

(B) Bone marrow from either WT or transplanted mice with JAK2V617F were treated with or without TG101348 120 mg/kg and fixed immediately and stained with indicated antibodies. Levels of phosphorylation were determined using flow cytometry.

(C) Human PV BFU-E colonies were picked after 14 days in culture and then put on slides. Indicated cells were treated with TG101348 for 15 min and then fixed and stained for pSTAT5 (red) and DAPI (blue). Images were taken and overlaid using a confocal immunofluorescence microscope and a representative image chosen for display.

time and labor intensive, such an approach enables accurate assessment of red cell response, in addition to providing insights into potential toxicities associated with long-term therapy. We observed that the drug was well tolerated, with statistically significant reductions in HCT and WBC, with concomitant evidence of response by histopathologic analysis, including attenuation of reticulin fibrosis, flow cytometric evidence for reduction in erythroid and myeloid progenitors, and reduction of colony-forming activity of these populations in vitro.

Thus, these in vivo data suggest that there is promise for this approach to treatment of *JAK2V617F*-positive MPD. In addition, there is a strong precedent for use of small-molecule tyrosine kinase inhibitors in BCR-ABL-positive CML, PDGFRB-rearranged CMML, PDGFRA-rearranged CEL, gastrointestinal stromal cell tumors, and non-small-cell lung carcinomas(Apperley et al., 2002; Cools et al., 2003; Druker et al., 2001a, 2001b; Lynch et al., 2004; Pao et al., 2004; van Oosterom et al., 2001). However, there are particular challenges in applying this strategy for treatment of *JAK2V617F*-positive MPDs.

PV, ET, and PMF are relatively indolent diseases that progress to AML at a lower frequency than CML, estimated to be ~5% over the lifetime of affected individuals. Furthermore, although PV, ET, and PMF are associated with potentially serious and/or fatal complications including thrombosis and hemorrhage, these are also relatively infrequent (Harrison et al., 2005). Thus, a JAK2 inhibitor for treatment of PV, ET, or PMF should be safe for prolonged treatment, and strategies need to be developed that will allow for reliable prediction and monitoring of response to therapy and identification of surrogate endpoints for response that will enable expeditious testing of JAK2 inhibitors.

As regards safety, in addition to the standard toxicities, attention has focused on potential off-target effects of JAK2 inhibitors on JAK3. PV, ET, or PMF patients are not immunocompromised as a consequence of their primary disease. If one assumes that, as for BCR-ABL-positive CML, long-term therapy is likely to be required for maintenance of remission, any effect on T cell number of function due to JAK3 inhibition could be deleterious in this patient population. In this light, efforts to develop JAK2 inhibitors

that are highly selective for JAK2 compared to JAK3, such as TG101348, are warranted.

Prediction and monitoring of response to therapy will also be of central importance based on the paradigm for treatment of BCR-ABL-positive disease, where it is now appreciated that molecular assessments of disease burden and of initial response to therapy are highly predictive of long-term responses. To this end, a need for reliable assays that measure disease burden can be anticipated in clinical trials of JAK2 inhibitors. Our findings indicate that genomic copy burden correlates with clinical response to disease, and such measurements should be considered as part of clinical trial design for treatment in humans. In addition, in PV, ET, or PMF with *JAK2V617F*, in contrast with CML, there is an advantage of assays for EEC (Prchal and Axelrad, 1974) that provides an opportunity for functional assessment of the malignant clones to therapy. Our data indicate that such assays may also be of value in clinical trials. Finally, assessment of the activation state of STAT5, or other effectors of activated JAK2 such as ERK or S6K, may be of value as pharmacodynamic marker of response. These type of surrogate endpoints for response have been validated in the context of CML (Prchal and Axelrad, 1974) and may enable more rapid assessment of the clinical potential of JAK2 inhibitors in smaller trials with fewer number of patients.

This animal model does not address the potential of JAK2 inhibitors for treating thrombocythemia associated with these MPD, in that thrombocytosis is not observed in the *JAK2V617F* murine model (Wernig et al., 2006). However, MPLW515L does induce robust thrombocytosis in the murine bone marrow transplant model and is sensitive to inhibition with JAK2 inhibitors, so this system may prove suitable for assessing platelet responses (Pikman et al., 2006).

Lastly, these findings raise the question of which patient population should be tested initially for efficacy and safety of JAK2 inhibitors. Although there are several potential approaches, it would seem most appropriate to treat individuals with severe manifestations of disease that are currently refractory to conventional therapies, such as progressive myelofibrosis. In this light, it

is promising to observe that, at least in this murine model of disease, JAK2 inhibition resulted in attenuation of myelofibrosis.

EXPERIMENTAL PROCEDURES

Reagents

TG101348 (N-tert-butyl-3-(5-methyl-2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenylamino)pyrimidin-4-ylamino)benzenesulfonamide) was synthesized by TargeGen Inc. (San Diego, CA, USA). Stock solutions were made in dimethylsulfoxide (DMSO), and subsequently diluted in RPMI-1640 medium for use. Anti-phospho-Stat5 (Tyr694) (polyclonal) and anti-Stat5 (polyclonal) were purchased from Cell Signaling (Beverly, MA, USA), and anti-β-actin (monoclonal) was purchased from Novus Biologicals Inc. (Littleton, CO, USA).

Cell Lines

Human erythroleukemic cell line (HEL) was purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI-1640 medium (GIBCO BRL, Gaithesburg, MD, USA), supplemented with 10% fetal bovine serum (FBS) penicillin at 37°C and 5% CO_2. The V617F mutation was introduced by site-directed mutagenesis (QuikChange kit, Stratagene, La Jolla, CA) into a plasmid that carries the full length human *JAK2* cDNA (Invitrogen, Carlsbad, CA). Once confirmed by DNA sequencing, the *JAK2V617F* cDNA was subcloned into a retroviral vector (Stratagene). Recombinant retroviral particles were produced in HEK293 cells and subsequently transduced into Ba/F3 cells. Permanently transduced Ba/F3 cells that express human JAK2V617F were selected and maintained by 1 mg/ml G418.

IC$_{50}$ Determinations by Cell-free Kinase Activity Assays

IC$_{50}$ values for TG101348 were determined commercially using the InVitrogen (Carlsbad, CA, USA) kinase profiling service for a 223 kinase screen that included JAK2 and JAK2V617F or Carna Biosciences (Kobe, Japan) for the screen of all Janus kinase family members including JAK1 and Tyk2. ATP concentration was set to approximately the Km value for each kinase.

XTT Assay for Cell Proliferation, Apoptosis, and DNA Laddering Assay

Approximately 2×10^3 cells were plated into microtiter-plate wells in 100 μl RPMI-1640 growth media with indicated concentrations of inhibitor. Following 72 hr incubation with TG101348, 50 μl of XTT dye (Roche; Basel, Switzerland) were added to each well and incubated for 4 hr in a CO_2 incubator. The colored formazan product was measured by spectrophotometry at 450 nm with correction at 650 nm. The concentration in which 50% of the effect (i.e., inhibition of proliferation) is observed (IC$_{50}$) was determined using the GraphPad Prism 4.0 software. All experiments were performed in triplicate, and the results were normalized to growth of untreated cells. Induction of apoptosis of EpoBa/F3 JAK2V617F, Ba/F3p210, HEL, and K562 cells was determined by DNA fragmentation with DMSO and increasing concentrations of inhibitor.

Western Blot Analysis

Cells were treated with DMSO and increasing concentrations of inhibitor for 4 hr in RPMI-1640 before collected in 1× Cell Lysis Buffer (Cell Signaling, Beverly, MA, USA), containing 1 mM PMSF, and protease inhibitor cocktail tablets (Roche). Protein lysates were quantified with Pierce Biotechnology BCA assay (Rockford, IL, USA). Similar protein amounts were mixed with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) plus β-mercaptoethanol, boiled for 5 min, and separated on a 4%–15% Tris-HCL gradient electrophoresis gel (Bio-Rad Laboratories). Gels were blotted onto a 0.45 μm nitrocellulose membrane (Bio-Rad), which was blocked in 5% nonfat dry milk and incubated with primary antibodies in either blocking solution or 5% BSA. The membranes were subsequently incubated with a mixture of donkey anti-rabbit IgG conjugate with infrared fluorophore (700 nm emission, LICOR) and goat anti-mouse IgG conjugated with infrared fluorophore (800 nm emission). Following washing with PBS, the membranes were scanned on a LICOR Odyssey scanner to detect total (red) and phospho-STAT5 (green) proteins.

Pharmacokinetic Properties of TG101348 in C57Bl/6 Mice

Fifty-four C57Bl/6 mice were divided into 3 groups with 18 mice at each dose level. Single oral doses of 30, 100, and 200 mg/kg were administered. Animals were allowed food and water ad libitum. Composite sampling was employed to generate plasma concentration-time profiles for TG101348 over the following time course (n = 3/ time point): 0.5, 1, 3, 5, 7, and 24 hr postdose. Plasma samples were processed by addition of a 2-fold excess of acetonitrile containing internal standard followed by centrifugation. The supernatants were isolated for analysis. Processed plasma samples were quantitated by LC/MS/MS against external calibration standards prepared in naive mouse plasma. Matrix calibration standards and quality control (QC) samples were prepared by adding stock solutions of TG101348 into blank mouse plasma. The concentrations of the external calibration curve ranged from 1.9 to190 nM. Study samples above the upper calibration limit were diluted into the calibration range with blank mouse plasma and reanalyzed.

The LC/MS/MS system consisted of a Sciex API3000 triple quadrupolar mass spectrometer (MDS Sciex), an Agilent 1100 HPLC system (Agilent Technologies, Inc), and a CTC autosampler (Leap Technologies). The LC separations were performed on a Zorbax SB 75 × 2.1 mm and a 3.5 μm reverse phase HPLC column (Agilent Technologies, Inc). The column temperature was kept at 40°C. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The flow rate was kept constant at 0.40 ml/min. Following a 20 μl sample injection, mobile B was held at 10% for 0.5 min followed by a linear increase to 90% mobile phase B over 1.5 min. The mass spectrometric detection of TG101348 and internal standard was achieved using electrospray ionization operating in positive ionization mode. The molecular ion transitions were monitored in MRM mode for TG101348 and internal standard.

Murine Model and Analysis of Mice after Treatment with TG101348

The murine BM transplant model was generated and analyzed exactly as previously described (Wernig et al., 2006). Briefly, C57BL/6 mice (Taconic, Germantown, NY) were intravenously injected with 1×10^6 whole bone marrow expressing JAK2V617F. Full development of disease was assessed with differential peripheral blood counts at day 26 after bone marrow transplantation. TG101348 was administered by oral gavage twice daily (b.i.d.) at 60 mg/kg, 120 mg/kg, or placebo from day 28 on for 42 days. Differential blood counts were assessed by retro-orbital nonlethal eyebleeds using EDTA glass capillary tubes before study initiation, during the study, and at study endpoints. C57/Bl6 mice were sacrificed at study endpoint or at times indicated based on an IUCAC-approved protocol that includes assessment of morbidity by > 10% loss of weight, scruffy appearance, lethargy, and/or splenomegaly extending across the midline. For histopathology, tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin or, to assess for fibrosis, stained with reticulin. Images of histological slides were obtained on a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) equipped with a SPOT RT color digital camera model 2.1.1 (Diagnostic Instruments, Sterling Heights, MI). Images were analyzed in Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). For flow cytometry, cells were washed in PBS, washed in 2% fetal bovine serum, blocked with Fc-Block (BD PharMingen, San Diego, CA) for 10 min on ice, and stained with monoclonal antibodies in PBS and 2% FCS for 30 min on ice. Antibodies used were allophycocyanin (APC)-conjugated ter119, Gr-1, CD4, and B220 and phycoerythrin (PE)-conjugated Mac1, CD8 (all 1:200), and CD71(1:100) rat anti-mouse (BD PharMingen). After washing, cells were resuspended in PBS and 2% FCS containing 0.5 μg/ml 7-amino-actinomycin D (7-AAD) (BD PharMingen) to allow discrimination of nonviable cells. Flow cytometry was performed on a FACS Calibur cytometer (BD Biosciences, San Jose, CA), at least 10,000 events were acquired, and data were analyzed using FloJo software. The results are presented as graphs and representative dot plots of viable cells selected on the basis of scatter and 7-AAD staining.

Colony Assays

To assess colony-forming capacity during in vivo drug treatment, 1×10^5 BM and spleen cells from JAK2V617F mice treated with 60 mg/kg or 120 mg/kg of drug or placebo were harvested at study endpoint, plated in duplicate in methylcellulose (M3434, Stem Cell Technologies) according to the manufacturer's

protocol, and counted after 7 days in culture. For colony assays with human MPD patient samples, whole bone marrow and peripheral blood of PV and ET patients positive for *JAK2V617F*, 1×10^5 cells/ml of bone marrow as well as peripheral blood were plated in methylcellulose (H4534 Stem Cell Technologies) without Epo or supplemented with 3 units of Epo (Cell Sciences). For human colony assays incubated with mouse serum, serum was collected from animals 2 hr postdosing with 60 mg/kg, 120 mg/kg, or placebo.

DNA Isolation and Measurement of JAK2V617F Virus Sequence to Detect Genomic Disease Burden by Quantitative Genomic PCR

Genomic DNA was isolated from peripheral blood, bone marrow, and spleen of drug-treated animals using the QIAamp DNA Blood Mini kit (QIAGEN). Quantitative PCR was performed using primers for retroviral sequence of JAK2V617F MSCV-IresGfp, 5'-AGG CGC CG AAT TAG ATC T and 3'-GAG GTT GCC TCC ATT TCT GTC. Primers were designed using Primer Express software (Applied Biosystems). Sample DNA was amplified with Taqman 2× PCR master mix or 2× SYBR green PCR master mix (Applied Biosystems). Reactions were run in duplicate with 2.5 μl cDNA in a total reaction volume of 25 μl using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Ratio of the means of relative copy number of the JAK2V617F MSCV-IresGfp virus sequence was calculated relative to Gapdh using a standard curve technique.

Flow Cytometric Staining of Intracellular Phosphorylated STAT5, ERK, and S6K

Freshly isolated BM from wild-type JAK2- and JAK2V617F-transplanted animals was harvested 3 hr after dosing with TG101348 120 mg/kg as control placebo-treated wild-type JAK2 and JAK2V617F bone marrow was used, fixed immediately with 2% PFA (Polysciences, Inc., Warrington, PA), and permeabilized with 90% ice-cold methanol or cultured for 4 hr in RPMI + 1% BSA at 37°C stimulated with IL-3, SCF, and IL-6 (StemCell technologies) or EPO (Cell Sciences) and treated with TG101348 (0.6 μM). Staining was performed as previously described (Krutzik and Nolan, 2003). Briefly, cells were incubated with anti-phospho-STAT5 PE (phycoerythin) (BD pharmingen) 1:50, anti-phospho-ERK 1:100 (Cell signaling), and anti-phospho-S6 1:100 (Cell signaling), respectively. Following a final wash, the samples were sorted by flow cytometry on a FACS Aria and analyzed using FloJo software. Human cells were analyzed on a ZEISS LSM510-META upright laser confocal microscope using 62× magnification and analyzed with LSM software from Zeiss.

Statistical Analysis, Animal Studies, and Patient Sample Collection

Pooled data are displayed as mean ± SD. To assess statistical significance among the two groups, the paired Student's t test was used. Animal studies were conducted using an IUCAC-approved animal protocol at our institution. In addition, studies involving human material were obtained under auspices of informed consent, and the study was approved by Institution Review Board.

SUPPLEMENTAL DATA

The Supplemental Data include four supplemental figures and three supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/4/311/DC1/.

ACKNOWLEDGMENTS

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