Efficacy of vorinostat in a murine model of polycythemia vera

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Abstract

The discovery of the JAK2V617F mutation in most patients with Ph-negative myeloproliferative neoplasms (MPNs) has led to the development of JAK2 kinase inhibitors. However, JAK2 inhibitor therapy has shown limited efficacy and dose-limiting hematopoietic toxicities in clinical trials. In this report, we describe the effects of vorinostat, a small molecule inhibitor of histone deacetylase, against cells expressing JAK2V617F and in an animal model of polycythemia vera (PV). We found that vorinostat markedly inhibited proliferation and induced apoptosis in cells expressing JAK2V617F. In addition, vorinostat significantly inhibited JAK2V617F-expressing mouse and human PV hematopoietic progenitors. Biochemical analyses revealed significant inhibition of phosphorylation of JAK2, Stat5, Stat3, Akt, and Erk1/2 in vorinostat-treated JAK2V617F-expressing HEL cells. Expression of JAK2V617F and several other genes including GATA1, KLF1, FOG1, SCL, C/EPBα, PU.1 and NF-E2 was significantly down-regulated whereas SOCS1 and SOCS3 expression was up-regulated by vorinostat treatment. More importantly, we observed that vorinostat treatment normalized the peripheral blood counts and markedly reduced splenomegaly in Jak2V617F knock-in mice compared with placebo treatment. Vorinostat treatment also decreased the mutant allele burden in mice. Thus, our results suggest that vorinostat may have therapeutic potential for treatment of PV and other JAK2V617F-associated MPNs.
Introduction

Myeloproliferative neoplasms (MPNs) are a group of clonal hematopoietic malignancies that include chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocytemia (ET) and primary myelofibrosis (PMF) \(^{1,2}\). These diseases are characterized by excessive proliferation of myeloid/erythroid lineage cells. A somatic point mutation (V617F) in the JAK2 tyrosine kinase has been found in most patients with PV and 50-60% patients with ET and PMF \(^{3-6}\). JAK2V617F is a constitutively active tyrosine kinase that can transform factor-dependent hematopoietic cell lines to cytokine independence \(^{3,4}\). Expression of JAK2V617F mutant activates multiple downstream signaling pathways, such as signal transducer and activator of transcription (Stat), extracellular signal-regulated kinase (Erk), and phosphatidylinositol 3-kinase/Akt pathways \(^{3,7,8}\).

Current therapies for MPNs include phlebotomy and myelosuppressive therapy (e.g., hydroxyurea, anagrelide) for PV and ET, transfusions and supportive care for PMF. These empiric treatments are unlikely to cure or offer remission to patients with MPNs. Thus, there is a clear need for new therapies for MPNs. The discovery of the JAK2V617F mutation in PV, ET and PMF has led to the development of inhibitors of JAK2. Several JAK2 inhibitors are undergoing clinical trials. Although JAK2 inhibitors are effective in reducing splenomegaly and improving constitutional symptoms, significant hematopoietic toxicities including anemia and thrombocytopenia are observed in a majority of patients treated with the JAK2 inhibitors \(^{9,10}\), consistent with the known function of JAK2 in normal hematopoiesis \(^{11,12}\). Ruxolitinib, a JAK1/JAK2 inhibitor, has been approved for treatment of myelofibrosis. However, a recent report on long-term
outcomes with Ruxolitinib treatment shows improvement in constitutional symptoms but no significant benefit in survival of myelofibrosis patients. In addition, there is an increased rate of discontinuation of Ruxolitinib therapy due to severe hematopoietic toxicities or lack of response. It is also possible that drug resistance may emerge in some patients treated with JAK2 inhibitors similar to that observed with ABL inhibitor imatinib in CML patients. Therefore, identifying additional new therapies targeting JAK2V617F or pathways downstream of JAK2V617F would be beneficial for treatment of patients with MPNs.

Acetylation is an important post-translational modification that serves as a key modulator of chromatin structure and gene transcription, and provides a mechanism for coupling extracellular signals with gene expression. This process is regulated by two classes of enzymes- the histone acetyltransferases (HATs) and the histone deacetylases (HDACs) that catalyze the acetylation or deacetylation of histones. Inhibition of HDAC activity has been linked to hematopoietic stem cell (HSC) proliferation and self-renewal. Aberrant acetylations of histones and other cellular proteins have been found in leukemia, lymphoma and solid tumors. Pharmacological inhibition of HDACs has shown promise in treating hematologic malignancies and other forms of cancer.

Several HDAC inhibitors including trichostatin A (TSA), valproic acid, depsipeptide, vorinostat, ITF2357 (givinostat) and panobinostat have been shown to cause death of cancer cells in vitro and in vivo. Vorinostat (also known as SAHA or Zolinza®), a small molecule inhibitor of class I and II HDACs, has been shown to induce growth arrest and promote apoptosis of a variety of cancer cells. It is an FDA approved drug for treatment of refractory cutaneous T-cell lymphoma (CTCL).
Vorinostat has also demonstrated activity against leukemias and solid tumors in preclinical and Phase I clinical studies\textsuperscript{15,21,28,29}. Increased HDAC activity has been found in patients with PMF\textsuperscript{30}. In vitro treatment of PMF CD34+ cells with 5-azacytidine plus TSA or vorinostat resulted in a significant decrease in the proportion of JAK2V617F homozygous colonies and marked reduction of JAK2V617F-positive SCID repopulating cells\textsuperscript{23,31}. Moreover, a beneficial effect of HDAC inhibition was observed in a patient with JAK2V617F-positive advanced myelofibrosis\textsuperscript{32}. Other HDAC inhibitors including ITF2357 (givinostat) and panobinostat also showed potent anti-proliferative and pro-apoptotic activity against murine and human cells expressing JAK2V617F\textsuperscript{24,33}. Thus, inhibition of HDAC could be useful in treating MPNs. In the present studies, we have tested the efficacy of vorinostat in an animal model of Jak2V617F-positive MPN\textsuperscript{7}. We have reported earlier that expression of Jak2V617F in knock-in mice reproducibly produced all the features of human PV\textsuperscript{7}. We have utilized this Jak2V617F knock-in mouse model to test the \textit{in vivo} effects of vorinostat. Our results show that vorinostat treatment significantly improves peripheral blood counts and attenuates splenomegaly in knock-in mice expressing Jak2V617F.

**Methods**

**Reagents and antibodies**

Vorinostat was kindly provided by Merck & Co., Inc. Polyinosine-polycytosine (pI:pC) was obtained from Amersham/GE healthcare. p-JAK2, p-Stat5, p-Stat3, p-Akt, p-Erk1/2, JAK2 and caspase-3 antibodies were purchased from Cell Signaling Technologies. Acetyl histone H4 antibody was obtained from Millipore and acetyl tubulin antibody was
from Enzo Life Sciences. PARP antibody was obtained from BD Pharmingen. Stat5, Stat3, Akt, Erk2, c-Myc, and Pim-1 antibodies were purchased from Santa Cruz Biotechnology. HSP90 antibody was obtained from StressGen Biotechnologies.

**Cell cultures**

HEL, K562 and murine BA/F3-EpoR-JAK2V617F cells were maintained in RPMI-1640 medium with 10% FBS plus penicillin/streptomycin. UKE cells were maintained in RPMI-1640 medium plus penicillin/streptomycin supplemented with 10% FBS, 10% donor horse serum and 1 μM hydrocortisone. Primary erythroblasts were generated from the bone marrow of MxCre;Jak2V617F/+ mice as previously described.

**Cell proliferation and viability assays**

Viable cells were plated at 3,000 cells (for BA/F3-EpoR-JAK2V617F cells) or 10,000 cells (HEL, UKE and K562 cells) per well in 96-well plates. Cells were treated with DMSO or vorinostat (0.0625 μM to 1.0 μM) for 48 hours. Cell proliferation was measured by WST assay using a Quick Cell Proliferation Assay Kit (Biovision, CA).

For cell viability measurement, 5 x 10^5 cells were cultured in RPMI-1640 medium with 10% FBS in the presence of DMSO or vorinostat (0.5 μM, or 1.0 μM). Viable cell number was assessed by trypan blue exclusion every 24 hours for 5 days.

**Apoptosis and cell cycle analysis**

The proportion of apoptotic cells was determined by Annexin V staining. The cells were treated with DMSO or vorinostat for 24 hours. Cells were harvested and stained with
APC-conjugated Annexin V and propidium iodide (PI), according to the manufacturer’s instruction (eBioscience, San Diego, CA), and analyzed by flow cytometry.

For cell cycle analysis, cells were treated with DMSO or 1.0 μM vorinostat for 24 hours. Cells were fixed in 100% ethanol and stained with PI. Cells were recorded using a BD LSRII flow cytometer and the cell cycle distribution was analyzed by Modfit software.

**Colony-forming Assay**

BA/F3-EpoR-JAK2V617F, HEL, and K562 cells were plated (250 cells/dish) in duplicates in methylcellulose medium (M3234, Stem Cell Technologies) without any cytokine in the presence of DMSO or vorinostat (0.5 μM or 1.0 μM). Colonies were counted on day 5. To detect erythropoietin (Epo)-independent colony-forming unit erythroid (CFU-E) colonies, spleen cells from MxCre;Jak2V617F/+ mice were plated (1 X 10^5 cells/dish) in duplicate in methylcellulose medium (M3234, Stem Cell Technologies) without any cytokine in the presence of DMSO or vorinostat (0.5 μM or 1.0 μM). CFU-E colonies were counted after 2 days following staining with benzidine solution (Sigma).

**Flow cytometry**

BM and spleen cells from placebo and vorinostat-treated mice were stained with APC-conjugated Ter119 and PE-conjugated CD71 monoclonal antibodies (eBioscience, San Diego, CA) for 20 min on ice. Flow cytometry was performed with an LSRII (Beckton-
Deckinson, San Diego, CA) and analyzed by using FlowJo software (TreeStar, Ashland, OR).

**Immunoblotting and immunoprecipitation**

For immunoblot analysis, cells were harvested after treatment with vorinostat (0.5 μM or 1.0 μM) for 24 hours. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE. Immunoblotting was performed using phospho-specific or total antibodies as indicated. For loading controls, blots were reprobed with anti-total Erk2 antibodies.

For immunoprecipitation, one milligram of total protein was incubated with protein A Sepharose beads along with either anti-Hsp90 or anti-JAK2 antibody for 3 hours. The bound beads were washed 5 times in lysis buffer and separated by SDS-PAGE, followed by immunoblotting with anti-JAK2 or anti-Hsp90 antibody.

**Real-time quantitative PCR**

Total RNA was extracted from the HEL cells after treatment with vorinostat (0.5 μM or 1.0 μM) using the RNeasy Mini Kit (Qiagen, CA). Reverse transcription was carried out with 200 ng of total RNA using QuantiTect reverse transcription kit (Qiagen, CA). Quantitative real-time PCR for JAK2, SOCS1, SOCS3, GATA1, KLF1, FOG1, SCL, C/EBPα, PU.1 and NF-E2 was performed using the SYBR Green PCR Master mix (Applied Biosystems). The primers used for real-time PCR are listed in the Supplemental Table 1. 18S was used for normalization of the expression levels of JAK2, SOCS1, SOCS3, GATA1, KLF1, FOG1, SCL, C/EBPα, PU.1 and NF-E2. Quantitative real-time
PCR was performed using a LightCycler 480 (Roche Applied Science) and analyzed with associated software.

**Primary MPN Cells**

Peripheral blood samples were collected from PV and ET patients and healthy volunteers after informed consent was obtained according to the Declaration of Helsinki and the guidelines of the Institutional Review Board of the SUNY Upstate Medical University. CD34+ cells were isolated using a magnetic-activated cell isolation kit (Miltenyi Biotech) according to the manufacturer’s instructions. For hematopoietic progenitor assay, purified CD34+ cells were plated (1 X 10^3 cells/dish) in the presence of vehicle (DMSO) or vorinostat in methylcellulose medium (H4034, Stem Cell Technologies) containing cytokines (SCF, G-CSF, GM-CSF, IL-3, and EPO). Colonies were counted after 14 days. In addition, CD34+ cells from JAK2V617F-positive PV patients and healthy volunteers were cultured in StemSpan H3000 medium (StemCell Technologies) supplemented with cytokines to enrich for erythroid progenitor cells as described previously. Primary MPN cells were then treated with DMSO or vorinostat in the presence of cytokines for 48 hours and cell proliferation was measured by WST assay.

**Animal studies with vorinostat**

A conditional Jak2V617F knock-in mouse was generated as described previously. Expression of Jak2V617F was induced in MxCre;Jak2V617F/+ mice by injection with polyinosine:polycytosine (pI:pC). Peripheral blood counts were measured four weeks after pI:pC induction to confirm the establishment of PV disease in these mice.
Vorinostat was prepared in 50% polyethylene glycol (PEG-400) solution and administered by intraperitoneal injection. Two groups of MxCre;Jak2V617F/+ mice were treated: one group received placebo and another group received 200mg/kg vorinostat for 5 days in a week for a period of 2 weeks. At the study end point, mice were assessed by their peripheral blood counts. Bone marrow and spleen from placebo and vorinostat-treated mice were analyzed by flow cytometry and histopathology. To determine the effects of vorinostat on Jak2V617F allele burden, BM from pl:pC induced Jak2V617F knock-in mice (CD45.2+) were mixed with the BM from wild type (CD45.1+) mice at a ratio of 3 to 1 (75% V617F versus 25% WT) and injected into lethally-irradiated CD45.1+ recipient animals. Four weeks after transplantation, peripheral blood counts were measured to confirm the establishment of PV disease. Mice were treated with either placebo or vorinostat (200 mg/kg) for two weeks as described above. The ratio of V617F to WT progenitors was determined in the peripheral blood and the BM of the recipient animals by determining the percentage of CD45.2+ (calculated as CD45.2+/CD45.1+ plus CD45.2+) cells in the myeloid (Gr-1+) populations. All animal studies were approved by the Committee for the Humane Use of Animals of SUNY Upstate Medical University.

**Blood and tissue analysis**

Peripheral blood counts were measured using Hemavet 950FS (Drew Scientific). Blood smears were stained with Wright-Giemsa. For histopathologic analysis, mouse tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (4 μm) were stained with hematoxylin and eosin (H&E).
Statistical analysis

Results are expressed as mean ± SEM, and data were analyzed by the two-tailed Student’s t-test. P < 0.05 was considered to be statistically significant.

Results

Vorinostat significantly inhibits proliferation of cell lines expressing JAK2V617F

We first determined the effects of vorinostat on proliferation of hematopoietic cell lines expressing JAK2V617F. BA/F3-EpoR cells stably expressing JAK2V617F (BA/F3-EpoR-JAK2V617F), human erythroleukemia (HEL) or UKE cells harboring JAK2V617F mutation, or wild type JAK2 expressing BCR-ABL-transformed K562 cells were treated with various doses of vorinostat (0.0625-1.0 μM) for 48 hours. Cell proliferation was assessed by WST assay. Interestingly, proliferation of JAK2V617F-expressing BA/F3-EpoR-JAK2V617F, HEL and UKE cells was significantly reduced upon treatment with vorinostat in a dose-dependent manner (Figure 1A). In contrast, proliferation of BCR-ABL-positive K562 cells was modestly affected by vorinostat only at higher concentration (Figure 1A).

We also tested the effects of vorinostat on cell viability. Similar to the effects on proliferation, cell viability was remarkably reduced in BA/F3-EpoR-JAK2V617F, HEL and UKE cells upon vorinostat (1.0 μM) treatment (Figure 1B). However, K562 cells continued to proliferate and the viable cell number was increased even after treatment with vorinostat (1.0 μM) for 5 days (Figure 1B). These data demonstrate that JAK2V617F mutant expressing cells are particularly sensitive to vorinostat treatment.
We next assessed the effects of vorinostat on colony-forming ability of cells expressing JAK2V617F. The colony-forming assay was performed in methylcellulose medium in the absence of cytokine. Vorinostat treatment at 0.5 μM reduced colony formation of JAK2V617F-expressing BA/F3-EpoR-JAK2V617F, UKE and HEL cells by ~40-60% (Figure 1C). Vorinostat at 1.0 μM almost completely inhibited colony formation by BA/F3-EpoR-JAK2V617F, HEL and UKE cells (Figure 1C). However, vorinostat treatment resulted in only modest inhibition of colony formation of K562 cells even at higher concentration (1.0 μM) (Figure 1C). Thus, vorinostat inhibits the clonogenic outgrowth of cells expressing JAK2V617F.

Vorinostat induces apoptosis and cell cycle arrest in cells expressing JAK2V617F

To determine whether vorinostat-mediated inhibition of cell growth is associated with the induction of apoptosis, we performed Annexin V staining after treatment of cells with various concentrations of vorinostat (0.25-1.0 μM) for 24 hours. We observed that vorinostat treatment (0.25-1.0 μM) induced significant apoptosis in BA/F3-EpoR-JAK2V617F, HEL and UKE cells in a dose-dependent manner (Figure 2A). In contrast, vorinostat treatment exhibited much less cytotoxic effect against BA/F3-EpoR cells expressing JAK2WT or the BCR-ABL-positive K562 cells (Figure 2A).

Cell cycle analysis revealed that vorinostat (1.0 μM) treatment caused significant reduction in the proportion of cells in the S-phase accompanied by an increase in the G0/G1 fraction in JAK2V617F-expressing HEL cells (Figure 2B). In contrast, vorinostat (1.0 μM) treatment had no significant effect in the cell cycle distribution in K562 cells.
(Figure 2B). Together, these results suggest that vorinostat induces significant apoptosis and cell cycle arrest in cells bearing the JAK2V617F mutation.

**Vorinostat inhibits primary erythroblasts expressing JAK2V617F**

Expression of JAK2V617F in hematopoietic stem cells/progenitors results in skewing towards erythroid lineage and expansion of erythroid progenitors\(^7,36\). So, we asked whether vorinostat could inhibit the growth of primary erythroblasts expressing JAK2V617F. Primary erythroblasts were derived from the bone marrow of Jak2V617F knock-in mice as previously described\(^34\). Jak2V617F-expressing primary erythroblasts were plated in a 96 well plate in the presence of vehicle (DMSO) or vorinostat at various concentrations (0.0625-1.0 \(\mu\)M) for 48 hours, and proliferation was measured by WST assay. Proliferation of murine erythroblasts expressing Jak2V617F was significantly inhibited by vorinostat in a dose-dependent manner (Figure 3A).

Expression of JAK2V617F results in Epo-independent erythroid colonies in the BM and spleens of mice and humans\(^7,37\), a hallmark feature of PV. So, we tested the effects of vorinostat on JAK2V617F-evoked Epo-independent CFU-E colony formation in the spleens of mice expressing Jak2V617F. As shown in Figure 3B, vorinostat treatment at 0.25 \(\mu\)M reduced Epo-independent CFU-E colonies by \(\sim70\%\). At higher doses of vorinostat (0.5-1.0 \(\mu\)M), Epo-independent CFU-E colonies were almost completely inhibited in the spleens of mice expressing Jak2V617F (MxCre;Jak2V617F/) (Figure 3B). Thus, vorinostat markedly inhibited Epo-independent erythroid colony formation mediated by JAK2V617F. We also compared the effects of vorinostat with JAK inhibitor on Epo-independent erythroid colony formation
mediated by JAK2V617F. Whereas vorinostat at 1 μM completely inhibited Epo-independent CFU-E colony formation, treatment with JAK inhibitor I at 2 μM concentration, which almost completely inhibited JAK2 kinase activity (as described in ^4 and data not shown), exhibited ~50% reduction in Epo-independent CFU-E colonies in the spleens of Jak2V617F mice (Figure 3C). These results suggest that vorinostat could be more potent than JAK inhibitor in inhibiting Epo-independent erythroid colony formation mediated by JAK2V617F.

Next, we asked if vorinostat has any toxic effect towards normal wild type hematopoietic progenitors. To address this question, we compared bone marrow progenitors from wild type mice treated with vorinostat (0.5-1.0 μM) to those with vehicle treatment by performing hematopoietic progenitor colony-forming assays in the presence of cytokine. Treatment with vorinostat (0.5-1.0 μM) exhibited little effect on erythroid (CFU-E, BFU-E) and myeloid (CFU-GM) colony formation in the bone marrow of wild type mice (Figure 3D). Together, these results suggest that vorinostat preferentially inhibit the clonogenic growth of primary erythroid progenitors expressing Jak2V617F without exhibiting significant toxicity towards wild-type Jak2-expressing normal hematopoietic progenitors in mice.

Vorinostat inhibits clonogenic growth of PV hematopoietic progenitors expressing JAK2V617F

We next examined the effects of vorinostat on the clonogenic growth of MPN primary hematopoietic progenitors. We isolated CD34+ cells from patients with JAK2V617F-positive PV and ET and from wild type JAK2-expressing healthy volunteers. Treatment
with vorinostat (0.5-1.0 μM) significantly reduced hematopoietic progenitor colonies in JAK2V617F-positive PV CD34+ cells (Figure 3E). In contrast, no significant inhibition of hematopoietic progenitor colonies was observed in healthy control samples (Figure 3E). However, vorinostat treatment only modestly inhibited hematopoietic progenitor colonies in ET samples. Overall, the effect of vorinostat was significantly greater on hematopoietic progenitors from patients with PV than with ET (Figure 3E).

We also have evaluated the effects of vorinostat on the growth of primary MPN cells. We cultured the PV and normal control CD34+ cells in a serum-free medium with cytokines that enriches the erythroid cells 35. We found that erythroid cells from MPN patients were significantly more (>2 fold) inhibited by vorinostat treatment than healthy control erythroid cells (Figure 3F). Thus, primary MPN cells are more sensitive to vorinostat than normal cells.

**Vorinostat downregulates expression of JAK2V617F and affects its downstream targets**

To gain insight into the mechanism of inhibition of cells expressing JAK2V617F by vorinostat, we performed immunoblotting experiments. Treatment with vorinostat significantly inhibited the phosphorylation of JAK2, Stat5, Stat3, Akt, and Erk1/2 in JAK2V617F-positive HEL cells (Figure 4A). Immunoblotting for total JAK2 protein revealed that expression of JAK2 protein was significantly downmodulated by vorinostat in HEL cells (Fig. 4A). Expression of Stat5, Stat3 and Akt was also reduced by vorinostat treatment in HEL cells, although Erk expression was unaffected by vorinostat treatment in these cells (Figure 4A). Immunoblotting with antibodies against acetylated histone H4
and acetylated tubulin showed that vorinostat treatment resulted in hyperacetylation of both histone and non-histone proteins in HEL cells (Fig. 4A). A marked reduction in c-Myc and Pim-1 levels in JAK2V617F-positive HEL cells was observed upon vorinostat treatment (Figure 4B). In accordance with increased apoptosis in vorinostat-treated HEL cells, we also observed cleavage of caspase 3 and PARP in HEL cells upon treatment with vorinostat (Fig. 4B). Together, these results suggest that vorinostat inhibits the JAK2V617F mutated cells by inhibiting the JAK2/Stat pathway.

To determine whether downregulation of JAK2V617F in vorinostat-treated HEL cells occur at the transcriptional or post-translational level, we analyzed the expression of JAK2 mRNA in HEL cells after treatment with vorinostat by quantitative real-time PCR. As shown in Figure 4C, treatment with vorinostat (0.5-1 μM) resulted in an approximately 40-50% decrease in JAK2 mRNA in HEL cells. However, treatment of HEL cells with vorinostat at 1 μM resulted in greater inhibition of JAK2V617F protein expression (Figure 4A), indicating that downmodulation of JAK2 levels in HEL cells upon vorinostat treatment may also occur at the protein level. Previous studies have suggested that heat shock protein 90 (HSP90) serves as a molecular chaperone for protein tyrosine kinases including JAK2. So, we tested the interaction of HSP90 and JAK2V617F in HEL cells by immunoprecipitation with HSP90 or JAK2 antibody in the presence or absence of vorinostat. Immunoprecipitates of HSP90 and JAK2 showed interaction between HSP90 and JAK2 in HEL cells, and that interaction was significantly reduced by vorinostat treatment (Figure 4D).

We also assessed the effects of vorinostat on the expression of suppressor of cytokine signaling (SOCS) molecules, which are known to negatively regulate the JAK-
Stat pathway. As shown in Figure 4E, expression of SOCS1 and SOCS3 were significantly increased in HEL cells treated with vorinostat. These results provide evidence that vorinostat inhibits JAK2V617F-expressing hematopoietic cells by regulating the expression of the modulators of the JAK2/Stat pathway.

Since vorinostat treatment inhibited MPN hematopoietic progenitors expressing JAK2V617F (Figures 3), we evaluated the effects of vorinostat on expression of several transcription factors that control hematopoiesis. Expression of transcription factors was analyzed in JAK2V617F-expressing HEL cells after treatment with vorinostat by quantitative real-time PCR. We found that expression of GATA1, KLF1, FOG1, SCL, C/EBPα, PU.1 and NF-E2 was significantly reduced in HEL cells treated with vorinostat (Figure 4F). GATA1, KLF1, FOG1, SCL and NF-E2 are known to regulate erythropoiesis, whereas PU.1 and C/EBPα are important for granulopoiesis/monopoiesis. Thus, vorinostat-evoked inhibition of JAK2V617F-expressing MPN cells could be due to reduced expression of these transcription factors.

**Vorinostat induces significant hematologic response in a murine model of polycythemia vera**

Since vorinostat significantly inhibited cells expressing JAK2V617F, we asked if vorinostat could be useful in treating PV using our Jak2V617F knock-in mouse model. To assess the effects of vorinostat on Jak2V617F-induced PV, we first established PV disease in MxCre;V617F/+ mice by injecting pI:pC. Four weeks after induction, the disease was confirmed by peripheral blood counts. Animals were then randomized to begin treatment with placebo (vehicle) or with vorinostat (200 mg/kg/per day by i.p.
injection) for 2 weeks. MxCre;V617F/+ mice receiving placebo exhibited elevated levels of red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT) and white blood cells (WBC) (Figure 5A-D), as expected. The increase in RBC, Hb, HCT and WBC counts in MxCre;V617F/+ mice was significantly reduced upon treatment with vorinostat (200 mg/kg/day) for 2 weeks (Figure 5A-D). The platelet count (Figure 5E), however, was not affected by vorinostat treatment. Vorinostat treatment also resulted in significant decrease in spleen size compared to placebo-treated MxCre;V617F/+ mice (Figure 5F). Conversely, vorinostat treatment (200 mg/kg/day) did not cause any significant decrease in RBC, Hb and HCT in control wild type animals (Supplemental Figure 1).

Histopathologic evaluation of the peripheral blood smears showed marked decrease in immature and mature forms of RBCs to normal levels in vorinostat-treated mice compared with placebo-treated animals (Figure 6A). Bone marrow from placebo-treated MxCre;V617F/+ mice showed hypercellularity with trilineage hyperplasia, which was significantly reduced upon vorinostat treatment (Figure 6B). Whereas placebo-treated MxCre;V617F/+ mice exhibited effacement of normal splenic architecture with increased numbers of megakaryocytes and clusters of immature erythroid precursors, vorinostat therapy normalized the splenic architecture and reduced extramedullary hematopoiesis (Figure 6C). Consistent with histopathologic analyses, flow cytometric analysis of bone marrow and spleen revealed a marked decrease in the proportion of erythroid precursors (CD71+/Ter119+) in mice treated with vorinostat compared with placebo-treated mice (Figure 6D). Together, these results suggest that vorinostat could be efficacious in treating PV.
Vorinostat treatment decreases JAK2V617F mutant allele burden in a murine model

Since we observed that vorinostat treatment significantly improved blood parameters and reduced splenomegaly in Jak2V617F knock-in mice (Figure 5A-F), we asked whether vorinostat treatment could reduce the Jak2V617F mutant allele burden. To address this question, we generated a chimeric mouse model by mixing BM from the Jak2V617F knock-in mice (CD45.2+) with the BM from wild type (CD45.1+) mice at a ratio of 3 to 1 (75% V617F versus 25% WT) and injected it into lethally irradiated wild type CD45.1+ recipient animals, as outlined in Figure 7A. As expected, the transplanted chimeric mice exhibited high hematocrit levels and high percentage (~70%) of CD45.2+ Jak2V617F-expressing cells in their peripheral blood within 4 weeks after transplantation (Figure 7B,C). Vorinostat treatment (200 mg/kg) for two weeks significantly reduced hematocrit levels in the transplanted animals (Figure 7B). In addition, vorinostat treatment caused significant reduction in Jak2V617F-expressing cells (determined by the ratio of CD45.2+/CD45.1+) in the peripheral blood and BM of the chimeric mice (Figure 7C,D). Together, these results provide strong evidence that vorinostat treatment can reduce the mutant allele burden in vivo.

Discussion

The JAK2V617F mutation is the most common somatic mutation found in PV, ET and PMF. Several small molecule JAK2 inhibitors have been developed for treatment of these MPNs, and some of these agents are currently undergoing clinical trials. Recent results from the clinical trials suggest that JAK2 inhibitor therapy can reduce splenomegaly and
constitutional symptoms, but can cause significant hematologic toxicities in MPN patients\textsuperscript{9,10,13}. Complete remissions similar to those seen in CML with the BCR-ABL inhibitor imatinib cannot be achieved with the JAK2 inhibitors. Thus, it is very likely that JAK2 inhibitor alone would not be sufficient to effectively treat MPNs. So, there is a clear need for development/identification of alternative therapeutic approaches for treatment of MPN patients.

In this report, we evaluate the efficacy of HDAC inhibitor vorinostat in hematopoietic cell lines and primary hematopoietic progenitors expressing JAK2V617F and in a murine model of PV. We tested vorinostat for several reasons. Vorinostat is a FDA approved drug for treatment of refractory CTCL and is currently under investigation in several other neoplasms. We demonstrate that vorinostat treatment significantly inhibits the growth of murine and human hematopoietic cells and progenitors expressing JAK2V617F. Other HDAC inhibitors including ITF2357 (givinostat) and panobinostat also showed activity against JAK2V617F-expressing cells\textsuperscript{24,33}. Treatment with vorinostat \textit{in vivo} resulted in significant improvement in peripheral blood counts with normalization of RBC, hemoglobin and hematocrit in the Jak2V617F knock-in mice (Figure 5A-D). We also observed marked improvements in bone marrow and spleen architecture, and significant reduction in splenomegaly (Figure 5F and Figure 6). Moreover, vorinostat treatment reduced the mutant allele burden in mice (Figure 7). Thus, our data show that vorinostat may have therapeutic potential for treatment of PV.

We did not observe anemia in control animals treated with vorinostat (Supplemental Figure 1). We have treated the control and Jak2V617F knock-in mice for short-term only. However, a toxicological study on long-term effects of vorinostat
treatment in normal rats and dogs show decreased food consumption, weight loss and some reduction in hematologic parameters in rats and primarily gastrointestinal toxicity with little or no reduction in WBC, RBC and platelet counts in dogs after 26 weeks of treatment with vorinostat at high doses. It is possible that the toxicity profile of vorinostat in animals could be different than in humans.

Biochemical analyses provide some clues to the inhibition of JAK2V617F-expressing cells by vorinostat. Phosphorylation of JAK2, Stat5, Stat3, Akt and Erk1/2 was markedly inhibited by vorinostat in JAK2V617F-positive HEL cells (Figure 4A). Interestingly, vorinostat treatment (1 μM) almost completely inhibited expression of JAK2V617F protein (Figure 4A). Thus, down-regulation of JAK2V617F by vorinostat may result in inhibition of JAK2V617F-positive MPN cells. We observed 50-60% reduction in JAK2V617F mRNA upon vorinostat treatment (Figure 4C), whereas JAK2V617F protein expression was profoundly inhibited by vorinostat treatment (Figure 4A), suggesting that vorinostat inhibits the JAK2V617F expression both at the mRNA and protein levels. One possible explanation for the reduction of JAK2V617F at the protein level is that vorinostat treatment may disrupt the binding of JAK2V617F to HSP90 and render the JAK2V617F susceptible to proteasomal degradation. It has been suggested that mutant oncoprotein kinases (such as, BCR-ABL, FLT3-ITD) are more dependent on chaperon association with HSP90 than unmutated kinases. Recent studies indicate that JAK2V617F is also an HSP90 client protein. Indeed, we observed dissociation of JAK2V617F with HSP90 upon vorinostat treatment (Figure 4D). However, the mechanism by which vorinostat inhibits transcription of JAK2V617F is unclear.
Myc and Pim 1 are known as downstream targets of JAK2V617F and are required for JAK2V617F-mediated transformation \(^{46}\). We observed marked reduction in c-Myc and Pim-1 in vorinostat-treated HEL cells (Figure 4B). Also, significant cleavage of caspase 3 and PARP was observed in HEL cells treated with vorinostat (Figure 4B) consistent with increased apoptosis in HEL cells upon vorinostat treatment.

A recent study has shown that hyperacetylation of SOCS1 and SOCS3 promoters by TSA treatment increases their expression in colorectal cancer cells \(^{47}\). We also observed significant increase in SOCS1 and SOCS3 expression in vorinostat-treated HEL cells (Figure 4E). Thus, vorinostat treatment not only decreases expression of JAK2V617F/Stat5/Stat3 but also increases expression of the negative regulators (SOCS1 and SOCS3) of the JAK/Stat pathway.

We also observed significant decrease in expression of several transcription factors including GATA1, KLF1, FOG1, SCL, C/EBP\(\alpha\), PU.1 and NF-E2 in vorinostat-treated HEL cells (Figure 4F). GATA1, KLF1, FOG1, SCL and NF-E2 control erythropoiesis \(^{41,42}\), whereas PU.1 and C/EBP\(\alpha\) are important for granulopoiesis/monopoiesis \(^{43}\). Interestingly, upregulation of NF-E2 and PU.1 expression was observed in MPN \(^{48,49}\). In addition, it has been found recently that overexpression of NF-E2 induces MPN in mice and vorinostat treatment can reduce the NF-E2 expression \(^{50}\). Thus, it is plausible that vorinostat may inhibit the growth of JAK2V617F-expressing hematopoietic progenitors by inhibiting the expression of transcription factors that control erythropoiesis and myelopoiesis. Future studies will determine how inhibition of HDAC by vorinostat regulates the expression of these transcription factors.
We observed that the growth of JAK2V617F-expressing primary erythroid precursors from mice and humans was remarkably inhibited by vorinostat treatment (Figure 3). Interestingly, vorinostat was more potent than JAK inhibitor I at inhibiting erythroid progenitors expressing JAK2V617F (Figure 3B,C). We also observed that the effect of vorinostat was significantly greater on hematopoietic progenitors from PV than ET (Figure 3E). Furthermore, we have demonstrated that vorinostat treatment provides significant hematologic response in a Jak2V617F knock-in mouse model of PV. More importantly, vorinostat treatment significantly reduced the mutant allele burden in mice (Figure 7). Taken together, our results suggest that vorinostat therapy may offer significant clinical benefit to patients with PV and possibly other JAK2V617F-positive MPNs.

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Authorship

Contribution: H.A. performed research, analyzed data and wrote the paper; S.A. performed research; A.G., A.B. and S.G. procured and assisted in performing studies on MPN primary cells. R.E.H. performed histopathologic analysis and revised the paper; G.M. designed and performed research, analyzed data and wrote the paper.
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References


Figure legends

Figure 1. Effects of vorinostat on cell proliferation and clonogenic outgrowth. (A) BA/F3-EpoR cells stably expressing JAK2V617F, HEL and UKE cells harboring JAK2V617F mutation or BCR-ABL-transformed K562 cells expressing wild-type JAK2 were treated with varying concentrations of vorinostat for 48 hours. Cell proliferation was measured by WST assay. Columns represent mean of three independent experiments; bars represent SEM. (B) Cells were treated with DMSO (control) or vorinostat, and viable cells were counted by trypan blue exclusion over five days. Results shown represent mean ±SEM from three independent experiments. (C) BA/F3-EpoR-JAK2V617F, HEL, UKE and K562 cells were left untreated or treated with vorinostat (0.5 or 1.0 μM) and plated in methylcellulose medium (Methocult M3234) in the absence of cytokine. Cytokine-independent colonies were counted on day 5. Results shown represent mean ±SEM from three independent experiments. Note that vorinostat significantly inhibited clonogenic growth of JAK2V617F-transformed cells.

Figure 2. Vorinostat induces apoptosis in cells expressing JAK2V617F. BA/F3-EpoR cells expressing JAK2WT, JAK2V617F, and human HEL, UKE and K562 cells were treated with DMSO or varying concentrations of vorinostat (0.25-1.0 μM) for 24 hr. (A)
Apoptosis was determined by staining with propidium iodide (PI) and annexin V followed by flow cytometric analysis. Results shown are representative of three independent experiments. Note that vorinostat treatment significantly increased apoptosis in JAK2V617F-positive cells. 

(B) Cell cycle distribution of HEL and K562 cells after 24 hr exposure to vorinostat (1.0 μM). Cell cycle analysis was performed by PI staining and analyzed by Modfit software. Results from three independent experiments are shown as mean ±SEM. Asterisks indicate significant differences (p<0.05) between vehicle and vorinostat-treated cells. Note that vorinostat-treated HEL cells were arrested at G0/G1 phase accompanied by significant decrease in the percentage of S phase.

Figure 3. Effects of vorinostat on mouse and human primary hematopoietic progenitors or precursors expressing JAK2V617F. (A) Primary erythroblasts derived from the BM of Jak2V617F knock-in mice were treated with DMSO or vorinostat for 48 hours, and cell proliferation was measured by WST assay. Note that proliferation of erythroblasts expressing Jak2V617F was significantly inhibited by vorinostat treatment. (B, C) Spleen cells from Jak2V617F knock-in mice were plated in methylcellulose medium (Methocult M3234) without any cytokine in the presence of DMSO or vorinostat (0.25-1.0 μM) (B) or JAK inhibitor I (0.50-2.0 μM) (C). CFU-E colonies were scored after 2 days by staining with benzidine solution. Results are shown as percentage of vehicle (DMSO)-treated controls. Data are presented as mean ±SEM from three independent experiments. (D) BM cells from wild type (control) mouse were plated in complete methylcellulose medium (Methocult M3434) in the presence of DMSO or vorinostat (0.5 or 1.0 μM). CFU-E colonies were counted 2 days after plating, and BFU-
E and CFU-GM colonies were counted on day 7. Results are shown as percentage of vehicle (DMSO)-treated controls. Data are presented as mean ±SEM from three independent experiments. Note that vorinostat treatment exhibited little or no toxicity against control BM progenitors. (E) Effects of vorinostat on MPN hematopoietic progenitors. CD34+ progenitor cells isolated from PV, ET and normal healthy control blood samples were plated (1 X 10^3 cells/dish) in duplicates in the presence of DMSO or vorinostat in methylcellulose medium (H4034, Stem Cell Technologies) containing cytokines (SCF, G-CSF, GM-CSF, IL-3, and EPO). Hematopoietic progenitor colonies were counted after 14 days. Results are shown as mean ± SEM of total colonies scored. Data are expressed as percentage of vehicle (DMSO)-treated controls. Note that PV CD34+ progenitor cells were significantly inhibited by vorinostat treatment compared with ET or healthy control CD34+ progenitor cells. ET CD34+ progenitor cells were only modestly inhibited by vorinostat treatment compared with control progenitors. Asterisks indicate significant differences by Student’s t-test (** indicates p<0.005; * indicates p<0.05). (F) Effects of vorinostat on the growth of primary MPN cells. Primary erythroid cells derived from PV or normal CD34+ cells were treated with DMSO or vorinostat in the presence of cytokines for 48 hours and cell proliferation was measured by WST assay. Data are expressed as percentage of vehicle (DMSO)-treated controls. Asterisks indicate significant differences by Student’s t-test (** indicates p<0.005; * indicates p<0.05). Note that primary erythroid cells from MPN patients were significantly more (>2 fold) inhibited by vorinostat than healthy control erythroid cells.
Figure 4. Vorinostat down-regulates expression of JAK2V617F and affects its downstream signaling. (A) HEL cells bearing JAK2V617F mutant were treated with the indicated concentrations of vorinostat for 24 hours. Acetylation of histone H4 and tubulin was detected by anti-acetyl-histone H4 and acetyl-tubulin immunoblottings. Activation of JAK2, Stat5, Stat3, Akt and Erk1/2 was detected by immunoblotting using phospho-specific antibodies. Membranes were re-probed for total proteins. (B) HEL cells were left untreated or treated with indicated concentrations of vorinostat, and c-Myc, Pim-1, PARP and caspase 3 levels were analyzed by immunoblotting. Levels of Erk2 served as the loading control. (C) HEL cells were treated with indicated concentrations of vorinostat for 24 hours. Total RNA was extracted and analyzed by the quantitative real-time PCR. Relative expression of JAK2 was normalized to 18S expression. Results are shown as mean ± SEM from three independent experiments. (D) HEL cells were treated with the indicated concentrations of vorinostat for 24 hours, and lysates were immunoprecipitated with anti-HSP90 or anti-JAK2 antibody and immunoblotted with anti-JAK2 or anti-HSP90 antibody. (E, F) HEL cells were treated with vorinostat (1.0 μM) or DMSO for 24 hours. Total RNA was extracted and reverse transcribed. Relative expression of SOCS1 and SOCS3 mRNA was determined by quantitative real-time PCR and normalized to 18S expression (E). Relative expression of GATA1, KLF1, FOG1, SCL, C/EBPα, PU.1 and NF-E2 mRNA was determined by quantitative real-time PCR and normalized to 18S expression (F). Results shown represent mean ± SEM from three independent experiments. Asterisks indicate significant differences by Student’s t-test (* indicates p<0.05; ** indicates p<0.005).
**Figure 5.** Vorinostat treatment normalizes peripheral blood counts and improves extramedullary hematopoiesis in Jak2V617F knock-in mice. Treatment with vorinostat at 200 mg/kg for 2 weeks resulted in significant improvement in RBC (A), hemoglobin (B), hematocrit (C) and WBC (D) counts in Jak2V617F knock-in mice (n=10) compared with placebo (vehicle)-treated mice (n=10). (E) Platelet counts in peripheral blood of placebo- and vorinostat-treated animals (n=10). (F) Spleen weight was significantly reduced in Jak2V617F knock-in mice upon 2 weeks of vorinostat treatment (n=7).

**Figure 6.** Histopathological characterization and flow cytometric analysis of BM and spleen of Jak2V617F knock-in mice treated with placebo or vorinostat. (A) Peripheral blood smears show normalization of RBC and reticulocytes in vorinostat treated Jak2V617F knock-in mice. Arrows indicate reticulocyte and immature nucleated RBC. H&E staining of the bone marrow (B) and spleen (C) sections from placebo-treated Jak2V617F knock-in mice show hypercellularity with trilineage hyperplasia, which was markedly reduced upon vorinostat treatment. (D) Percentage of erythroid precursors, determined by Ter119 and CD71 positive cells, was significantly reduced in both BM and spleens of Jak2V617F knock-in mice treated with vorinostat compared with those of placebo-treated mice. Results shown represent mean ±SEM from 7 mice in each group.

**Figure 7.** Vorinostat treatment reduces the mutant allele burden in mice. (A) Experimental design for analysis of mutant allele burden upon vorinostat treatment. BM from pI:pC induced MxCre;Jak2V617F/+ mice (CD45.2+) were mixed with the BM from
wild type (CD45.1+) mice at a ratio of 3 to 1 (75% V617F versus 25% WT) and injected into lethally irradiated wild type CD45.1+ recipient animals. Four weeks after transplantation, peripheral blood counts were measured to confirm the establishment of PV disease, and the chimerism was assessed in the peripheral blood leukocytes by determining the ratio of CD45.2+/CD45.1+ cells using flow cytometry. Chimeric mice were treated with either placebo or vorinostat (200 mg/kg) for two weeks. The ratio of V617F (CD45.2+) to WT (CD45.1+) progenitors was determined by flow cytometry. (B) Treatment with vorinostat significantly reduced hemaotcrit levels in chimeric mice (n=4). (C) Representative flow cytometry plots on the ratio of V617F (CD45.2+) to WT (CD45.1+) cells in peripheral blood leukocytes before and after treatment with placebo or vorinostat are shown (left). Bar graphs (right) show that the ratio of V617F to WT in Gr-1+ cells was dramatically decreased after vorinostat treatment (n=4). (D) Representative flow cytometry plots on the ratio of V617F (CD45.2+) to WT (CD45.1+) cells in the BM of chimeric mice after treatment with placebo or vorinostat are shown (left). Bar graphs (right) show that the ratio of V617F to WT in Gr-1+ cells was significantly decreased in the BM after vorinostat treatment (n=4). Results shown represent mean ± SEM. Asterisks indicate significant differences by Student’s t-test (* indicates p<0.05; ** indicates p<0.005).
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Figure 7
Efficacy of vorinostat in a murine model of polycythemia vera

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