Growth Factors, Cytokines, and Cell Cycle Molecules

The Small Molecule Inhibitor G6 Significantly Reduces Bone Marrow Fibrosis and the Mutant Burden in a Mouse Model of Jak2-Mediated Myelofibrosis

Annet Kirabo,* Sung O. Park,* Heather L. Wamsley,† Meghanath Gali,‡ Rebekah Baskin,* Mary K. Reinhard,§ Zhizhuang J. Zhao,¶ Kirpal S. Bisht,‡ György M. Keserü,** Christopher R. Cogle,** and Peter P. Sayeski*

From the Department of Physiology and Functional Genomics,* and the Division of Hematology and Oncology,** Department of Medicine, University of Florida College of Medicine, Gainesville, Florida; the Department of Physiological Sciences,† University of Florida College of Veterinary Medicine, Gainesville, Florida; the Department of Chemistry,‡ University of South Florida, Tampa, Florida; the Department Animal Care Services,§ University of Florida, Gainesville, Florida; the Department of Pathology,¶ University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; and the Department of General and Analytical Chemistry,** Budapest University of Technology and Economics, Budapest, Hungary

Philadelphia chromosome–negative myeloproliferative neoplasms, including polycythemia vera, essential thrombocytosis, and myelofibrosis, are disorders characterized by abnormal hematopoiesis. Among these myeloproliferative neoplasms, myelofibrosis has the most unfavorable prognosis. Furthermore, currently available therapies for myelofibrosis have little to no efficacy in the bone marrow and hence, are palliative. We recently developed a Janus kinase 2 (Jak2) small molecule inhibitor called G6 and found that it exhibits marked efficacy in a xenograft model of Jak2-V617F–mediated hyperplasia and a transgenic mouse model of Jak2-V617F–mediated polycythemia vera/essential thrombocytosis. However, its efficacy in Jak2-mediated myelofibrosis has not previously been examined. Here, we hypothesized that G6 would be efficacious in Jak2-V617F–mediated myelofibrosis. To test this, mice expressing the human Jak2-V617F cDNA under the control of the vav promoter were administered G6 or vehicle control solution, and efficacy was determined by measuring parameters within the peripheral blood, liver, spleen, and bone marrow. We found that G6 significantly reduced extramedullary hematopoiesis in the liver and splenomegaly. In the bone marrow, G6 significantly reduced pathogenic Jak/STAT signaling by 53%, megakaryocytic hyperplasia by 70%, and the Jak2 mutant burden by 68%. Furthermore, G6 significantly improved the myeloid to erythroid ratio and significantly reversed the myelofibrosis. Collectively, these results indicate that G6 is efficacious in Jak2-V617F–mediated myelofibrosis, and given its bone marrow efficacy, it may alter the natural history of this disease. (Am J Pathol 2012, 181:858–865; http://dx.doi.org/10.1016/j.ajpath.2012.05.033)

The BCR-ABL1–negative myeloproliferative neoplasms (MPNs) encompassing polycythemia vera (PV), essential thrombocytosis (ET), and myelofibrosis have a high prevalence in the United States. There are approximately 22 cases of PV, 24 cases of ET, and 1.46 cases of myelofibrosis for every 100,000 people, which amounts to approximately 68,000 patients with PV, 74,000 with ET, and 4500 with myelofibrosis in the United States.1,2 MPNs are characterized by similar pathological syndromes, including excess production of blood cells from the bone marrow, pruritus, splenomegaly, and extramedullary hematopoiesis. Among all of the MPNs, myelofibrosis has the most severe prognosis. It is characterized by profound structural remodeling and fibrosis in the bone marrow leading to severe anemia, weakness, and fatigue. Other characteristics of myelofibrosis are hypercellularity, osteosclerosis, and megakaryocytic hyperplasia in the bone marrow.3 The natural history and structural changes in the marrow contribute toward the poor prognosis of myelofibrosis and make it a rather complex disease to treat.

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Address reprint requests to Peter P. Sayeski, Ph.D., Department of Physiology and Functional Genomics, University of Florida College of Medicine, PO Box 100274, Gainesville, FL 32610. E-mail: psayeski@ufl.edu.
The identification of Janus kinase 2 (Jak2) somatic mutations in a large percentage of MPN patients has resulted in the initiation of molecularly targeted therapies for the treatment of MPNs. However, despite the high incidence of MPNs in humans and a reasonable understanding of the molecular mechanisms that underlie these disorders, currently available therapies are limited. They include cytoreductive agents such as hydroxyurea and the pan-Jak1/2 small molecule inhibitor, ruxolitinib. Although these therapies provide some temporary relief of associated symptomologies, they are not curative in any way. Furthermore, in the case of ruxolitinib, other than alleviation of constitutional symptoms, the palliative relief is not durable, and it has a high discontinuation rate due to a lack of efficacy characterized by an inability to reduce mutant clones in the bone marrow or improve patient survival. Thus, there is still a need to develop molecularly targeted Jak2 treatment options for MPNs that are effective in eliminating the etiology of the disease within the bone marrow.

We recently developed a small molecule Jak2 inhibitor called G6. We demonstrated that it has excellent therapeutic efficacy in Jak2-V617F–mediated disease pathogenesis as it significantly reduced or eliminated mutant cells from the bone marrow using mouse models of Jak2-V617F–mediated hyperplasia and Jak2-V617F–mediated PV/ET. However, its efficacy in Jak2-mediated myelofibrosis has not previously been examined. The studies described herein were therefore designed to determine whether G6 could provide therapeutic efficacy to the bone marrow in a mouse model of Jak2-mediated myelofibrosis. For this, transgenic mice expressing the human Jak2-V617F cDNA under the control of the vav promoter, and fully manifesting the myelofibrosis phenotype were administered either G6 or vehicle control solution for 28 consecutive days. The mice were then euthanized, and efficacy was determined by performing postmortem analysis on peripheral blood, liver, spleen, and bone marrow. We found that in addition to improving a number of constitutional symptoms associated with myelofibrosis, G6 provided significant therapeutic efficacy in the bone marrow in the form of a reduced Jak2 mutant burden, decreased pathogenic Jak/signal transducer and activator of transcription (STAT) signaling, a significant improvement of the myeloid to erythroid (M/E) ratio, and a significant reversal of bone marrow fibrosis. Collectively, these results indicate that G6 is efficacious in Jak2-V617F–mediated myelofibrosis, and given its bone marrow efficacy, it may alter the natural history of this disease.

Materials and Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida. Male mice, 11 to 12 months of age and expressing the human Jak2-V617F cDNA under the control of the vav promoter, were used for these experiments. The mice were generated on a C57BL/6 background strain as previously described. Following acquisition of baseline peripheral blood samples obtained via submandibular bleeding, the mice were given daily intraperitoneal injections of either vehicle (n = 9) or 10 mg/kg/day of G6 (n = 7) for 28 consecutive days. Terminal peripheral blood samples were then obtained after 28 days of treatment.

Blood Sample Analysis

Complete blood counts were obtained using a HESKA Vet ABC-Diff Hematology analyzer (Heska, Loveland, CO). Peripheral blood smears were also processed using Dip Quick (Jorgensen Laboratories, Loveland, CO) and analyzed by a veterinary pathologist.

Histological Analysis

Samples from the liver, spleen, and bone marrow were fixed in buffered formalin. The samples were then paraffin embedded and sectioned (4 μm). The femurs were decalcified for 16 hours before embedding. Tissues sections were H&E stained and then examined by a board-certified laboratory animal pathologist who was blinded to specific treatment groups. Bone marrow evaluation was completed according to the guidelines established by Elmore. The tissue sections were evaluated for fibrosis, cellularity, M/E ratios, megakaryocytic counts, and extramedullary hematopoiesis (EMH). The bone marrow sections were also stained with reticulin (American MasterTech Scientific, Lodi, CA), and the degree of fibrosis was rated by a veterinary pathologist who was blind to specific treatment groups: 0 = normal to minimal, 1 = minimal to mild, 2 = mild to moderate, and 3 = moderate to extensive, as previously described. The reticulin staining was also quantified via computer-assisted morphometric analysis using the NIS-Elements D software package (Nikon, Tokyo, Japan). Phospho-STAT5 immunohistochemistry was carried out on bone marrow sections using anti–phospho-STAT5 (Ab32364; Abcam, Cambridge, UK) antibody as previously described.

Determination of Jak2-V617F Mutant Burden in the Bone Marrow

Bone marrow samples were obtained from the femur, and RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNase-free DNase was used to eliminate DNA contamination. cDNA was synthesized from 2 μg of RNA using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was carried out in a Multicolor Real-Time PCR Detection System using TaqMan gene expression assays (Applied Biosystems). All PCR amplifications were performed in triplicate for human JAK2 (Hs01078124_m1) and mouse Jak2 (Mm00434577_m1). Parallel measurements of mouse β-actin cDNA were also done as an internal control, and all calculations for the copy number of the human Jak2 sequence were done relative to β-actin. The mutant burden was calculated by determining the ratio of human Jak2-V617F to endogenous mouse Jak2-WT transcripts.
Results

Effect of G6 on the Peripheral Blood of Myelofibrosis Mice

Myelofibrosis is a complex disease that presents variable clinical features, including anemia, extramedullary hematopoiesis, splenomegaly, myeloid hyperplasia, and marrow fibrosis. To determine whether G6 has therapeutic efficacy in the treatment of myelofibrosis, we used mice expressing the human Jak2-V617F cDNA in hematopoietic tissues.\textsuperscript{17} These mice exhibit most of the phenotypic syndromes associated with myelofibrosis and constitutive Jak2 signaling. Baseline peripheral blood counts were obtained, and the mice were then randomly assigned to one of the two groups: vehicle control or G6 treatment. Examination of the baseline samples revealed significant variations in the different cellular components when compared to nontransgenic mice (Table 1). Peripheral blood samples were again obtained after 28 days of either vehicle or G6 treatment, and despite the high variability in the counts, one significant observation was that myelofibrosis mice receiving vehicle control solution experienced an anemic effect during the 28-day treatment period characterized by a 38% decrease in red cell counts, a 30% decrease in hemoglobin, and a 36% decrease in hematocrit levels (Table 1). Although myelofibrosis mice receiving G6 also experienced anemia over the same 28-day treatment period, the effect was significantly less severe from that observed in mice receiving vehicle control solution, namely, an 18% decrease in red cell counts, a 9% decrease in hemoglobin, and a 15% decrease in hematocrit levels.

G6 Reduces Extramedullary Hematopoiesis in Jak2-V617F-Mediated Myelofibrosis

Jak2-V617F–mediated myelofibrosis is often associated with increased EMH in the liver. Here, we wanted to determine whether G6 could demonstrate therapeutic efficacy by reducing the number of EMH sites in this tissue.
ducing the spleen size in these mice, we determined the spleen-weight to body-weight ratios in wild-type mice, myelofibrosis mice treated with vehicle, and myelofibrosis mice treated with G6. Figure 2A shows representative spleens from each condition, and Figure 2B shows the spleen-weight to body-weight ratios plotted as a function of treatment group. We found that when compared to wild-type mice, myelofibrosis mice treated with vehicle solution had a >250% increase in the spleen-weight to body-weight ratios, and this was significantly reduced with G6 treatment. Figure 2C shows representative H&E-stained spleens from each condition, and Figure 2D indicates the number of megakaryocytes per high-power field plotted as a function of treatment group. We found that when compared to wild-type mice, the spleens from myelofibrosis mice treated with vehicle control solution had a 150% increase in the number of megakaryocytes per field, and this was significantly reduced with G6 treatment.

**G6 Alleviates Megakaryocytic and Myeloid Hyperplasia in the Bone Marrow**

Myelofibrosis is often characterized by marrow megakaryocytic and myeloid hyperplasia.3 As a consequence, bone marrow biopsies from myelofibrosis patients usually exhibit an abnormal M/E ratio. To gain some understanding of the potential efficacy of G6 in the bone marrow, histological sections were prepared from each mouse and examined. Figure 3A shows representative sections for each condition. Figure 3B shows the average number of megakaryocytes per high-power field in each treatment group. Figure 3C indicates the average M/E ratios of each condition. *P < 0.05 versus vehicle-treated myelofibrosis (MF) mice; **P < 0.001 versus wild type.
When compared to age-matched wild-type mice, myelofibrosis mice treated with vehicle control solution were found to have marked megakaryocytic hyperplasia and myelofibrosis, and this was reversed with G6 treatment. To quantify these results, the numbers of megakaryocytes per high-power field were plotted as a function of condition (Figure 3B). We found that when compared to the wild-type mice, the myelofibrosis mice treated with vehicle control solution had a >200% increase in the number of megakaryocytes per high-power field, and G6 significantly reduced this number. Furthermore, when compared to wild-type controls, the M/E ratio in the myelofibrosis mice was increased >250%, and this too was significantly reduced with G6 treatment (Figure 3C).

G6 Reduces Bone Marrow Reticulin Fibrosis

Myelofibrosis is characterized clinically by scarring of the bone marrow, and this is a major contributor to the poor prognosis in patients affected by this condition. Thus, identification of drugs that can reverse this deleterious outcome could significantly improve prognosis. To determine whether G6 reduces bone marrow fibrosis in this model, we carried out reticulin staining of the bone marrow sections of wild-type mice, myelofibrosis mice treated with vehicle control solution, and myelofibrosis mice treated with G6. Figure 4A shows representative images of the reticulin staining at ×40 and ×100 magnifications. Qualitatively, the myelofibrosis mice treated with vehicle control solution exhibited marked reticulin staining, and this was greatly reduced in the G6-treated mice. To quantify these data, we used two independent approaches. First, we measured the levels of reticulin stain using computer-assisted morphometric analysis. Second, we determined the levels of fibrosis via a standardized pathological index score. We found that when compared to age-matched wild-type controls, bone marrow sections obtained from myelofibrosis mice treated with vehicle control solution had significantly elevated levels of both reticulin stain (Figure 4B) and pathological index score (Figure 4C). However, G6 treatment significantly reduced these two parameters in the myelofibrosis mice (Figure 4, B and C).

G6 Reduces Phosphorylation of STAT5 in the Bone Marrow

Aberrant Jak/STAT signaling is a hallmark of Jak2-V617F–driven MPNs, including myelofibrosis. Here, we wanted to determine whether treatment with G6 reduces the increased activation of the Jak/STAT signaling pathway as indicated by phosphorylation of the proliferative marker STAT5. To this end, we carried out anti–phospho-STAT5 immunohistochemistry staining of the bone marrow sections from wild-type, myelofibrosis mice treated with vehicle, and myelofibrosis mice treated with G6. Figure 5A shows representative images of the anti–phospho-STAT5 immunohistochemistry at ×40 and ×100 magnifications. Qualitatively, we found that the bone marrow sections from the myelofibrosis mice treated with vehicle control solution had a significant increase in phospho-STAT5 staining when compared to the wild-type mice, and this was reduced with G6 treatment. To quantify these data, computer-assisted morphometric analysis was used, and the relative amounts of anti–phospho-STAT staining were plotted as a function of treatment group (Figure 5B). We found that G6 treatment resulted in a 53% reduction in the amount of phospho-STAT5 signal in the bone marrow, and this was significant.

G6 Significantly Reduces the Jak2-V617F Mutant Burden in the Bone Marrow

Several Jak2 inhibitors have been tested in mouse models of Jak2-V617F–mediated myelofibrosis (MF). We wanted to determine whether treatment with G6 reduces the increased activation of the Jak/STAT signaling pathway as indicated by phosphorylation of the proliferative marker STAT5. To this end, we carried out anti–phospho-STAT5 immunohistochemistry staining of the bone marrow sections from wild-type, myelofibrosis mice treated with vehicle, and myelofibrosis mice treated with G6. Figure 5A shows representative images of the anti–phospho-STAT5 immunohistochemistry at ×40 and ×100 magnifications. Qualitatively, we found that the bone marrow sections from the myelofibrosis mice treated with vehicle control solution had a significant increase in phospho-STAT5 staining when compared to the wild-type mice, and this was reduced with G6 treatment. To quantify these data, computer-assisted morphometric analysis was used, and the relative amounts of anti–phospho-STAT staining were plotted as a function of treatment group (Figure 5B). We found that G6 treatment resulted in a 53% reduction in the amount of phospho-STAT5 signal in the bone marrow, and this was significant.

G6 Significantly Reduces the Jak2-V617F Mutant Burden in the Bone Marrow

Several Jak2 inhibitors have been tested in mouse models of Jak2-V617F–mediated MPNs. However, these inhibitors are limited by their inability to decrease the Jak2-V617F mutant burden in the bone marrow. To assess this in our model using G6, we measured the levels of mutant Jak2-V617F transcripts and endogenous Jak2-WT mouse transcripts in the bone marrow. Figure 6A shows that G6 treatment reduced the levels of mutant Jak2
transcripts by 75% when compared to myelofibrosis mice that received vehicle control solution. To determine whether this reduction was due to nonspecific elimination of cells from the marrow by G6, we also measured the levels of endogenous Jak2-WT mRNA transcripts (Figure 6B). We found that G6 treatment slightly reduced the levels of Jak2-WT mRNA, but this was not significantly different from myelofibrosis mice that received vehicle control solution. Consequently, we found that the mutant burden, defined as the ratio of mutant Jak2-V617F to endogenous wild-type Jak2 mRNA transcripts, was reduced by 68% with G6 treatment, and this was significant (Figure 6C).

**Discussion**

MPNs are a group of related diseases that are characterized by a dysregulated clonal myeloproliferation resulting in excess production of terminally differentiated blood cells. Myelofibrosis has the most unfavorable natural history and worst prognosis of the MPNs because of the structural changes that occur in the bone marrow. Although currently available therapies alleviate symptoms such as splenomegaly, abnormal blood counts, and/or reduction of inflammatory cytokines, unfortunately, they lack bone marrow efficacy in the form of histopathologic, cytogenetic, or molecular remissions. Given this current backdrop, it is not surprising that there are continued calls for the further development of Jak2 small molecule inhibitors with particular emphasis on bone marrow efficacy. In addition to demonstrating efficacy in the form of amelioration of anemia, reduced EMH, and reduced splenomegaly, we demonstrate here significant bone marrow efficacy characterized by a 70% reduction in megakaryocytic hyperplasia, a significant correction of the M/E ratio, a 53% reduction in pathogenic phospho-STAT5 signaling, a 68% reduction in the Jak2 mutant burden, and a 67% decrease in the amount of reticulin staining. When taken together, these data indicate an overall improvement in the bone marrow and a significant reversal of myelofibrosis.

We recently reported that G6 is very efficacious in eliminating the Jak2 mutant burden from the bone marrow in a human erythroleukemia cell xenograft model of Jak2-V617F–mediated hyperplasia. Specifically, G6 at doses of 1 mg/kg/day and 10 mg/kg/day decreased the mutant burden by 90% and 95%, respectively. Although the human erythroleukemia cell xenograft model exhibited many of the bone marrow pathologies observed in Jak2-V617F–positive patients such as a low mutant burden in the context of the marrow niche, it was limiting in that it lacked some MPN features such as myeloid neoplasia. More recently, we demonstrated G6 bone marrow efficacy using a mouse model of Jak2-V617F–mediated PV/ET. Although this model manifested marked myeloid neoplasia, it lacked major deleterious structural changes in

**Figure 5.** G6 reduces phosphorylation of STAT5 in the bone marrow. A: Representative images of anti-phospho-STAT5 immunohistochemistry at the indicated magnifications. B: Quantification of the anti-phospho-STAT5 staining plotted as a function of treatment group. *P < 0.05 versus vehicle-treated myelofibrosis mice; **P < 0.001 versus wild type. MF, myelofibrosis.

**Figure 6.** G6 significantly reduces the Jak2-V617F mutant burden in the bone marrow. A: The amount of human Jak2-V617F mRNA transcripts from the bone marrow of mice for the indicated groups. B: Levels of endogenous wild-type Jak2 mRNA transcripts from the bone marrow of the same animals. C: The Jak2-V617F mutant burden (the ratio of mutant to wild type) plotted as a function of treatment group. **P < 0.001 versus myelofibrosis (MF) mice treated with vehicle control solution. ns, not significant.
the marrow such as myelofibrosis. Therefore, in this current study, we used a transgenic mouse model of Jak2-V617F myelofibrosis to determine whether G6 could reverse the damaging structural changes in the bone marrow. In conjunction with the two previously published models, our data here in the form of repeated measures of bone marrow efficacy, with particular emphasis on significantly reduced bone marrow fibrotic index scores, indicate that G6 facilitates disease remission.

Another important aspect of this work is the continued ability of G6 to exhibit in vivo efficacy. Although a number of previous Jak2 small molecule inhibitors demonstrated Jak2 inhibitory potential in vitro, those inhibitory potentials failed to fully manifest themselves when the compounds were tested in vivo. For example, INCB18424 (Ruxolitinib/Jakafi), CYT387, CEP-701, and INCB16562 all demonstrated good in vitro efficacy, but had limited efficacy in vivo.20,21,25,26 The demonstration here that Jak2 possesses marked in vivo efficacy using a third model of Jak2-mediated disease is significant and underscores the possibility that this compound may be efficacious in some human disorders that are caused by aberrant Jak2 signaling.

Perhaps the most significant observations of this work are the reductions in the Jak2 mutant burden and the reductions in the myelofibrosis. Comparison of the current G6 data with that of other Jak2 inhibitors is notable. For instance, we demonstrate here that IP administration of G6 at 10 mg/kg/day reduced the Jak2 mutant burden by 68% (Figure 6). By contrast, oral administration of the Jak2 inhibitor CYT387 at doses of 50 and 100 mg/kg/day displayed no histopathologies beyond those observed in the marrow such as myelofibrosis.21 In the case of ruxolitinib, initiating an oral dose of 180 mg/kg/day on the same day that Jak2-V617F cells were implanted into the bone marrow of recipient mice reduced the Jak2 mutant burden by only 33%, indicating that this drug modestly prevents engraftment of Jak2 mutant cells into the marrow.20 In instances where human Jak2-dependent disease was fully established in the marrow before treatment with ruxolitinib was initiated, the drug had no effect on bone marrow mutant burdens.9 Lastly, oral administration of TG101348 at doses of 120 and 240 mg/kg/day reduced the Jak2 mutant burden in the marrow by 0% and 67%, respectively.22 We are, of course, aware that G6 was administered via i.p. injection, whereas the others were delivered orally, and this can explain some of the observed differences in efficacy. However, we also realize that G6 has the potential to significantly impact the bone marrow in terms of reduced mutant burdens, whereas these other compounds are restricted. With respect to the improvement of myelofibrosis, Jak2 inhibitors that have been studied in preclinical and clinical trials have been very limited in this regard. In the case of ruxolitinib, it has no effect in the bone marrow, and hence, it cannot alter the natural history of myelofibrosis.12,13 Furthermore, other than alleviation of constitutional symptoms, the palliative relief of this drug is not durable, and it has a high discontinuation rate due to a lack of efficacy characterized by an inability to reduce mutant clones in the bone marrow or improve patient survival.10,11 This in part has fueled calls for the development of Jak2 inhibitors that can halt or even reverse fibrosis.23,24 We show here that G6 significantly reduced both the reticulin stain and the fibrotic index score of mice when compared to mice that received vehicle control solution (Figure 4). As such, our results demonstrate that the seemingly irreversible structural damage in the bone marrow of these mice is reversed with G6.

Putative Jak2 inhibitors comprise a number of diverse chemical structures including tyrphostins, pyrazines, pyrimidines, azaindoles, aminoindazoles, deazapurines, benzoxazoles, and quinoxalines.27 G6 is the only known Jak2 inhibitor belonging to a group of diarylethene compounds known as stilbenoids. Stilbenoids are naturally occurring compounds found in a wide variety of plants, such as grapes, and they act to defend against disease pathogens and DNA damage in the form of UV exposure.28 Within animals, stilbenoids provide therapeutic benefit to pathological conditions such as inflammation, cancer, oxidative stress, cardiovascular diseases, and viral diseases.29–31 Stilbenoids have also been reported to inhibit tyrosine kinases such as LMP2.32,33 However, no study has reported any therapeutic efficacy of stilbenoid compounds in myelofibrosis. We believe the ability of G6 to demonstrate in vivo efficacy in the form of decreased Jak2/STAT signaling and reduced myelofibrosis may lie in its unique chemical scaffold and its reactive groups.12,13 Furthermore, given that stilbenoids are naturally occurring, they generally have low toxicity profiles. This is supported in this current work in that myelofibrosis mice that were treated with G6 displayed no histopathologies beyond those observed in the hematopoietic tissues that were consistent with constitutive Jak2 signaling. As such, this absence of cytotoxicity suggests that G6 may be well tolerated in humans.

In conclusion, we show that the small molecule stilbenoid Jak2 inhibitor, G6, provides therapeutic efficacy in a mouse model of Jak2-mediated myelofibrosis by resolving the considerable structural remodeling in the bone marrow. This therapeutic efficacy was also accompanied by a significant reduction in the Jak2 mutant burden and an elimination or alleviation of a number of associated symptomologies. As such, these results suggest that G6 may be therapeutically potent in altering the natural history of myelofibrosis and, therefore, may be a viable candidate for progression into clinical trials for the treatment of this debilitating disorder.

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References


