

ORIGINAL ARTICLE

TG101209, a small molecule JAK2-selective kinase inhibitor potently inhibits myeloproliferative disorder-associated JAK2V617F and MPLW515L/K mutations

A Pardanani¹, J Hood², T Lasho¹, RL Levine³, MB Martin², G Noronha², C Finke¹, CC Mak², R Mesa¹, H Zhu², R Soll², DG Gilliland^{3,4} and A Tefferi¹

¹Division of Hematology, Mayo Clinic, Rochester, MN, USA; ²Research and Development, TargeGen Inc., San Diego, CA, USA; ³Brigham and Women's Hospital and the Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA and ⁴Howard Hughes Medical Institute, Harvard Medical School, Boston, MA, USA

JAK2V617F and MPLW515L/K represent recently identified mutations in myeloproliferative disorders (MPD) that cause dysregulated JAK-STAT signaling, which is implicated in MPD pathogenesis. We developed TG101209, an orally bioavailable small molecule that potently inhibits JAK2 (IC₅₀ = 6 nM), FLT3 (IC₅₀ = 25 nM) and RET (IC₅₀ = 17 nM) kinases, with significantly less activity against other tyrosine kinases including JAK3 (IC₅₀ = 169 nM). TG101209 inhibited growth of Ba/F3 cells expressing JAK2V617F or MPLW515L mutations with an IC₅₀ of ~200 nM. In a human JAK2V617F-expressing acute myeloid leukemia cell line, TG101209-induced cell cycle arrest and apoptosis, and inhibited phosphorylation of JAK2V617F, STAT5 and STAT3. Therapeutic efficacy of TG101209 was demonstrated in a nude mouse model. Furthermore, TG101209 suppressed growth of hematopoietic colonies from primary progenitor cells harboring JAK2V617F or MPL515 mutations.

Leukemia advance online publication, 31 May 2007;
doi:10.1038/sj.leu.2404750

Keywords: myeloproliferative disorder; mutation; JAK2V617F; kinase inhibitor

Introduction

Acquisition of somatic mutations such as JAK2V617F¹ results in constitutive activation of JAK-STAT signaling, which is thought to play a primary role in the pathogenesis of myeloproliferative disorders (MPD) including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). In normal hematopoiesis, ligand-induced activation of a spectrum of hematopoietic cytokine receptors, including receptors for erythropoietin, thrombopoietin (MPL) and granulocyte colony stimulating factor, converges upon Janus kinase 2 (JAK2). The importance of JAK2 in hematopoiesis has been demonstrated in mice that are genetically deficient in JAK2, and have severe defects in erythropoiesis.² Dysregulated JAK-STAT signaling may be important in JAK2V617F-negative MPD as well, in that other activating JAK2 alleles have been identified in these patients, including JAK2 exon 12 mutations, JAK2D620E, JAK2ΔIREED, in addition to activating mutations in MPL at position W515.^{3–6}

Expression of JAK2V617F *in vivo* in a murine bone marrow transplant assay results in a phenotype resembling PV;^{7,8} in contrast, MPLW515L expression in a similar assay results in a PMF-like phenotype.⁹ Inhibition of JAK2 with small molecule 'tool' compounds that lack potential for clinical development, and that are not selective among JAK family members, induce apoptotic cell death in hematopoietic cell lines transformed

either with JAK2V617F¹⁰ or with MPLW515L,⁹ and, similarly, may decrease the hematocrit in mouse models of JAK2V617F-induced disease.⁸

MPD are currently not captured in the surveillance, epidemiology and end results (SEER) database or other cancer registries, but incidence of PV, ET and PMF has been estimated in the 1–5/100 000 per year range.¹¹ Because of relatively long survival after diagnosis, it has been estimated that the prevalence of MPD is on the order of 80 000–100 000 cases in the United States, significantly higher than that of BCR-ABL-positive chronic myeloid leukemia (CML). Although the MPD are relatively indolent, most patients ultimately develop one or more complications related to their disease, including thrombosis, hemorrhage, massive splenomegaly, or progression to acute leukemia. Current therapy is empirically derived, and not without attendant side effects; most patients are not candidates for stem cell transplantation given their advanced age at the time of diagnosis.

We developed TG101209, a small molecule, selective JAK2 kinase inhibitor using structure-based drug design. We present results from pre-clinical studies showing that TG101209 is a potent inhibitor of JAK2V617F and MPLW515L mutations, both *in vitro* and *in vivo*.

Materials and methods

Reagents

TG101209 (*N*-tert-butyl-3-(5-methyl-2-[4-(4-methyl-piperazin-1-yl)-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-Jak2 [Tyr1007/1008] (polyclonal), anti-Jak2 [24B11] (monoclonal), anti-phospho-Stat5 [Tyr694] (polyclonal), anti-Stat5 (polyclonal), anti-phospho-Stat3 [Tyr705] (monoclonal) and anti-Stat3 [124H6] (monoclonal) were purchased from Cell Signaling (Beverly, MA, USA), and anti-β-actin (monoclonal) from Novus Biologicals Inc. (Littleton, CO, USA).

Molecular Modeling

See Supplementary Information.

Cell lines

Human erythroleukemia (HEL), Ba/F3, CTLL-2 and normal human dermal fibroblasts (NHDF) cells were purchased from American Type Culture Collection (Rockville, MD, USA). Ba/F3 cells expressing JAK2V617F (Ba/F3-EpoR-V617F)¹² and

Correspondence: Professor A Tefferi, Division of Hematology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA.

E-mail: tefferi.ayalew@mayo.edu

Received 12 April 2007; accepted 18 April 2007

MPLW515L (Ba/F3-W515L)⁹ mutants, and the human leukemia cell lines HEL, K562, CHRF-288-11 and CMK were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and L-glutamine at 37°C and 5% CO₂. CTLL-2 and Ba/F3 cells were grown in the same media further supplemented with 20 U/ml recombinant mouse IL-2 and 1 ng/ml IL-3, respectively (Hoffmann-LaRoche, Nutley, NJ, USA). Green fluorescent protein (GFP) was introduced into Ba/F3-V617F cells by lentiviral transduction using pLenti6-GFP (Invitrogen, Carlsbad, CA, USA), followed by selection with blasticidin and confirmation of GFP expression using flow cytometry analysis (Ba/F3-V617F-GFP).

Cell-free kinase activity assays (IC₅₀ determinations)
see Supplementary Information.

XTT assay for cell proliferation

In brief, approximately 2×10^3 cells were plated into microtiter-plate wells in 100 μ l RPMI-1640 growth media with indicated concentrations of inhibitor. The relative growth of cells was quantified at 24-h intervals using Cell Proliferation Kit II (XTT) as per manufacturer's guidelines (Roche, Indianapolis, IN, USA). After incubation, 20 μ l of XTT was added to the wells and allowed to incubate for 4–6 h. The colored formazan product was measured spectrophotometrically at 450 nm with correction

at 650 nm, and IC₅₀ values were determined using the GraphPad Prism 4.0 software. Data were subjected to a non-linear regression-fit analysis and IC₅₀ values were determined as the concentration, that inhibited proliferation by 50%. All experiments were done in triplicate and the results normalized to growth of untreated cells.

Cell cycle and apoptosis assays

Cell cycle was analyzed with the FITC BrdU Kit (BD Pharmingen, San Diego, CA, USA), as per manufacturer's instructions. Cells were incubated with indicated concentrations of inhibitor for 24 h, and subsequently pulsed with BrdU for 30 min at 37°C. The cells were washed in staining buffer (1 \times Dulbecco's phosphate-buffered saline (DPBS) + 3% FBS), fixed/permeabilized with Cytotfix/Cytoperm buffer and washed with Perm/Wash buffer, with all incubations carried out on ice. After permeabilization, cells were treated with 30 μ g DNase for 1 h at 37°C, and then stained with FITC-conjugated anti-BrdU antibody and 7-AAD before flow cytometric analysis. DNA contents were analyzed with the use of a FACS Canto flow cytometer with FACS Diva software (BD Biosciences, San Jose, CA, USA).

Induction of apoptosis of HEL and K562 cells was determined by analyzing the binding of Annexin V and the incorporation of PI, following incubation with indicated concentrations of inhibitor for 0, 24, 48 and 72 h. The cells were washed with 1 \times PBS, resuspended in apoptosis buffer (1 mM HEPES/NaOH, pH, 7.4, 140 mM NaCl and 2.5 mM CaCl₂) and stained with 5 μ l

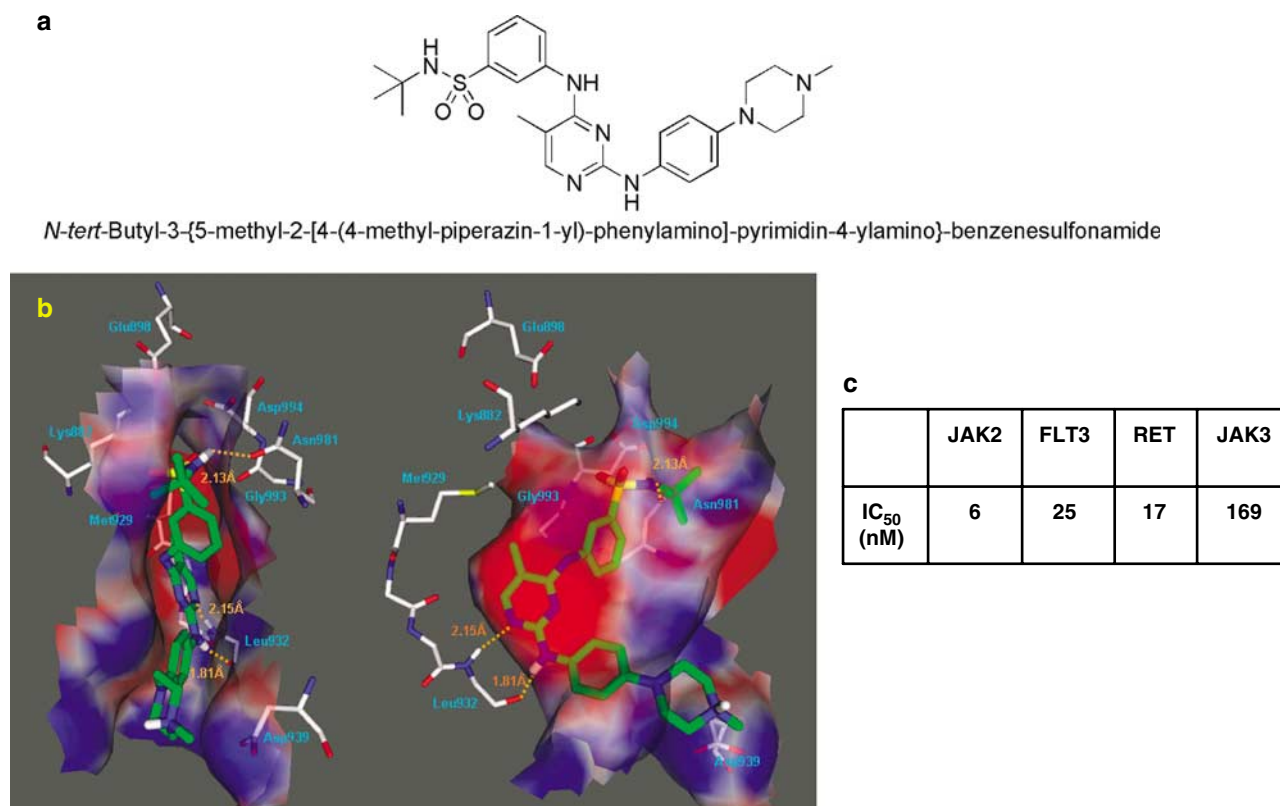


Figure 1 Molecular structure of TG101209 and its selective inhibition of JAK2 kinase. (a) Chemical structure of TG101209 (molecular formula, C₂₆H₃₅N₇O₂S; molecular weight, 509.7; melting point 243°C). (b) Molecular model depicting docking of TG101209 in the JAK2 ATP pocket. The shaded surface illustrates hydrophobic (red) and hydrophilic (blue) portions of the protein. Key inhibitor–protein interactions, including the hydrogen bond with the hinge Leu932 residue, the hydrophobic contacts in the shallow angular pocket lined by residues Met929, the methylene groups of Lys882, and the initial portion of the DFG (AspPheGly) (Asp994 shown) activation loop, as well as the hydrogen bond with the NH of the sulfonamide from TG101209 with Asn981 are shown. (c) TG101209 inhibitory activity (IC₅₀) against select kinases in the *in vitro* kinase assay.

Annexin V APC (BD Biosciences, San Diego, CA, USA) for 15 min at room temperature. Cells were resuspended in Apoptosis Buffer and 5 μ l of PI was added. Samples were analyzed on a FACScalibur cytometer (BD Biosciences, San Jose, CA, USA).

Western blot analysis

Cells were treated with inhibitor for 18 h in RPMI-1640 before harvesting in 1 \times Cell Lysis Buffer (Cell Signaling, Beverly, MA, USA), supplemented with 1 mM PMSF, and complete protease inhibitor cocktail tablets (Roche). Lysates were clarified by centrifugation and protein was quantified with the Pierce Biotechnology BCA assay (Rockford, IL, USA). Equal amounts of protein were combined with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) plus β -mercaptoethanol, boiled for 5 min, and loaded onto 4–15% Tris–HCl gradient electrophoresis gels (Bio-Rad Laboratories). Gels were transferred to 0.45 μ m trans-blot nitrocellulose membrane (Bio-Rad), the membrane blocked in 5% nonfat dry milk, and blotted overnight with primary antibodies diluted in either blocking solution or 5% bovine serum albumin. After washing, the primary antibodies were revealed using the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling) and detected by the Phototope chemiluminescence kit (Cell Signaling). In conjunction, blots were probed with anti- β -actin antibody from Novus Biologicals Inc. to confirm equal loading of protein.

Murine tumor model and drug treatment effect

Severe combined immunodeficiency (SCID) mice (Harlan, Indianapolis, IN, USA) were intravenously injected with 10×10^6 sorted GFP-positive Ba/F3 cells expressing JAK2V617F (Ba/F3-V617F-GFP). TG101209 was administered by oral gavage at the indicated doses beginning day +3 after tumor cell infusion and ending on day +20. On day +11 following

tumor cell injection, 1 ml blood was collected by terminal cardiac bleeding from the mouse that received vehicle, and 0.1 ml of blood was collected by non-lethal retro-orbital collection from each of the three six-mouse groups dosed with 10, 30 or 100 mg/kg b.i.d. (twice daily) of TG101209, and samples pooled within the dose groups. Blood mononuclear cells were isolated by a Ficoll (Sigma-Aldrich, St Louis, MO, USA) cushion centrifugation method (600 RCF and 30 min). The isolated cells were subjected to FACS analysis to determine the percentage of GFP-positive tumor cells (that is, Ba/F3-V617F-GFP cells).

In a parallel study, two additional animals were treated as described above with the exception that they were given a single 100 mg/kg dose of drug on day 11 and humanely killed 7 h later for analysis of STAT5 phosphorylation in the tumor-bearing spleen. Spleens were homogenized in a FastPrep machine (Qbiogen, Irvine, CA, USA). Subsequently, 100 μ g of each spleen homogenate was subjected to electrophoretic separation and the protein blot was probed with an anti-phospho-STAT5 (Tyr694/699) (Upstate), as well as with an anti-STAT5 antibody (Cell Signaling), and visualized by the enhanced chemoluminescence method.

Patient accrual and sample collection

The current study was approved by the Mayo Clinic Institutional Review Board. All patients provided verbal and written informed consent, and research was carried out according to the principles of the Declaration of Helsinki.

Clonogenic assays/single-colony analysis

CD34⁺ cells, peripheral blood mononuclear cells (PBMC), and granulocytes were isolated as described previously¹³ Flow cytometry analysis has shown CD34⁺ cell purity to be at least 95% in our hands. Freshly isolated CD34⁺ cells were plated in a

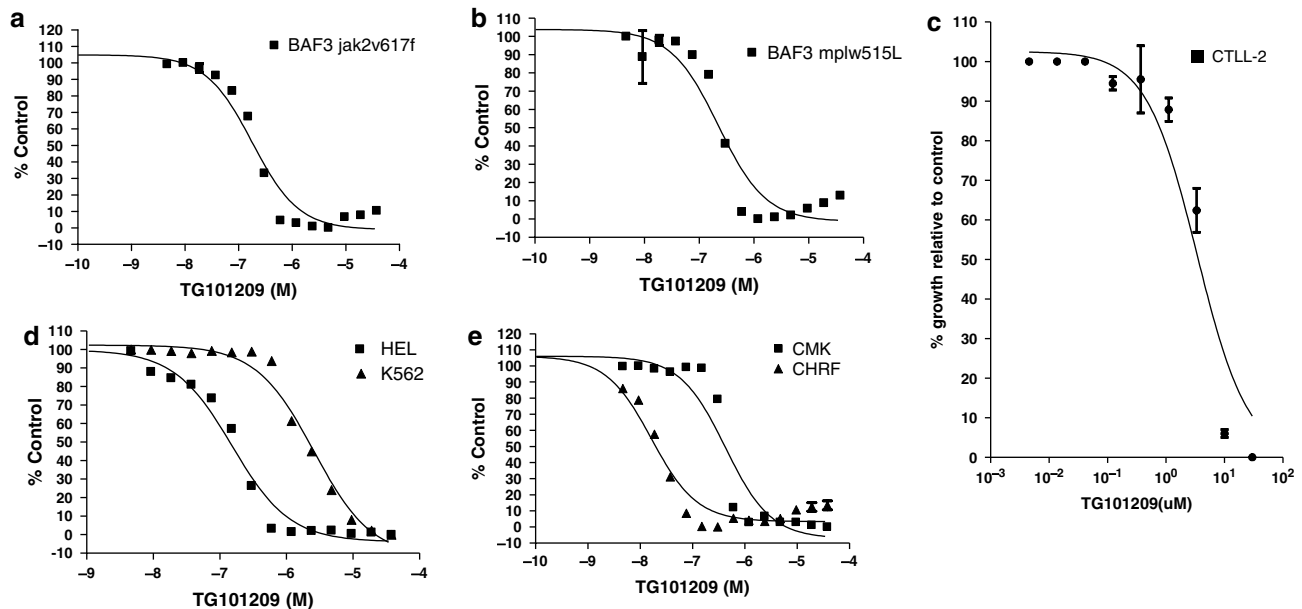


Figure 2 TG101209 inhibits JAK2V617F- and MPLW515L-dependent cell proliferation, and selectively inhibits JAK2 versus JAK3 kinase-dependent cell proliferation. Dose–response curves of (a) Ba/F3 cells stably expressing JAK2V617F (IC₅₀ = 170 nM), (b) MPLW515L (IC₅₀ = 220 nM), or (c) CTLL-2 cells (IC₅₀ = 3400 nM). The percentage of growth, relative to the growth of cells in the absence of drug, is plotted for increasing concentrations of TG101209 (4.6–38 400 nM). (d) Dose–response curves comparing HEL cells (■) and K562 cells (▲) with increasing concentrations of TG101209 (IC₅₀ = 152 and 2000 nM for HEL and K562 cells, respectively). (e) Dose–response curves comparing CHR2F-288-11 cells (JAK2T875N-positive) (▲) and CMK cells (JAK3A572V-positive) (■) with increasing concentrations of TG101209 (IC₅₀ = 16 and 420 nM for CHR2F-288-11 and CMK cells, respectively).

methylcellulose-based, semisolid medium (MethoCult media, Stem Cell Technologies, Vancouver, BC). Clonogenic assays were set up in duplicate or triplicate (catalog# H4435), as per manufacturer's guidelines; 1×10^3 CD34⁺ cells were plated for cytokine-supported colony growth. The plates were incubated at 37°C in a 5% carbon dioxide and 95% air mixture for 10–12 days, and colonies were scored using standard morphological criteria. In the presence of TG101209, only appreciably hemoglobinized colonies were scored as 'erythroid'. Individual well-separated colonies were harvested for DNA sequencing as described previously.¹⁴

Genotyping of mutant alleles was performed by direct DNA sequencing as described previously for *JAK2V617F*¹⁵ and *MPL515*.¹⁶

Results

TG101209 is a selective JAK2 kinase inhibitor in vitro

We utilized rational structure-based techniques to design and optimize a new series of pyrimidine-based inhibitors to target JAK2. We started from a $\sim 5 \mu\text{M}$ IC₅₀ hit, and made use of two crystal structures available in the literature (2B7A.pdb for JAK2 and 1YVJ.pdb for JAK3)^{17,18} to design molecules guided by

molecular modeling. With this approach, the series was rapidly optimized to yield low-nanomolar (nM) IC₅₀ concentration inhibitors, and TG101209, which is one such JAK2-selective inhibitor, was chosen for further characterization. The molecular structure of the small molecule inhibitor TG101209 is shown in Figure 1a. A molecular model showing TG101209 docked and minimized in the ATP pocket of JAK2 kinase, highlighting the key interactions is shown in Figure 1b. The specificity of TG101209 was tested against a wide range of kinases using purified proteins. TG101209 inhibited JAK2, FLT3, RET and JAK3 kinases with an IC₅₀ of 6–169 nM in *in vitro* kinase assays (Figure 1c). TG101209 was most active against JAK2 (IC₅₀ = 6 nM), and exhibited selectivity for JAK2 relative to JAK3 (28 × greater inhibition of JAK2) in this assay. We assessed TG101209 activity against a total of 63 kinases at 500 nM. Of these, only six tyrosine kinases, including the four aforementioned kinases, were inhibited by $\geq 80\%$ (Supplementary Table 1).

TG101209 inhibits JAK2V617F- and MPLW515L-dependent cell proliferation, and selectively inhibits JAK2 versus JAK3 kinase-dependent cell proliferation

We next tested the ability of TG101209 to inhibit the interleukin (IL)-3-independent growth of Ba/F3 cells that were stably

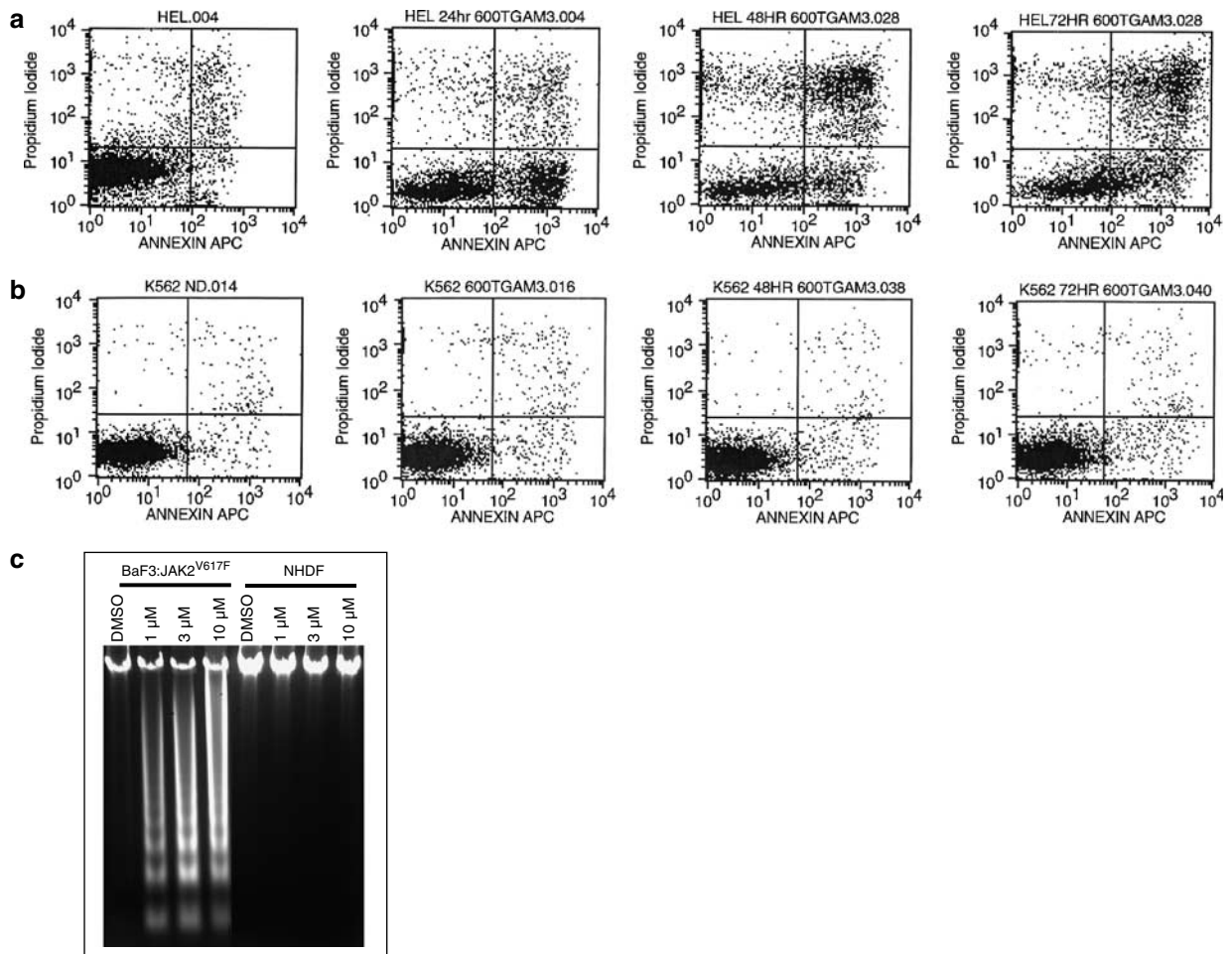


Figure 3 TG101209 induces apoptosis in JAK2V617F-expressing HEL and Ba/F3 cells. HEL or K562 cells were cultured in growth medium in the presence of 600 nM TG101209. Cells were harvested daily and stained with Annexin V-FITC and propidium iodide (PI) followed by flow cytometric analysis. Shown are representative data from two independent experiments. TG101209 treatment increased the percentage of Annexin V positive HEL cells from a background level of 6–46% (24 h), 44% (48 h) and 68% (72 h) (a), and from 5 to 8% (24 h), 6% (48 h) and 8% (72 h) for K562 cells (b). Effect of TG101209 in inducing apoptosis of Ba/F3-V617F cells or normal human dermal fibroblasts (NHDF) after a 24-h incubation at the indicated concentrations. The extent of DNA fragmentation as a measure of apoptosis is measured by agarose gel electrophoresis (c).

transduced with constitutively activated JAK2V617F or MPLW515L mutants. Ba/F3 cells expressing each mutant were grown in the presence of increasing concentrations of inhibitor (4.6–38 400 nM) (Figure 2a and b). TG101209 inhibited the growth of Ba/F3-V617F and Ba/F3-W515L cells with an IC_{50} of 170 and 220 nM, respectively. As expected, parental Ba/F3 cells, which require IL-3 for growth, were also inhibited by TG101209 (IC_{50} = 470; data not shown), reflecting their dependence on signaling by *wild type* JAK2. In contrast, TG101209 had minimal effects on the growth of CTLL-2 cells, a clone of cytotoxic T cells that is dependent upon IL-2 for growth (IC_{50} = 3400 nM) (Figure 2c). Since IL-2 receptor signaling is known to be mediated by JAK1 and JAK3 kinases, but not JAK2,¹⁹ the less potent inhibitory effect on CTLL-2 cell growth is consistent with the compound's relatively selective inhibition of JAK2 kinase previously seen in the *in vitro* kinase assay. Furthermore, TG101209 had negligible inhibitory effects on the growth of NHDF (IC_{50} = 6500 nM; data not shown), confirming that the growth inhibition of the transformed Ba/F3 cells was not due to non-specific toxicity.

To extend these studies in a more clinically relevant system, TG101209 activity on the growth and survival of select human leukemia cell lines was examined. TG101209 activity was tested in HEL cells that are homozygous for the JAK2V617F allele.¹⁰

Incubation with increasing concentrations of TG101209 (4.6–38 400 nM) resulted in dose-dependent inhibition of HEL cell growth (IC_{50} = 152 nM) (Figure 2d). In control experiments, TG101209 inhibited BCR-ABL-positive K562 cell growth with an IC_{50} of 2000 nM (Figure 2d).

The relative specificity of TG101209 for inhibiting JAK2 versus JAK3 kinase was further studied by investigating the effects of TG101209 on the growth of CHRF-288-11 and CMK cells, which are acute megakaryoblastic leukemia (AMKL) cell lines that carry the JAK2T875N²⁰ and JAK3A572V²¹ mutations, respectively, with increasing concentrations of TG101209 (Figure 2e). TG101209 was 26 × more potent at inhibiting proliferation of CHRF-288-11 cells (IC_{50} = 16 nM) as compared to CMK cells (IC_{50} = 420 nM), thus confirming the specificity of TG101209 for JAK2 versus JAK3 in cell-based assays.

TG101209 induces apoptosis in JAK2V617F-expressing human erythroleukemia and Ba/F3 cells

To determine whether inhibition of proliferation of JAK2V617F-harboring HEL cells was accompanied by an increase in apoptosis, we assessed Annexin V binding by flow cytometry. Treatment of HEL cells with 600 nM TG101209 resulted in an increase in the percentage of Annexin V-positive cells from a

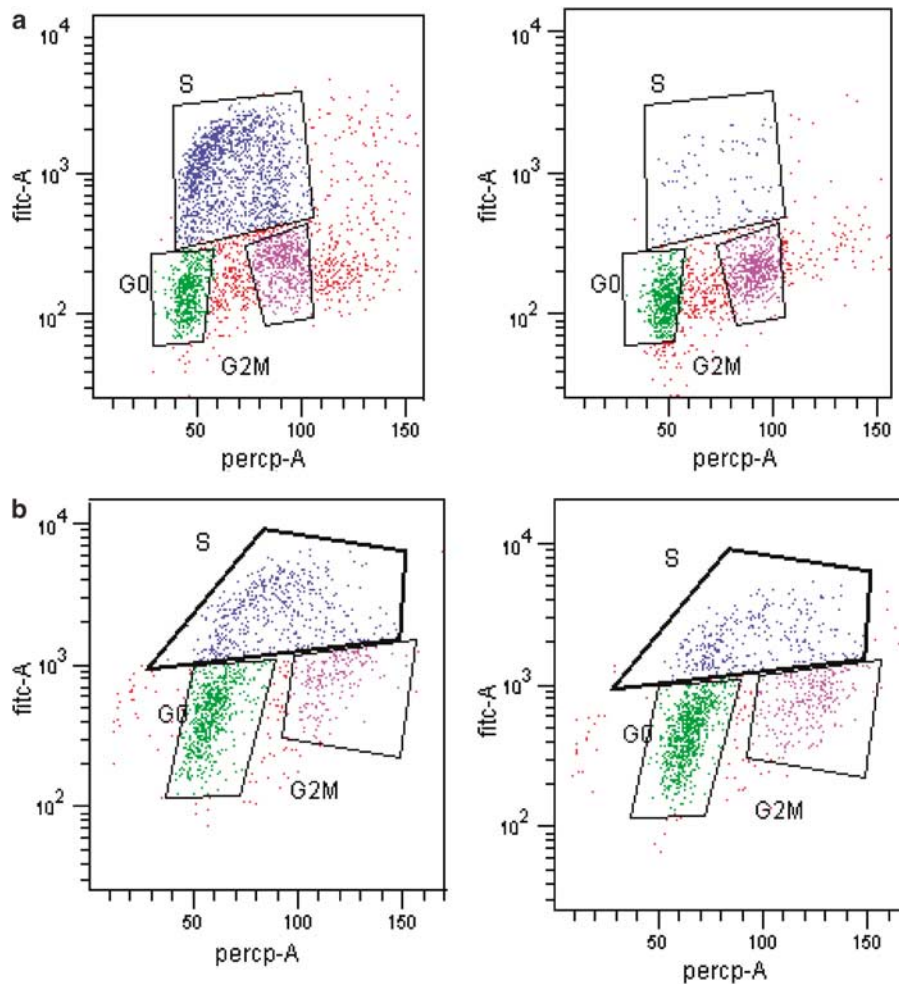


Figure 4 TG101209 induces cell cycle arrest of JAK2V617F-expressing HEL cells. Cells were analyzed for cell-cycle effects by BrdU/7-AAD staining, after TG101209 treatment for 24 h. Data shown are representative of two independent experiments (a) HEL cells without inhibitor (left panel), or with 1200 nM TG101209 (right panel). G₀/G₁ fraction increased from a background level of 15–50%. (b) K562 cells either without inhibitor (left panel), or with 1200 nM TG101209 (right panel). G₀/G₁ fraction increased from a background level of 45–50%.

background level of 6–46% at 24 h, 44% at 48 h and 68% at 72 h (Figure 3a). As expected, TG101209 treatment was less potent in inducing apoptosis in BCR-ABL-positive K562 cells in control experiments; the percentage of Annexin V-positive cells remained relatively constant from 0 to 72 h (~8%) (Figure 3b).

TG101209 also induced apoptosis of Ba/F3-V617F cells as assessed by a DNA laddering assay (Figure 3c). A progressive increase in DNA fragmentation indicating apoptosis was observed at 24 h for Ba/F3-V617F cells treated with increasing concentrations of TG101209. In contrast, no DNA fragmentation was observed in control cells (dermal fibroblasts) even at the highest TG101209 concentration used (10 μ M), thus demonstrating that induction of apoptosis in Ba/F3-V617F cells was due to JAK2V617F inhibition.

TG101209 induces cell cycle arrest of JAK2V617F-expressing human erythroleukemia cells

We next assessed whether TG101209 induced cell cycle arrest in hematopoietic cells transformed by JAK2V617F. HEL or K562 cells were treated with indicated concentrations of inhibitor, followed by pulse labeling with bromodeoxyuridine (BrdU) and staining with 7-AAD. Treatment of HEL cells with 1200 nM TG101209 resulted in an increase in the percentage of G₀/G₁ fraction cells from a background level of 15–50% at 24 h, thus indicating G₀/G₁ arrest (Figure 4a). As expected, TG101209 treatment of BCR-ABL-positive K562 cells had a considerably less effect – the G₀/G₁ fraction increased from 45 to 50% (Figure 4b).

TG101209 inhibits phosphorylation of JAK2V617F, STAT5 and STAT3

JAK2V617F has been previously shown to be constitutively autophosphorylated and expression of JAK2V617F kinase results in constitutive phosphorylation of downstream signaling pathways.¹⁰ We next examined whether TG101209 could inhibit phosphorylation of JAK2V617F, as well as of its downstream signaling effectors, STAT5 and STAT3. A dose-dependent decrease in phosphorylation was seen, with IC₅₀ of ~300 nM for phospho-JAK2V617F inhibition and 300–600 nM for phospho-STAT5 and phospho-STAT3 inhibition (Figure 5a, b and c). TG101209 also inhibited STAT5 phosphorylation in the Ba/F3-V617F cell line (IC₅₀ ~300 nM) (Figure 5d). These data suggest that the basis for JAK2V617F-specific effect on cell growth is the pharmacologic inhibition of this kinase by TG101209, with resulting downregulation of key downstream signaling intermediates. As reported previously,¹⁰ we did not find appreciable levels of phospho-JAK2 in K562 cells (data not shown), further indicating that these cells are not dependent on constitutive JAK2 signaling for their growth.

TG101209 effectively treats JAK2V617F-induced hematopoietic disease in a nude mouse model

To test the *in vivo* efficacy of TG101209 in inhibiting JAK2V617F, we used a mouse model of JAK2V617F-induced hematopoietic disease. Essentially, Ba/F3-V617F-GFP cells were injected into immunodeficient SCID mice. The animals develop a rapidly fatal (median latency 11 days), fully penetrant hematopoietic disease characterized by peripheral blood leukocytosis, and splenomegaly (mean spleen weight = 0.85 g versus 0.028 g in placebo-treated animals) (Figure 6a). Flow cytometric analysis of peripheral blood mononuclear cells from the affected animals (day 11) confirmed that most cells were derived from the

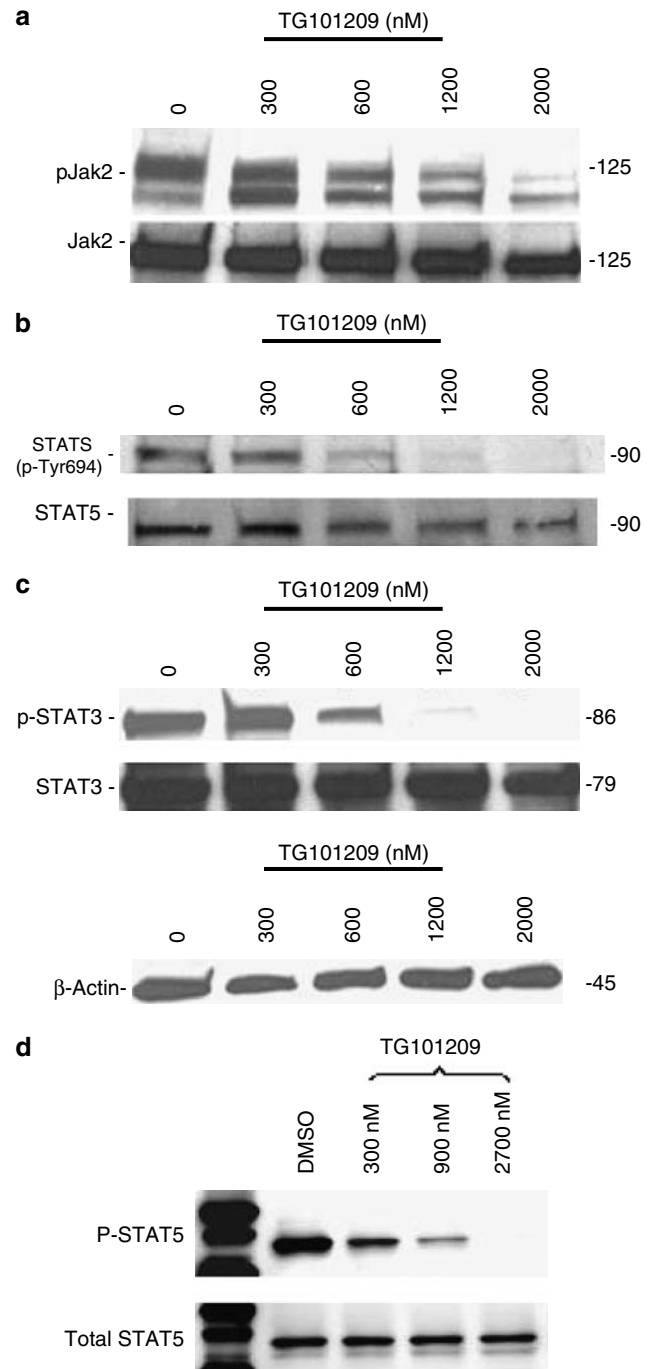


Figure 5 TG101209 inhibits phosphorylation of JAK2V617F, STAT5 and STAT3. Effect of increasing concentration of TG101209 on JAK2V617F phosphorylation (a), STAT5 phosphorylation (b) and STAT3 phosphorylation (c) in HEL cells. Western blot was performed using whole cell lysates from HEL cells, and were visualized with antibodies that detect the phosphorylated isoform, as well as the total amount (loading control) of each protein. β -actin served as an additional loading control in these experiments. TG101209 inhibits STAT5 phosphorylation in Ba/F3-V617F cells (d).

JAK2V617F-bearing clone (that is, GFP-positive) (Figure 6b). Next, the recipient mice were divided into four groups and treated with TG101209 by oral gavage at the following doses: 0 mg/day (vehicle control), or 20 mg/kg/day, 60 mg/kg/day, or 200 mg/kg/day, each in two divided doses (b.i.d.), from days +3

to +20 following tumor cell injection. In the placebo arm, all the animals died due to disease progression by day 11. In striking contrast, TG101209 at the highest dose level (100 mg/kg b.i.d.) was effective in treating JAK2V617F-induced disease as there was a statistically significant prolongation of survival in this group (10 days; $P < 0.02$), and the animals in this group were still alive at the previously defined study end point of 10 days past the time of the death of the final placebo-treated animal (Figure 6a). The animals at the lower dose levels (that is, 10 or 30 mg/kg b.i.d.) developed disease with the same latency and penetrance as the placebo-treated animals, without evidence of prolongation of survival (Figure 6a). Compared with placebo-treated animals, TG101209-treated animals exhibited a statistically significant, dose-dependent reduction in the circulating tumor cell burden at day +11 (75% GFP+ cells in placebo-treated versus 15% GFP+ cells in 100 mg/kg b.i.d. TG101209-treated animals; $P < 0.02$) (Figure 6b). The clinical benefit of TG101209 in this model correlated with inhibition of JAK2V617F activity *in vivo*, evident in the marked decrease

in STAT-5 phosphorylation demonstrable in splenic tumors, as early as 7 h after administration of a single dose of TG101209 (100 mg/kg) to the affected mice (Figure 6c).

TG101209 inhibits hematopoietic colony formation in vitro – comparing effects on progenitor cells from normal controls versus those harboring JAK2 V617F or MPLW515L/K

Since cell line studies may not accurately represent the true sensitivity of clonal bone marrow-derived cells, experiments were performed with primary cells harboring JAK2V617F ($n = 5$) or MPLW515L/K ($n = 3$) mutations, or neither mutation ($n = 1$) (Table 1). The clonogenic assay readout included observed changes in (i) erythroid and myeloid colony number, (ii) colony size/morphology and (iii) mutant colony burden, with TG101209 treatment. On the basis of cell line experiments (above), we used 0, 300 and 600 nM as the TG101209 working concentrations for this assay.

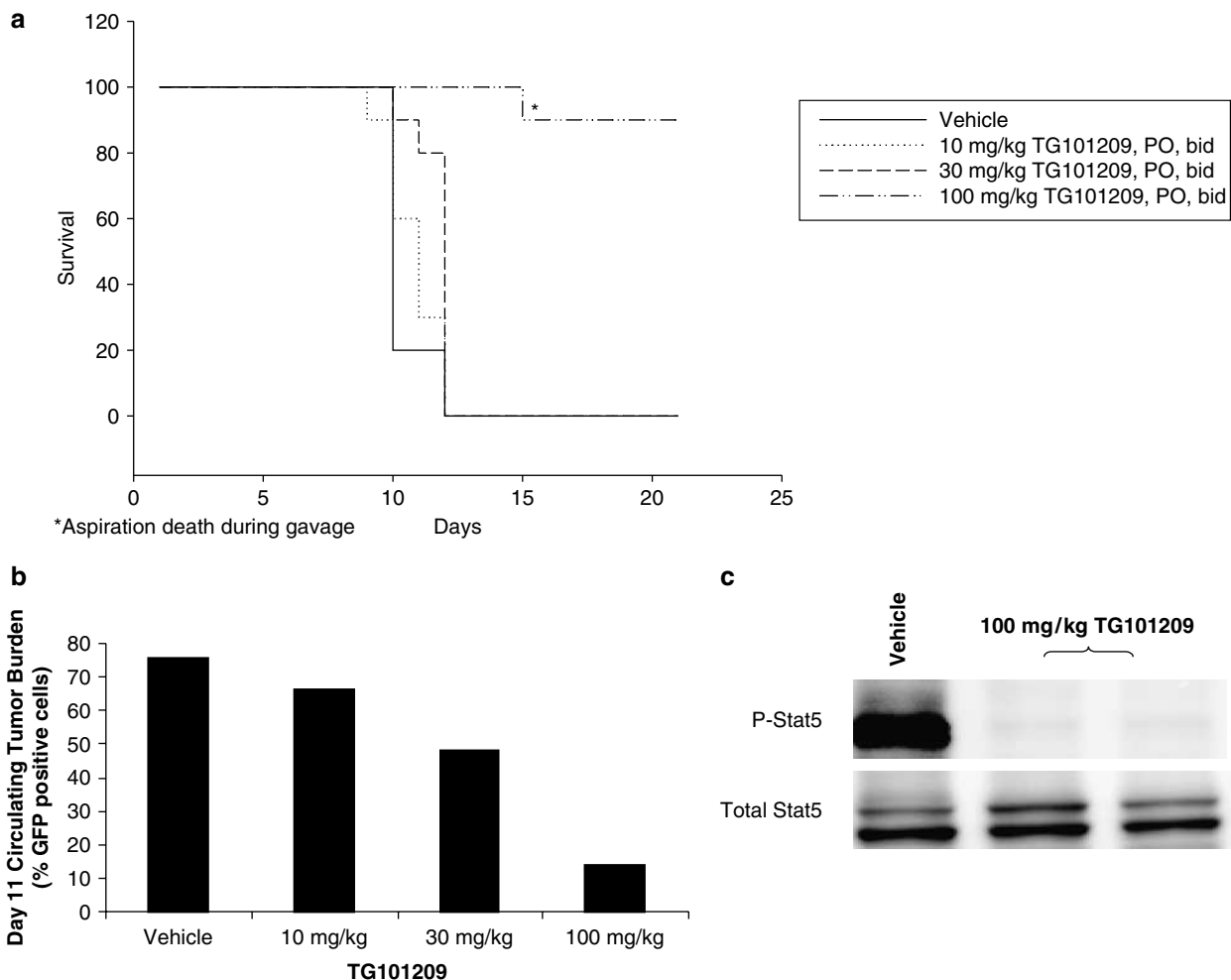


Figure 6 TG101209 effectively treats JAK2V617F-induced hematopoietic disease in a nude mouse model. (a) Kaplan–Meier plot of SCID mice injected with Ba/F3 cells expressing JAK2V617F and green fluorescent protein (Ba/F3-V617F-GFP), and treated with either placebo or TG101209 at indicated doses. There was a significant difference in survival between the cohort treated with 100 mg/kg b.i.d. TG101209 versus either placebo-treated cohort, or lower-dose cohorts (10 or 30 mg/kg b.i.d. TG101209). The asterisk indicates the single animal in the 100 mg/kg b.i.d. cohort that died from a disease-unrelated cause (tracheal aspiration during oral gavage). (b) Flow cytometry analysis of peripheral blood mononuclear cells (PBMC) from affected mice at day +11, indicating percentage of GFP-positive tumor cells in the placebo- and TG101209-treated animal cohorts. (c) TG101209 inhibits STAT5 phosphorylation *in vivo*. Western blot was performed using lysates of spleens harvested 7 h after the administering either placebo or a single dose of TG101209 (100 mg/kg) with antibodies against phosphorylated STAT5 and total STAT5 (loading control). Results from two independent experiments are shown.

CD34⁺ cells purified from the buffy coat of healthy donor phlebotomy units were used as controls for this assay. TG101209 inhibited cytokine-supported colony growth from normal CD34⁺ cells, with IC₅₀ of 1000 nM (erythroid colonies) and 600 nM (myeloid colonies) (Table 1). In terms of effect on colony size, burst forming unit-erythroid (BFU-E) colonies were smaller with TG101209 treatment, although hemoglobinization was relatively preserved even at 600 nM TG101209 (Figure 7a). The observed inhibitory effect on colony growth from normal CD34⁺ cells was predicted, given the known importance of wild type JAK signaling during normal hematopoiesis.²

The effect of TG101209 on hematopoietic colony growth was next studied in five PV patients (all *JAK2V617F*-positive; PV1-PV5) (Table 1). Incubation with TG101209 produced fewer colonies in PV patients, relative to normal controls (IC₅₀ of ~600 nM and 300–600 nM for erythroid and myeloid colonies, respectively). Similarly, TG101209 had a greater dose-dependent inhibitory effect on colony size of erythroid colonies in PV patients, as compared to normal controls (compare Figure 7a and b). Genotyping of individual colonies revealed a selective suppression of *JAK2V617F* harboring colonies in the presence of TG101209 for three of five PV patients (Table 1). This effect was more pronounced in some patients (for example, PV1; 50% versus 90% *JAK2V617F*-positive colonies in the presence and absence of inhibitor, respectively), but not others (PV5) (Table 1 and Figure 7d).

The effect of TG101209 on colony growth was also studied in four PMF patients (3 *MPLW515L/K*-positive; PMF1-PMF3). Incubation with TG101209 resulted in fewer colonies (IC₅₀ = 300–600 nM for erythroid and myeloid colonies), as compared

to PV patients, as well as normal controls (Table 1). Correspondingly, a greater dose-dependent inhibitory effect on colony size was also seen (compare Figure 7c with Figure 7a and b). Single-colony genotyping studies showed that, for two of the three PMF patients (PMF1 and PMF2), growth of colonies harboring *MPL515* mutations was selectively suppressed after incubation with TG101209 (Table 1 and Figure 7e).

We also studied TG101209 effects on colony growth in the absence of cytokines for three PV patients (PV2, PV4 and PV5). Here, erythropoietin-independent erythroid colony (EEC) growth was more sensitive to TG101209 as compared to cytokine-supported colonies (IC₅₀ < 300 nM) (data not shown). Characterization of EEC obtained in the presence of TG101209 (beyond colony number) was difficult given the dysmorphic nature of these colonies. Furthermore, genotyping of these colonies was frequently unsuccessful, presumably because most cells in these colonies are apoptotic.

Discussion

JAK2V617F has a primary role in the pathogenesis of PV, PMF, ET and potentially, that of other MPD as well. *JAK2V617F* is highly prevalent in the former three disorders, and confers cytokine hypersensitivity, and promotes endogenous erythroid colony formation, which are salient *in vitro* characteristics of PV-derived progenitor cells.¹⁴ Furthermore, *JAK2V617F* expression in murine disease models produces a MPD mimicking PV.^{7,8} Finally, genotype–phenotype correlation studies in MPD patients point to a significant association between *JAK2V617F*

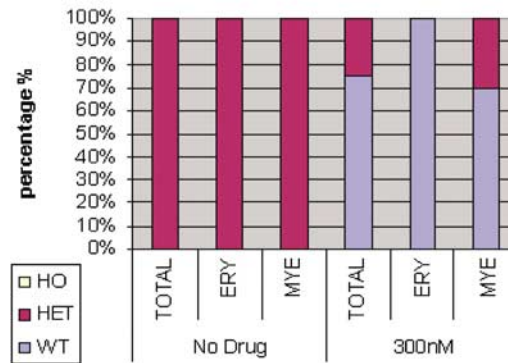
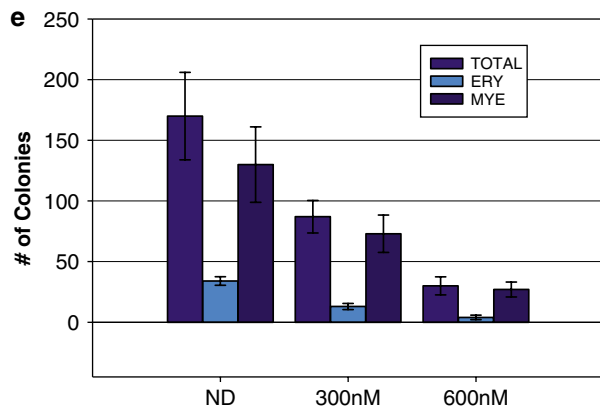
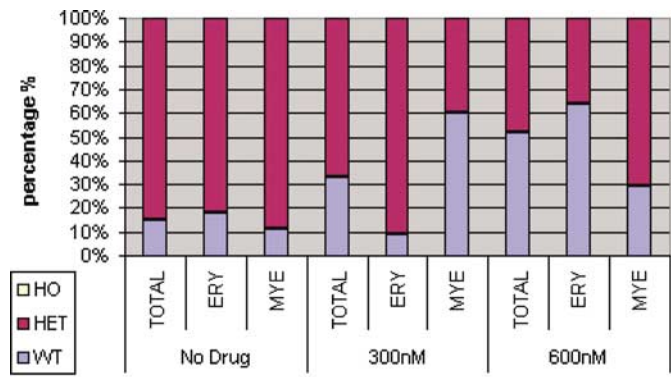
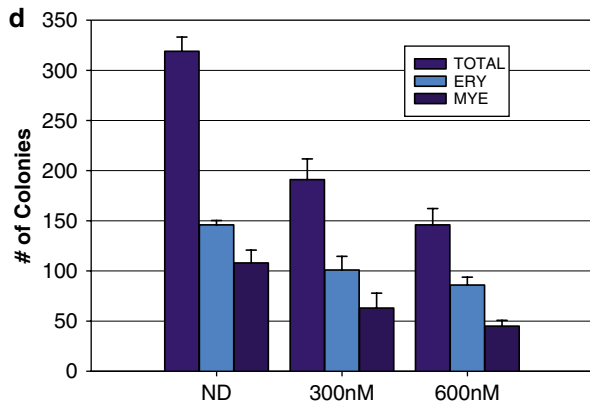
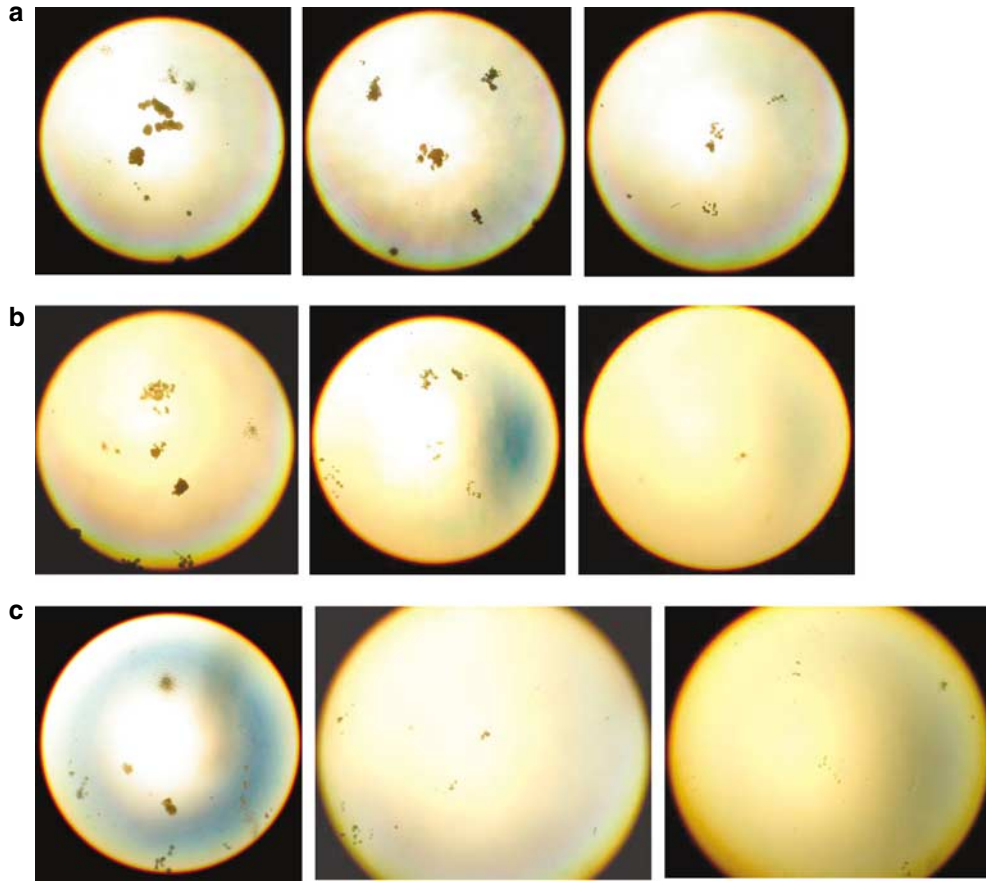
Table 1 TG101209 effect on cytokine-supported hematopoietic colony growth from MPD patients harboring *JAK2V617F* or *MPLW515*

Cytokine-supported colonies – mutation pattern as assessed by genotyping of 575 individual colonies

Patient	Mutation	IC ₅₀ (nM)		Total no. of colonies genotyped	Mutation pattern without inhibitor (% colonies genotyped)	Mutation pattern with inhibitor (% colonies genotyped)	% mutation-positive colonies without inhibitor		% mutation-positive colonies with inhibitor		
		Erythroid	Myeloid				Erythroid	Myeloid	Erythroid	Myeloid	
Normal	WT	1000	600	n/a	n/a	n/a	n/a				
PV1	<i>JAK2V617F</i>	~600	300–600	62	HET/WT (85/15)	HET/WT (57/43)	82	90	36	70	
PV2	<i>JAK2V617F</i>	~600	~300	111	HET/WT (65/35)	HET/WT (60/40)	70	55	60	20	
PV3	<i>JAK2V617F</i>		NA	59	HOM/WT (23/77)	HOM/HET/WT (8/8/84)	20	30	30	0	
PV4	<i>JAK2V617F</i>	~600	~600	61	HOM/HET/WT (4/4/92)	HOM/HET/WT (8/11/81)	7	10	17	10	
PV5	<i>JAK2V617F</i>	~300	300–600	47	HOM/HET/WT (74/4/22)	HOM/HET/WT (92/4/4)	64	92	82	100	
PMF1	<i>MPLW515K</i>	<300	~300	59	HET/WT (93/7)	HET/WT (17/83)	100	90	0	20	
PMF2	<i>MPLW515K</i>	300–600	~300	86	HET/WT (90/10)	HET/WT (39/61)	100	90	25	36	
PMF3	<i>MPLW515L</i>	300–600	300–600	90	HOM/HET (91/9)	HOM/HET/WT (71/22/7)	100	100	92	90	
PMF4	WT	>600	300–600	n/a	n/a	n/a	n/a				

Abbreviations: HET, heterozygous; HOM, homozygous; IC₅₀, inhibitor (TG101209) concentration that suppresses colony number by 50%; MPD, myeloproliferative disorders; n/a, not applicable; NA, not available; PMF, primary myelofibrosis; PV indicates polycythemia vera; WT, wild-type.

Figure 7 TG101209 inhibits the *in vitro* growth of hematopoietic colonies derived from progenitor cells harboring *JAK2V617F* or *MPLW515K* mutations. Representative photomicrographs of colonies derived from CD34⁺ cells plated in methylcellulose supplemented with cytokines are shown. CD34⁺ cells were obtained either from healthy controls (a), or patients harboring *JAK2V617F* (b), or *MPLW515K* (c) mutations and plated either in the absence of inhibitor (left panels), or presence of 300 nM TG101209 (middle panels), or 600 nM TG101209 (right panels). Bar graph comparing number of colonies (total, erythroid or myeloid) obtained either without inhibitor, or with 300 or 600 nM TG101209, for a representative patient carrying the *JAK2V617F* (d; left panel) or *MPLW515K* mutation (e; left panel). Genotyping of individual colonies shows that TG101209 selectively suppresses growth of colonies harboring *JAK2V617F* (d; right panel) or *MPLW515K* (e; right panel) mutations.



and specific clinical features, including higher hemoglobin and leukocyte counts, increased incidence of venous thrombosis, and potentially, inferior survival.^{22,23} These data therefore provide a strong rationale for targeting JAK2V617F as a therapeutic strategy in MPD.

In this report, we describe a potent, small molecule JAK2-selective kinase inhibitor that was developed rationally, using structure-based drug design. TG101209 is orally bioavailable (pharmacokinetics data are presented as Supplementary Information), and has significant *in vitro* activity against JAK2V617F. Correspondingly, TG101209 inhibited proliferation of leukemic cells carrying mutant JAK2 alleles through cell cycle effects and induction of apoptosis. Furthermore, TG101209 effectively treated an aggressive JAK2V617F-induced hematopoietic disease in mice, an effect that was correlated with inhibition of STAT5 phosphorylation *in vivo*. Finally, TG101209 suppressed growth of hematopoietic colonies from primary progenitor cells harboring JAK2V617F or MPL515 mutations.

A key question pertains to precisely which MPD patients will benefit from therapies targeting JAK2 signaling – from a pathogenetic standpoint, as well as from the standpoint of safety of such therapies. While JAK2V617F is the predominant disease associated allele in MPD, it is becoming clear that other alleles contribute to MPD phenotype. In general, the novel allele appears to substitute for JAK2V617F (for example, JAK2 exon 12 mutations, JAK2ΔIREED),^{4,6} although in some cases, such alleles act in conjunction with JAK2V617F (for example, JAK2D620E, MPLW515L/K).^{3,16} These mutant alleles effectively converge upon JAK2, leading to constitutive autophosphorylation of this kinase. These observations raise the possibility that most, if not all, MPD patients harbor mutations (some yet to be identified) that potentially serve as a molecular target for a selective JAK2 inhibitor. Indeed, our data revealed that *in vitro* growth of hematopoietic colonies from a PMF patient (PMF4; Table 1) without identifiable mutations was inhibited at least as effectively as that from patients harboring JAK2V617F or MPLW515 mutations.

Given that most MPD patients have indolent disease and may require only simple therapeutic interventions (for example, intermittent phlebotomy plus aspirin in a young low-risk PV patient), it may be that JAK2 targeted therapy is appropriate, at least initially, only for high-risk patients, where the disease is poorly controlled with conventional therapies (for example, the advanced PMF patient with significant cytopenias, where use of myelosuppressive agents is problematic). The extent to which a JAK2-selective inhibitor will be myelosuppressive and/or immunosuppressive in the setting of MPD therapy remains to be established. JAK3 kinase is predominantly expressed in hematopoietic cells, and associates with the common γ chain (γ c) shared by IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors, and thus plays a key role in development and homeostasis of the immune system. These data raise theoretical concerns for immunologic toxicities that may potentially be seen with 'off-target' JAK3 kinase inhibition. More recently, however, a novel inhibitor, MK-0457 or VX-680, that targets Aurora kinases, as well as BCR-ABL and JAK2 kinases, was used to treat three patients with CML or acute lymphoblastic leukemia with the T315I BCR-ABL mutation.²⁴ Here, MK-0457 given by a 5-day continuous i.v. infusion schedule, led to clinical responses. Encouragingly, there was no significant extramedullary toxicity seen with MK-0457 therapy in this preliminary study, which, by extrapolation, supports feasibility of TG101209 use in MPD patients from a drug safety standpoint. Furthermore, given that TG101209 more selectively targets JAK2 kinase, relative to JAK3 (28 ×), this ought to, in theory, limit its potential immunosup-

pressive/immunomodulatory effects. Whether this particular characteristic translates into a more favorable clinical safety profile, however remains to be demonstrated.

In summary, we have shown that a novel small molecule kinase inhibitor, TG101209, is effective both *in vitro* and *in vivo* against JAK2V617F and MPLW515L/K tyrosine kinases, and provides survival benefit in a mouse disease model. Thus, TG101209, the prototype of a new class of JAK2 kinase inhibitors, opens the prospect for effective molecularly-targeted therapy in MPD, and possibly other malignancies caused by dysregulated JAK-STAT signaling, regardless of whether the causative mutations occur at the cytokine receptor level or involve JAK2 itself.

Acknowledgements

This work was partially supported by research grants from the Myeloproliferative Disorders Foundation, (Chicago, IL, USA) and from TargeGen Inc., (San Diego, CA, USA).

References

- 1 Tefferi A, Gilliland DG. Oncogenes in myeloproliferative disorders. *Cell Cycle* 2007; **6**: 550–566.
- 2 Parganas E, Wang D, Stravopodis D, Topham DJ, Marine JC, Teglund S *et al*. Jak2 is essential for signaling through a variety of cytokine receptors. *Cell* 1998; **93**: 385–395.
- 3 Grunebach F, Bross-Bach U, Kanz L, Brossart P. Detection of a new JAK2 D620E mutation in addition to V617F in a patient with polycythemia vera. *Leukemia* 2006; **20**: 2210–2211.
- 4 Malinge S, Ben-Abdelali R, Settegrana C, Radford-Weiss I, Debre M, Beldjord K *et al*. A novel activating JAK2 mutation in a Down Syndrome patient with B-cell acute lymphoblastic leukemia. *Blood* 2007; **109**: 2202–2204.
- 5 Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M *et al*. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med* 2006; **3**: e270.
- 6 Scott LM, Tong W, Levine RL, Scott MA, Beer PA, Stratton MR *et al*. JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N Engl J Med* 2007; **356**: 459–468.
- 7 Wernig G, Mercher T, Okabe R, Levine RL, Lee BH, Gilliland DG. Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. *Blood* 2006; **107**: 4274–4281.
- 8 Zaleskas VM, Krause DS, Lazarides K, Patel N, Hu Y, Li S *et al*. Molecular pathogenesis and therapy of polycythemia induced in mice by JAK2 V617F. *PLoS One* 2006; **1**: e18.
- 9 Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M *et al*. MPLW515L Is a Novel Somatic Activating Mutation in Myelofibrosis with Myeloid Metaplasia. *PLoS Med* 2006; **3**: 1140–1151.
- 10 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**: 387–397.
- 11 Mesa RA, Silverstein MN, Jacobsen SJ, Wollan PC, Tefferi A. Population-based incidence and survival figures in essential thrombocythemia and agnogenic myeloid metaplasia: an Olmsted County Study, 1976–1995. *Am J Hematol* 1999; **61**: 10–15.
- 12 Levine RL, Loriaux M, Huntly BJ, Loh ML, Beran M, Stoffregen E *et al*. The JAK2V617F activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia. *Blood* 2005; **106**: 3377–3379.
- 13 Lasho TL, Mesa R, Gilliland DG, Tefferi A. Mutation studies in CD3+, CD19+ and CD34+ cell fractions in myeloproliferative disorders with homozygous JAK2(V617F) in granulocytes. *Br J Haematol* 2005; **130**: 797–799.
- 14 Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S *et al*. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* 2005; **365**: 1054–1061.

- 15 Steensma DP, Dewald GW, Lasho TL, Powell HL, McClure RF, Levine RL *et al*. The JAK2 V617F activating tyrosine kinase mutation is an infrequent event in both 'atypical' myeloproliferative disorders and myelodysplastic syndromes. *Blood* 2005; **106**: 1207–1209.
- 16 Pardanani AD, Levine RL, Lasho T, Pikman Y, Mesa RA, Wadleigh M *et al*. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood* 2006; **108**: 3472–3476.
- 17 Lucet IS, Fantino E, Styles M, Bamert R, Patel O, Broughton SE *et al*. The structural basis of Janus kinase 2 inhibition by a potent and specific pan-Janus kinase inhibitor. *Blood* 2006; **107**: 176–183.
- 18 Boggon TJ, Li Y, Manley PW, Eck MJ. Crystal structure of the Jak3 kinase domain in complex with a staurosporine analog. *Blood* 2005; **106**: 996–1002.
- 19 Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* 2006; **6**: 595–601.
- 20 Mercher T, Wernig G, Moore SA, Levine RL, Gu TL, Frohling S *et al*. JAK2T875N is a novel activating mutation that results in myeloproliferative disease with features of megakaryoblastic leukemia in a murine bone marrow transplantation model. *Blood* 2006; **108**: 2770–2779.
- 21 Walters DK, Mercher T, Gu TL, O'Hare T, Tyner JW, Loriaux M *et al*. Activating alleles of JAK3 in acute megakaryoblastic leukemia. *Cancer Cell* 2006; **10**: 65–75.
- 22 Wolanskyj AP, Lasho TL, Schwager SM, McClure RF, Wadleigh M, Lee SJ *et al*. JAK2 mutation in essential thrombocythemia: clinical associations and long-term prognostic relevance. *Br J Haematol* 2005; **131**: 208–213.
- 23 Campbell PJ, Griesshammer M, Dohner K, Dohner H, Kusec R, Hasselbalch HC *et al*. V617F mutation in JAK2 is associated with poorer survival in idiopathic myelofibrosis. *Blood* 2006; **107**: 2098–2100.
- 24 Giles FJ, Cortes J, Jones D, Bergstrom D, Kantarjian H, Freedman SJ. MK-0457, a novel kinase inhibitor, is active in patients with chronic myeloid leukemia or acute lymphocytic leukemia with the T315I BCR-ABL mutation. *Blood* 2007; **109**: 500–502.

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