Cotreatment with panobinostat and JAK2 inhibitor TG101209 attenuates JAK2V617F levels and signaling and exerts synergistic cytotoxic effects against human myeloproliferative neoplastic cells

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The mutant JAK2V617F tyrosine kinase (TK) is present in the majority of patients with BCR-ABL–negative myeloproliferative neoplasms (MPNs). JAK2V617F activates downstream signaling through the signal transducers and activators of transcription (STAT), RAS/mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3 (PI3)/AKT pathways, conferring proliferative and survival advantages in the MPN hematopoietic progenitor cells (HPCs). Treatment with the pan-histone deacetylase (HDAC) inhibitor panobinostat (PS) is known to inhibit the chaperone function of heat shock protein 90, as well as induce growth arrest and apoptosis of transformed HPCs. Here, we demonstrate that PS treatment depletes the autophosphorylation, expression, and downstream signaling of JAK2V617F. Treatment with PS also disrupted the chaperone association of JAK2V617F with hsp90, promoting proteasomal degradation of JAK2V617F. PS also induced apoptosis of the cultured JAK2V617F-expressing human erythroleukemia HEL92.1.7 and Ba/F3-JAK2V617F cells. Treatment with the JAK2 TK inhibitor TG101209 attenuated JAK2V617F autophosphorylation and induced apoptosis of HEL92.1.7 and Ba/F3-JAK2V617F cells. Cotreatment with PS and TG101209 further depleted JAK/STAT signaling and synergistically induced apoptosis of HEL92.1.7 and Ba/F3-JAK2V617F cells. Cotreatment with TG101209 and PS exerted greater cytotoxicity against primary CD34+ MPN cells than normal CD34+ HPCs. These in vitro findings suggest combination therapy with HDAC and JAK2V617F inhibitors is of potential value for the treatment of JAK2V617F-positive MPN. (Blood. 2009;114:5024-5033)

Introduction

Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) are a group of clonal hematopoietic disorders that includes polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF).[1,2] Recent studies have confirmed the pathogenetic involvement of an acquired, somatic, gain-of-function, activating, point mutation JAK2V617F in MPNs.[3-6] This represents a guanine to thymidine mutation in exon 14 resulting in a valine to phenylalanine substitution at codon 617 in the JH2 or pseudokinase domain of the JAK2 gene (a member of the Janus kinase [JAK] family of nonreceptor tyrosine kinases, JAK1, JAK2, JAK3, and TYK2).[2,6] Highly sensitive assays for JAK2 have determined that the JAK2V617F mutation is present in 90% of patients with PV and approximately 50% to 60% of patients with ET or PMF.[7] In addition, a subset of patients, most commonly with PV, are homozygous for the JAK2V617F allele, the result of copy-neutral loss of heterozygosity at the JAK2 locus, especially in patients with PV.[2,7,8] Mutations in exon 12 of JAK2 are present in almost all patients with PV who are JAK2V617F negative.[9,10] The JAK proteins function in the cytoplasm to relay signals initiated by membrane-bound cytokine receptors. Engagement of the receptor results in the phosphorylation of the receptor and JAK2, which recruits its substrate proteins such as signal transducers and activators of transcription (STATs).[11,12] STATs, especially STAT3 and STAT5, translocate to the nucleus and transactivate many genes involved in cell proliferation and survival (eg, Bcl-xL, cyclin D1, and PIM1).[8,11,12] The V617F mutation in JAK2 also activates the downstream signaling pathways through the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK). This contributes to diminished apoptosis of the hematopoietic progenitor cells (HPCs).[2,8] Overexpression of JAK2V617F in murine Ba/F3 cells with coexpression of the erythropoietin receptor (EpoR) confers in vitro cytokine-independent growth.[3,13] Recently, it was shown that enforced expression of JAK2V617F in human hematopoietic stem cells and myeloid progenitors directed differentiation toward the erythroid lineage, along with increased expression and phosphorylation of GATA-1 and decreased expression of PU.1.[14-16] JAK2V617F expression in retroviral models and in transgenic mice is sufficient to cause myeloproliferative disorders in the mice that recapitulate many clinicopathologic features observed in human PV, ET, and PMF.[17-21] Therefore, the mutant JAK2 represents an excellent target for therapeutic intervention in MPNs. Several orally bioavailable, small molecule, ATP-competitive, JAK2-selective inhibitors have been tested in preclinical studies and are undergoing clinical testing in MPNs.[22] Preclinical

studies have shown that treatment with JAK2-selective kinase inhibitors (eg, TG101209 [TG] and TG101348) attenuate phosphorylated (p)–JAK2 levels, as well as inhibit JAK2-induced p-STAT5, p-STAT3, p-AKT, and p-ERK1/2 levels in cultured and primary human MPN cells with JAK2V617F mutation.[22,23] In vivo studies in mouse models have also shown that mutant JAK2V617F represents a novel target for therapeutic intervention with JAK2-selective tyrosine kinase inhibitors in MPNs.[21,24] For example, TG101348 inhibits myeloproliferation and myelofibrosis in a murine model of JAK2V617F-induced polycythemia.[21,22] Early clinical trials of several JAK2-selective kinase inhibitors (eg, XL019, TG101348, and INCB18424) are under way in JAK2-driven MPNs with poor prognosis (eg, PMF).[22,25] Preliminary results suggest that selective JAK2 inhibitors are relatively well tolerated, ameliorate constitutional symptoms including pruritus and fatigue, and reduce splenomegaly, but have so far not shown the ability to reverse myelofibrosis or to eradicate the JAK2V617F mutant clone.[2,23,25] Short of allogeneic stem cell transplantation, curative therapies that confer a survival benefit are not available, thereby creating a need for better therapies for myelofibrosis (MF)–MPN.[26]

Panobinostat (PS; LBH589) is a cinnamic acid hydroxamate capable of inhibiting class I and class II histone deacetylases (HDACs), thereby inducing the acetylation of both histone and nonhistone proteins.[27-31] Treatment with HDAC inhibitors (HDIs), for example, PS, has been shown to induce cell cycle inhibition, growth arrest, and apoptosis of human leukemia cells, which is correlated with increased expression of p21, p27, and prodeath Bcl-2 family of proteins, as well as concomitant attenuation of p-AKT, c-RAF, and antiapoptotic Bcl-2 family of proteins.[27-32] Importantly, PS exerts a relatively sparing effect on normal bone marrow progenitor cells.[28,29] PS-mediated hyperacetylation and inhibition of the chaperone function of hsp90 directs its client proteins (eg, BCR-ABL, RAF-1, and AKT) to polyubiquitylation and proteasomal degradation.[27-31] Recently, ITF2357 (a class I and II HDI) was shown to selectively down modulate the levels of JAK2V617F protein and its downstream signaling through p-STAT3 and p-STAT5 in human MPN cells.[33] Importantly, ITF2357 also reduced splenomegaly and constitutional symptoms, and induced hematologic responses in some patients with PV/ET.[34] In the present studies, we determined the effects of PS alone and in combination with TG in mouse and human bone marrow cells containing the mutant JAK2V617F. Our findings demonstrate that PS treatment inhibits the expression levels, activity, and downstream progrowth and prosurvival signaling of JAK2V617F. In addition, our findings show that combined treatment with PS and TG induces synergistic apoptosis of HEL92.1.7 (HEL) cells and exhibits superior activity against primary MF-MPN cells.

Methods

Reagents and antibodies

Panobinostat (PS) was kindly provided by Novartis Pharmaceuticals Inc. TG101209 (TG) was kindly provided by TargeGen Inc. Cycloheximide was obtained from Sigma-Aldrich. Anti–p-JAK2 (Tyr1007/1008), anti-JAK2, anti-pSTAT3 (Tyr705), anti-pSTAT3 (Ser727), anti-STAT3 anti-pAKT (Ser473), anti-AKT, and polyclonal GATA-1 were obtained from Cell Signaling Technology. Monoclonal anti-pSTAT5 (Tyr694), monoclonal anti–c-RAF, and Bcl-x_L were obtained from BD Transduction Labs. Polyclonal anti-STAT5 was obtained from Santa Cruz Biotechnologies. Polyclonal anti–pGATA-1(Ser310) was obtained from Novus Biologicals. Rat monoclonal anti-hsp90 antibody was obtained from StressGen Biotech-

nologies. Polyclonal anti-pERK1/2 and anti-ERK1/2 were obtained from Invitrogen Life Sciences. Acetylated K69 hsp90 antibody was generated as previously described.[31] Monoclonal anti–β-actin was obtained from Sigma-Aldrich.

Cell lines and cell culture

HEL 92.1.7 (HEL) and murine Ba/F3-EpoR-JAK2V617F, and Ba/F3-EpoR cells were maintained in RPMI media with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% nonessential amino acids.[11,23] Ba/F3-hEpoR cells were supplemented with 10% WEHI preconditioned media. Logarithmically growing cells were exposed to the designated concentrations of TG101209 and/or panobinostat. After these treatments, cells or cell pellets were washed free of the drug(s) before performing the studies.

Primary MF-MPN cells

Primary peripheral blood and/or bone marrow aspirate MF-MPN samples were obtained with informed consent according to the Declaration of Helsinki from patients with high-risk MF ($\geq$ 3, according to the International Prognostic Scoring System [IPSS]).[1,35] This was sanctioned by a clinical protocol approved by the Institutional Review Board of the Medical College of Georgia. The samples were collected in heparinized tubes, and mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation (StemCell Technologies), washed once and resuspended in complete RPMI-1640, and counted to determine the number of cells isolated before their use in the experiments.[28] Banked, delinked, and deidentified donor peripheral blood CD34+ mononuclear cells procured from recipients who had since died and primary MF-MPN cells were purified by immunomagnetic beads conjugated with anti-CD34 antibody before use in the cell viability assay (StemCell Technologies). Human Primitive Hematopoietic Progenitor Cell Enrichment Kit (StemCell Technologies) was used to obtain stem cells. Briefly, the mononuclear fraction from MF-MPN was incubated with StemSep Enrichment Cocktail (containing monoclonal antibodies to lineage markers such as CD2, CD3, CD14, CD16, CD19, CD24, CD36, CD38, CD45RA, CD56, CD66b, and glycophorin A and conjugated to antidextran antibodies) and incubated for 15 minutes at room temperature. After this, the mixture was incubated with magnetic colloid, mixed, and further incubated for 15 minutes at room temperature. The mixture was loaded onto a primed column and placed inside a magnet to eliminate all cells that expressed markers in the Enrichment Cocktail. CD34+CD38−Lin− cells were obtained from the column flow through during column washing with 2% fetal bovine serum containing phosphate-buffered saline (PBS). CD34+CD38− stem cells were immunophenotyped by staining with CD34-phycoerythrin and CD38–fluorescein isothiocyanate then analyzed by flow cytometry for the percentage enrichment and purity using a FACSCalibur flow cytometer (BD Biosciences).

RNA isolation and quantitative polymerase chain reaction

Untreated or drug-treated HEL cells were centrifuged to pellet the cells. Total RNA was extracted using an RNAqueous RNA kit (Ambion) according to the manufacturer's protocol. RNA (2 μg) was reverse transcribed using a first-strand synthesis kit from Invitrogen. The resulting cDNAs were mixed with 10 μL of 2 × TaqMan Universal Master mix (Applied Biosystems) and TaqMan probes (containing primers and 5 pmol of 5-carboxyfluorescein fluorescently labeled probe) for the exon 8 to 9 and exon 23 to 24 boundary of JAK2 (Applied Biosystems). All samples and loading controls were plated in triplicate, centrifuged briefly, and then loaded onto a StepOne 9600 Real Time PCR system (Applied Biosystems). Amplifications were performed using the following PCR cycling conditions. Samples were heated to 95°C for 10 minutes, then 40 cycles of denaturation at 92°C (15 seconds) and annealing and extension step at 60°C (1 minute). Amplified products were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Assessment of apoptosis by annexin-V staining

Untreated or drug-treated cells were stained with annexin-V (Pharmingen) and propidium iodide and the percentage of apoptotic cells was determined
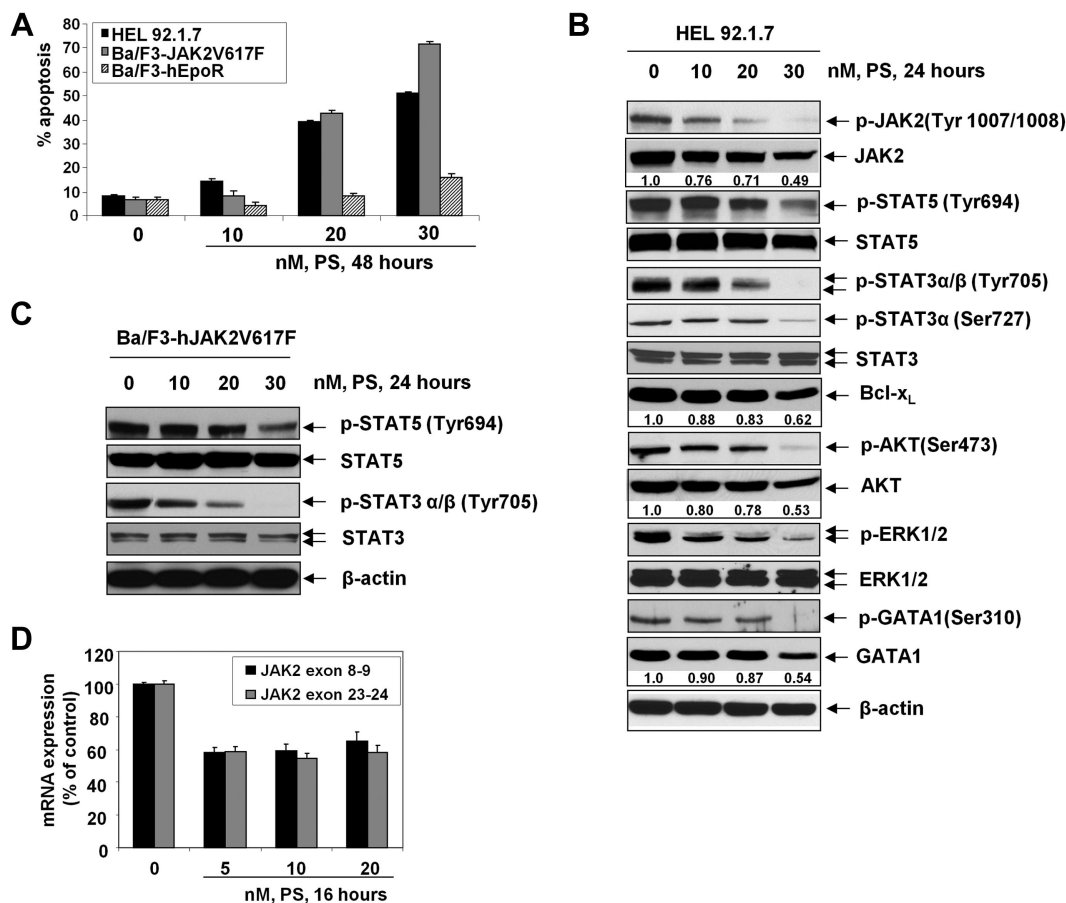
Figure 1. Treatment with panobinostat inhibits JAK2 mRNA expression and JAK/STAT signaling and induces apoptosis of myeloproliferative disorder (MPD) cells and Ba/F3 cells with ectopic overexpression of JAK2V617F. (A) HEL, Ba/F3-JAK2V617F, and Ba/F3-hEpoR cells were treated with the indicated concentrations of panobinostat (PS) for 48 hours. After this, the cells were stained with annexin-V and propidium iodide and the percentages of apoptotic cells were determined by flow cytometry. Columns represent mean of 3 independent experiments; bars represent SEM. (B) HEL cells were treated with the indicated concentrations of PS for 24 hours. Then, total cell lysates were prepared and immunoblot analyses were performed for pJAK2 (Tyr1007/1008), JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), pSTAT3 (Ser727), STAT3, Bcl-x$_L$, pAKT (Ser473), AKT, pERK1/2, ERK1/2, pGATA-1 (Ser310), and GATA-1. The expression levels of β-actin in the lysates served as the loading control. (C) Ba/F3-JAK2V617F cells were treated with the indicated concentrations of PS for 24 hours. After treatment, cell lysates were prepared and immunoblot analyses were performed for pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), and STAT3. The expression levels of β-actin in the lysates served as the loading control. (D) HEL cells were treated with the indicated concentrations of PS for 16 hours, then mRNA was isolated and reverse transcribed. Quantitative real-time PCR was performed on the cDNA using TaqMan probes for the exon 8 to 9 boundary and exon 23 to 24 boundary of JAK2. Relative expression of JAK2 mRNA was normalized to GAPDH expression.

by flow cytometry. To analyze synergism between TG101209 (TG) and panobinostat in inducing apoptosis, cells were treated with TG (200-800 nM) and panobinostat (5-20 nM) at a constant ratio of 40:1 for 48 hours. The percentage of apoptotic cells was determined by flow cytometry, as previously described.[27,29] The CI for each drug combination was calculated by median dose effect analyses,[36] using the combination index equation within the commercially available software Calcusyn (Biosoft). CI values of less than 1.0 represent synergism of the 2 drugs in the combination.

Assessment of percentage nonviable cells

After designated treatments, cells were stained with trypan blue (Sigma-Aldrich). The numbers of nonviable cells were determined by counting the cells that showed trypan blue uptake in a hemocytometer, and reported as percentage of untreated control cells.

Determination of JAK2 protein half-life

HEL cells (5×10^6) were left untreated or treated with 5 μg/mL cycloheximide (CHX), 50nM PS, or the combination of PS and cycloheximide for 0, 4, 8, 16, and 24 hours. Cell lysates were prepared and immunoblot analyses were performed for JAK2 and β-actin. Representative immunoblots were used for densitometric analysis to assess the percentage of JAK2 remaining in each treatment group.

Cell lysis and protein quantitation

Untreated or drug-treated cells were centrifuged, and the cell lysates were obtained from cell pellets and incubated on ice for 30 minutes, as previously described.[28] After centrifugation, an aliquot of each cell lysate was diluted 1:10 and protein quantitated using a BCA protein quantitation kit (Pierce), according to the manufacturer's protocol.

Immunoprecipitation of hsp90 and JAK2 and immunoblot analyses

After the designated treatments, immunoprecipitation and immunoblotting of hsp90 were performed as previously described.[30] For the immunoprecipitation of JAK2 from total cell lysates, 500 μg of total cell lysate was used with 0.5 μg of rabbit monoclonal anti-JAK2 antibody (Cell Signaling Technology). Protein A–agarose beads were used to pull down the immunoprecipitates. The beads were washed 4 times in lysis buffer, and then boiled in sodium dodecyl sulfate (SDS) sample buffer before SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblot analyses.

SDS-PAGE and Western blotting

Total cell lysate (100 μg) was used for SDS-PAGE. Western blot analyses of pJAK2 (Tyr1007/1008), JAK2, pSTAT3 (Tyr705), pSTAT3 (Ser727), STAT3, pSTAT5 (Tyr694), STAT5, pAKT (Ser473), AKT, Bcl-x$_L$, pGATA
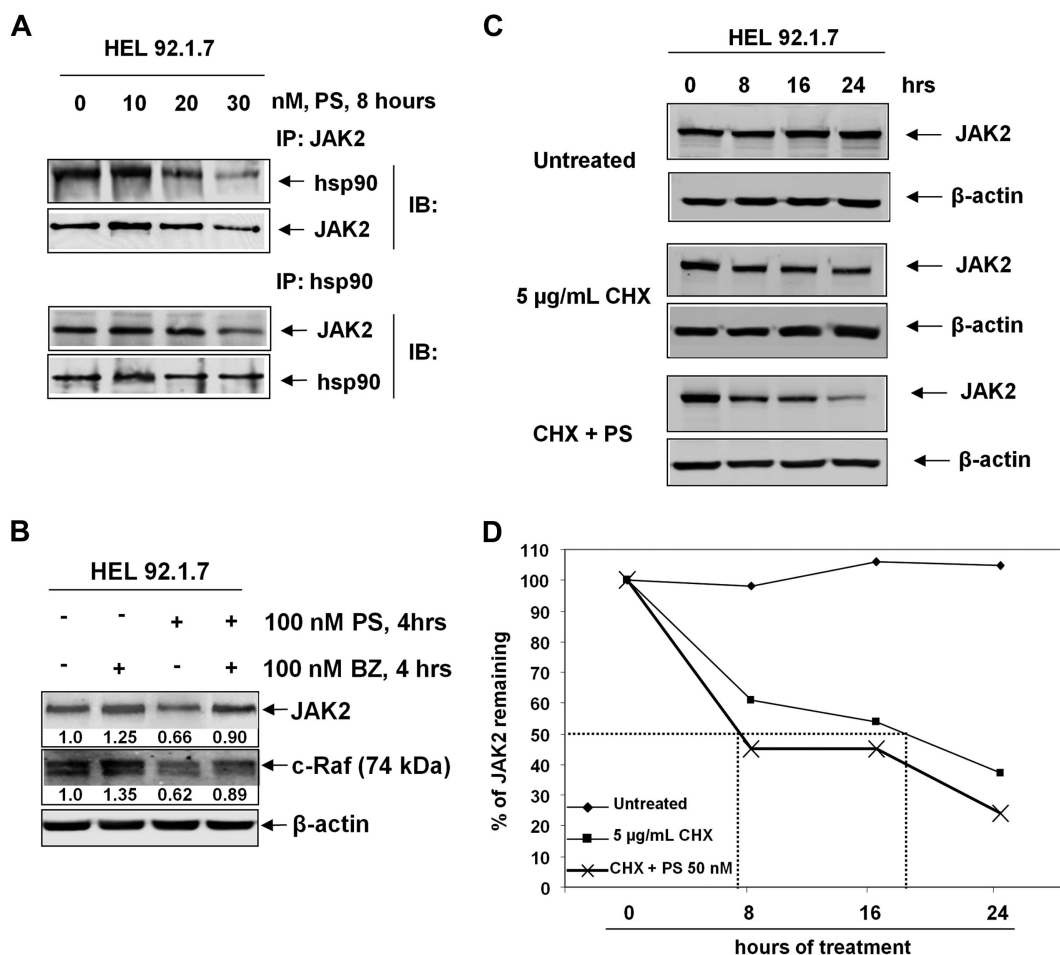
Figure 2. Treatment with PS inhibits JAK2 binding with hsp90, leading to proteasomal degradation of JAK2 in MPD cells. (A) HEL cells were treated with the indicated concentrations of PS for 8 hours. Then, cell lysates were prepared and hsp90 was immunoprecipitated. The immunoprecipitates were separated by SDS-PAGE and immunoblot analyses were performed for JAK2 and hsp90. Alternatively, JAK2 immunoprecipitates were prepared and immunoblot analyses were performed for hsp90 and JAK2. (B) HEL cells were treated with the indicated concentrations of PS and/or bortezomib (BZ) for 4 hours. After treatment, cell lysates were prepared and immunoblot analyses were performed for JAK2 and c-Raf (RAF1). The expression levels of β-actin in the lysates served as the loading control. (C) HEL92.1.7 cells were treated with the indicated concentrations of cycloheximide (CHX) and PS for 0, 8, 16, and 24 hours. Cell lysates were prepared and immunoblot analyses were performed for JAK2. The expression levels of β-actin in the lysates served as the loading control. (D) Graphic representation of data presented in panel C.

(Ser310), GATA, pERK1/2, and ERK1/2 were performed on total cell lysates using specific antisera or monoclonal antibodies, as previously described.[27-31] The expression level of β-actin was used as the loading control for the Western blots. Blots were developed with a chemiluminescent substrate enhanced chemiluminescence (Amersham Biosciences).

Statistical analysis

Significant differences between values obtained in a population of leukemic cells treated with different experimental conditions were determined using the Student t test. P values of less than .05 were assigned significance.

Results

PS inhibits JAK2V617F expression and signaling and induces apoptosis of mouse and human HPCs expressing JAK2V617F

We first determined the effects of clinically achievable concentrations of PS on the viability of the cultured human erythroleukemia HEL cells and the mouse pro-B Ba/F3-hEpoR and Ba/F3-hEpoR-JAK2V617F cells with or without the ectopic expression of JAK2V617F.[23] As demonstrated in Figure 1A, treatment with panobinostat (10-30 nM) induced apoptosis of HEL and Ba/F3-

JAK2V617F cells in a dose-dependent manner. Conversely, panobinostat exerted significantly fewer cytotoxic effects against Ba/F3-hEpoR cells without the expression of JAK2V617F (Figure 1A). We next determined the effects of PS on the expression and signaling downstream of JAK2V617F in HEL cells. Treatment with PS inhibited the autophosphorylation and levels of JAK2V617F in a dose-dependent manner with near-complete loss of JAK2 phosphorylation at Tyr1007/1008, after exposure to 30 nM of PS (Figure 1B). Inhibition of JAK2 activity by PS was associated with inhibition of p-STAT3 and p-STAT5, with greater reduction in STAT3 than STAT5 levels, respectively (Figure 1B). Treatment with PS also reduced the levels of p-GATA1, p-AKT, and p-ERK1/2, with concomitant decline in the levels of GATA1 and AKT but not ERK1/2 (Figure 1B). Inhibition of STAT-5 signaling was associated with decline in the levels of Bcl-xL, as has also been previously reported (Figure 1B).[26] Caspase-3 activity leading to apoptosis, as induced by PS, is also known to cause processing of Bcl-xL, which likely contributed to the decline in the levels of Bcl-xL.[37] Treatment with PS induced similar effects on p-JAK2, as well as on levels of p-STAT3 and p-STAT5 in Ba/F3-JAK2V617F cells (Figure 1C and data not shown). In contrast, PS treatment did not result in loss of p-JAK2 or p-STAT5 and p-STAT3 in
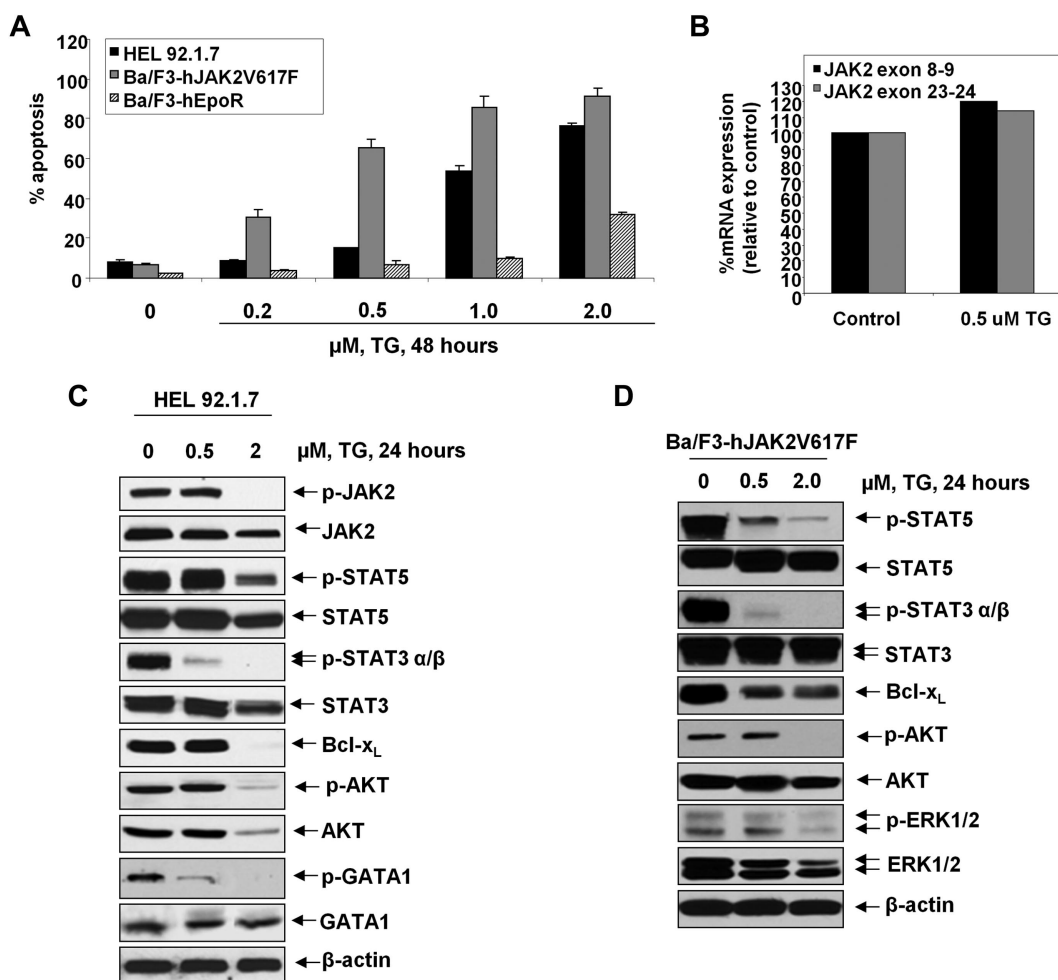
Figure 3. Treatment with TG inhibits JAK2 phosphorylation and downstream signaling and induces apoptosis in HEL and Ba/F3-JAK2V617F cells. (A) HEL, Ba/F3-JAK2V617F, and Ba/F3-hEpoR cells were treated with the indicated concentrations of TG for 48 hours. After treatment, the cells were stained with annexin-V and propidium iodide and the percentages of apoptotic cells were determined by flow cytometry. Columns represent mean of 3 independent experiments; bars represent SEM. (B) HEL cells were treated with the indicated concentration of TG for 16 hours. Total RNA was harvested and reverse transcribed, then used for quantitative real-time polymerase chain reaction for JAK2 expression. Relative expression of JAK2 was normalized to GAPDH. (C) HEL cells were treated with the indicated concentrations of TG for 24 hours. Then, cell lysates were prepared and immunoblot analyses were performed for pJAK2 (Tyr1007/1008), JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), STAT3, Bcl-x$_L$, pAKT (Ser473), AKT, pERK1/2, ERK1/2, pGATA-1 (Ser310), and GATA-1. The expression levels of β-actin in the lysates served as the loading control. (D) Ba/F3-JAK2V617F cells were treated with the indicated concentrations of TG for 24 hours. After treatment, cell lysates were prepared and immunoblot analyses were performed for pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), STAT3, Bcl-x$_L$, pAKT (Ser473), AKT, pERK1/2, and ERK1/2. The expression levels of β-actin in the lysates served as the loading control.

Ba/F3-hEpoR cells (supplemental Figure 1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). We next determined whether PS treatment also inhibited the mRNA expression of JAK2. As shown in Figure 1D, treatment with even a low level of PS (5 nM) resulted in approximately 40% depletion of the mRNA expression of JAK2 as determined by the real-time polymerase chain reaction with 2 independent primer/probe sets located at different exon boundaries within the mRNA. Exposure to higher concentrations of panobinostat did not further reduce the expression of JAK2 mRNA (Figure 1D). We have previously demonstrated that PS treatment induces hyperacetylation and inhibition of the chaperone function of hsp90, resulting in proteasomal degradation of hsp90 client proteins (eg, BCR-ABL, AKT, and RAF1).[30,31] Results of our present studies show that immunoprecipitates of hsp90 and JAK2 showed binding of JAK2 to hsp90 and were partially disrupted by exposure to PS (Figure 2A). Consistent with this, PS treatment promoted proteasomal degradation and partial depletion of JAK2 and RAF1, which was reversed by cotreatment with the proteasome inhibitor bortezomib (Figure 2B). These findings indicate that

PS-mediated depletion of JAK2 levels is due partly to inhibition of mRNA and partly to increased JAK2 protein degradation. We next determined the half-life of the JAK2 protein in HEL cells. Treatment with cycloheximide caused a time-dependent decline in the JAK2 protein levels with a 50% reduction in expression by 18 hours (Figure 2C). Cotreatment with PS and cycloheximide resulted in a more rapid decline in JAK2 expression levels (Figure 2C). The findings demonstrate that treatment with PS shortened the half-life of JAK2 by approximately 60%, from 18 to 7 hours (Figure 2C).

TG inhibits the activity and downstream signaling of JAK2V617F and induces apoptosis of mouse and human HPCs expressing JAK2V617F

We next determined the effects of TG in cultured bone marrow progenitor cells expressing JAK2V617F. Treatment with TG (0.2-2 μM) dose-dependently induced apoptosis of HEL cells (Figure 3A). TG also induced significantly more apoptosis of Ba/F3-JAK2V617F versus Ba/F3-hEpoR cells (Figure 3A). We

Figure 4. Cotreatment with PS enhances TG-mediated inhibition of JAK2 downstream signaling and induction of apoptosis in Ba/F3 cells with ectopic overexpression of JAK2V617F. (A) Ba/F3-JAK2V617F and Ba/F3-hEpoR cells were treated with the indicated concentrations of TG and/or PS for 48 hours. After treatment, the cells were washed with $1 \times$ PBS and stained with annexin-V and propidium iodide, and the percentages of apoptotic cells were determined by flow cytometry. Columns represent mean of 3 independent experiments; bars represent SEM. (B-C) Ba/F3-JAK2V617F and Ba/F3-hEpoR cells were treated with the indicated concentrations of TG and/or PS for 24 hours. After this, cell lysates were prepared and immunoblot analyses were performed for pJAK2 (Tyr1007/1008), JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), STAT3, pAKT (Ser473), and AKT. The expression levels of β-actin in the lysates served as the loading control. A vertical line has been inserted to indicate a repositioned gel lane.
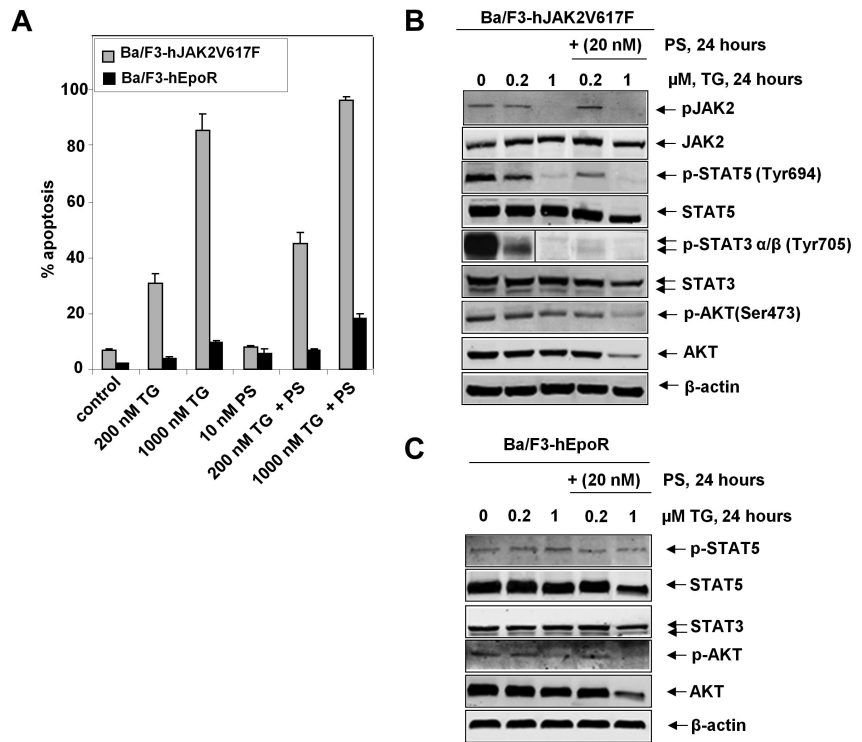
next determined the effect of TG on JAK2 expression and signaling. Unlike panobinostat, treatment with TG did not significantly alter the mRNA expression of JAK2 in HEL cells (Figure 3B). Although treatment with TG inhibited p-JAK2, p-STAT3, and p-STAT5, significant attenuation of p-JAK2, JAK2, STAT3, and STAT5 levels was observed in HEL cells only after exposure to 2.0 μM of TG. Treatment with TG also reduced Bcl-x_L, p-AKT, and p-GATA1 levels in HEL cells, correlating with TG-induced apoptosis of HEL cells (Figure 3C). Similar to the observations in HEL cells, TG also inhibited the downstream signaling due to JAK2V617F in the Ba/F3 cells (Figures 3D and 4B). Treatment with TG markedly depleted p-JAK2, p-STAT3, p-STAT5, and Bcl-x_L levels, without significantly depleting JAK2V617F, STAT3, and STAT5 levels. In addition, TG treatment attenuated p-AKT and p-GATA1 levels in Ba/F3-JAK2V617F cells. We also determined the effects of TG in Ba/F3-hEpoR cells. Treatment with TG had minimal effects on p-JAK2, p-STAT5, and p-AKT levels in the Ba/F3-hEpoR cells. However, TG treatment significantly inhibited p-STAT3 in the Ba/F3-hEpoR cells (supplemental Figure 1B).

Cotreatment with TG and PS causes greater inhibition of JAK/STAT activity and induces synergistic apoptosis of mouse and human HPCs expressing JAK2V617F

We next determined the effects of cotreatment with TG and PS in Ba/F3-JAK2V617F and HEL cells. Figure 4A demonstrates that cotreatment with TG (200 or 1000 nM) and PS (10 nM) induced more apoptosis of Ba/F3-JAK2V617F cells than either agent alone. Both TG and/or PS induced more apoptosis of Ba/F3-JAK2V617F versus Ba/F3-hEpoR cells (Figure 4A). Immunoblot analyses after treatment with TG or TG plus PS (20 nM) demonstrated that cotreatment with TG and PS caused greater depletion of p-STAT5 and p-AKT than TG alone in Ba/F3-JAK2V617F cells (Figure 4B). A similar effect was also observed against p-STAT3 (data not shown). Combined treatment with TG and PS also decreased the levels of STAT5 and AKT, especially at higher dose levels of TG

(Figure 4B). In contrast, cotreatment with TG and PS exerted similar but less pronounced effects than TG alone on p-STAT5, p-AKT, and p-STAT3 (not shown) in Ba/F3-hEpoR cells (Figure 4C). Cotreatment with 10 nM of PS also significantly increased apoptosis of HEL cells induced by 500 or 1000 nM of TG ($P < .05$; Figure 5A). Importantly, the combined treatment with PS and TG synergistically induced apoptosis of HEL cells, as determined by median dose effect analysis of Chou and Talalay (Figure 5B). CI values for the drug combinations were less than 1.0, indicating a synergistic interaction at concentrations that were below the median inhibitory concentration values for PS (5-20 nM) and TG (200-800 nM; Figure 5B). Consistent with these observations, cotreatment with 20 nM of PS markedly increased TG (0.2 or 1.0 μM)–mediated attenuation of p-JAK2, JAK2, p-STAT5, p-STAT3, p-AKT, and p-GATA1 levels in HEL cells (Figure 5C), compared with treatment with TG or PS alone (Figure 1B).

PS and TG inhibit JAK/STAT signaling and exert greater anti–MF-MPN effects than either agent alone in primary MF-MPN HPCs expressing JAK2V617F

We next determined the effects of PS and/or TG on the viability of primary CD34^{+} MF-MPN cells from patients with MF due to JAK2V617F and normal CD34^{+} HPCs. Treatment with TG caused a dose-dependent increase in the loss of viability of CD34^{+} primary MF-MPN cells (Figure 6A). A similar effect was also observed with PS treatment alone (data not shown), with approximately 50% of the cells determined to be nonviable after treatment with 20 nM of PS (Figure 6A). Cotreatment with 20 nM of PS significantly enhanced TG-induced cell death of CD34^{+} primary MF-MPN cells. In contrast, treatment with PS alone or cotreatment with PS and TG induced significantly less cell death in normal human CD34^{+} HPCs, compared with CD34^{+} primary MF-MPN cells (Figure 6A). In a representative sample yielding adequate numbers of CD34^{+} primary MF-MPN cells for immunoblot analysis, treatment with PS dose-dependently depleted JAK2, p-STAT5,
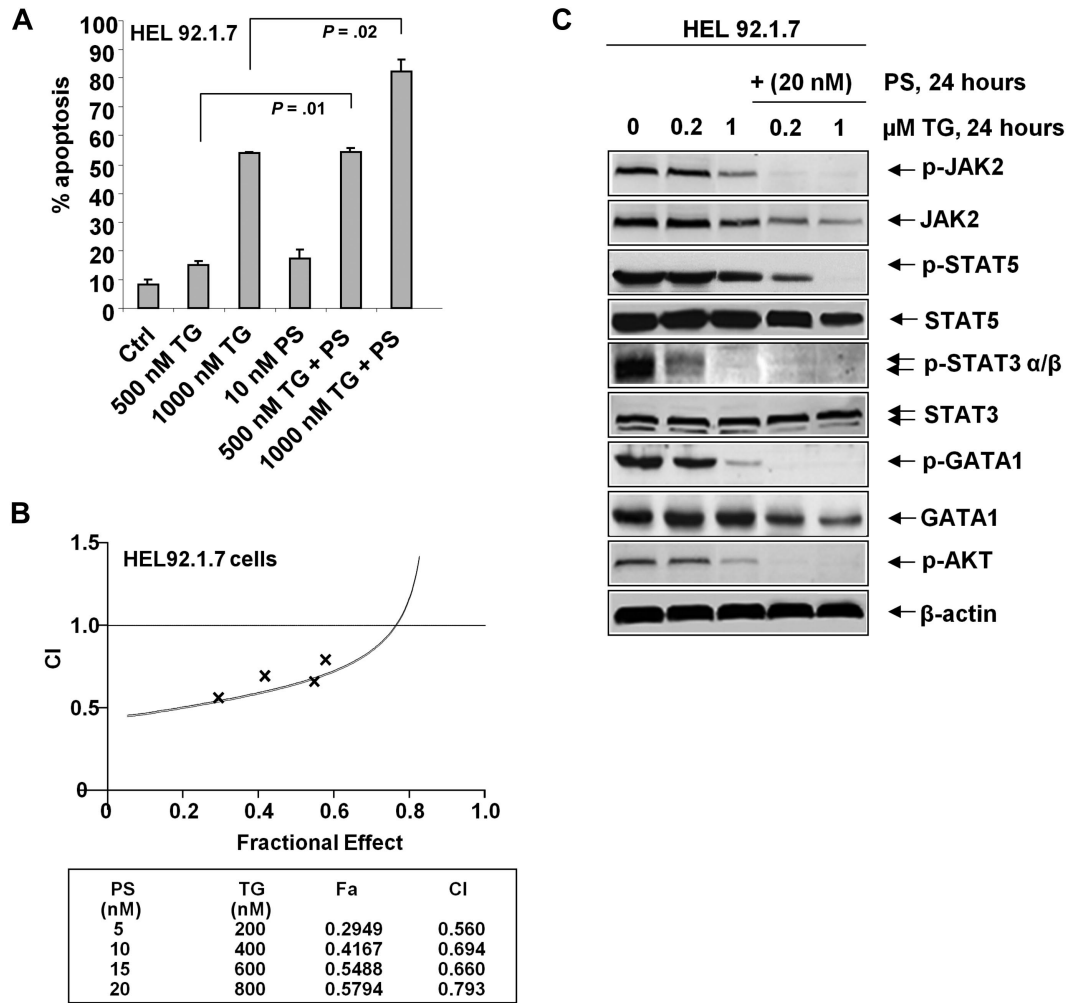
Figure 5. Cotreatment with PS enhances TG-mediated inhibition of JAK2 phosphorylation and downstream signaling and induces synergistic apoptosis of MPD cells. (A) HEL cells were treated with the indicated concentrations of TG and/or PS for 48 hours. Then, the cells were washed with 1 × PBS and stained with annexin-V and propidium iodide, and the percentages of apoptotic cells were determined by flow cytometry. Columns represent mean of 3 independent experiments; bars represent SEM. (B) HEL cells were treated with TG (200-800 nM) and PS (5-20 nM) for 48 hours. Apoptosis was determined by annexin-V staining and flow cytometry. Median dose effect and combination indices were obtained using Calcusyn software. CI values less than 1.0 indicate synergism of the 2 agents. (C) HEL cells were treated with the indicated concentrations of TG and/or PS for 24 hours. After treatment, cell lysates were prepared and immunoblot analyses were performed for pJAK2 (Tyr1007/1008), JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), STAT3, pGATA-1 (Ser310), and GATA-1 and pAKT (Ser473). The expression levels of β-actin in the lysates served as the loading control.

p-STAT3, p-ERK1/2, and p-AKT levels, without significantly affecting STAT5, STAT3, AKT, and ERK1/2 levels (Figure 6B). As previously reported for human breast cancer cells, treatment with PS also induced hyperacetylation of lysine (K) 69 on hsp90 in the primary CD34$^+$ MF-MPN cells (Figure 6B). In the same sample of primary CD34$^+$ MF-MPN cells, cotreatment with TG and PS resulted in greater inhibition of STAT5 and STAT3 phosphorylation than either agent alone, as determined by immunoblot analysis of total cell lysates (Figure 6C). CD34$^+$CD38$^-$Lin$^-$ cells from 3 MF-MPN patients were also treated with the indicated concentrations of TG101209 (TG) and/or panobinostat (PS) for 48 hours (Figure 6D). After treatment, the percentages of nonviable cells were determined by trypan blue dye uptake in a hemocytometer. As shown in Figure 6D, treatment with 20 nM of PS induced more cell death of MF-MPN stem cells than treatment with either 200 or 1000 nM of TG. In addition, cotreatment with PS and TG also induced significantly more cell death of bone marrow stem cells than either agent alone ($P < .05$). These findings demonstrate that combined treatment with PS and TG would also be effective in exerting lethal action against bone marrow–derived stem cells with mutant JAK2V617F expression.

Discussion

In the present studies, we demonstrate that treatment with PS inhibits the autophosphorylation and expression of JAK2V617F, as well as its downstream signaling in the cultured mouse Ba/F3, human erythroleukemia HEL cells, and primary MF-MPN HPCs. Although our studies neither identified the transcription factor involved nor elucidated the mechanism by which the transcription of JAK2V617F is affected, it is clear that PS depleted the mRNA levels as well as promoted the proteasomal degradation of JAK2V617F, which together contributed to the overall decline in the levels of JAK2V617F and its downstream signaling. These findings are consistent with the previously reported observation in which PS and other pan-HDAC inhibitors were shown to deplete BCR-ABL and FLT-3 levels in human leukemia cells both by transcriptional and posttranscriptional mechanisms.[27,29-31] Because these agents also inhibit HDAC6, pan-HDAC inhibitors such as PS induce hyperacetylation of hsp90, thereby inhibiting its chaperone function and promoting the polyubiquitylation and proteasomal
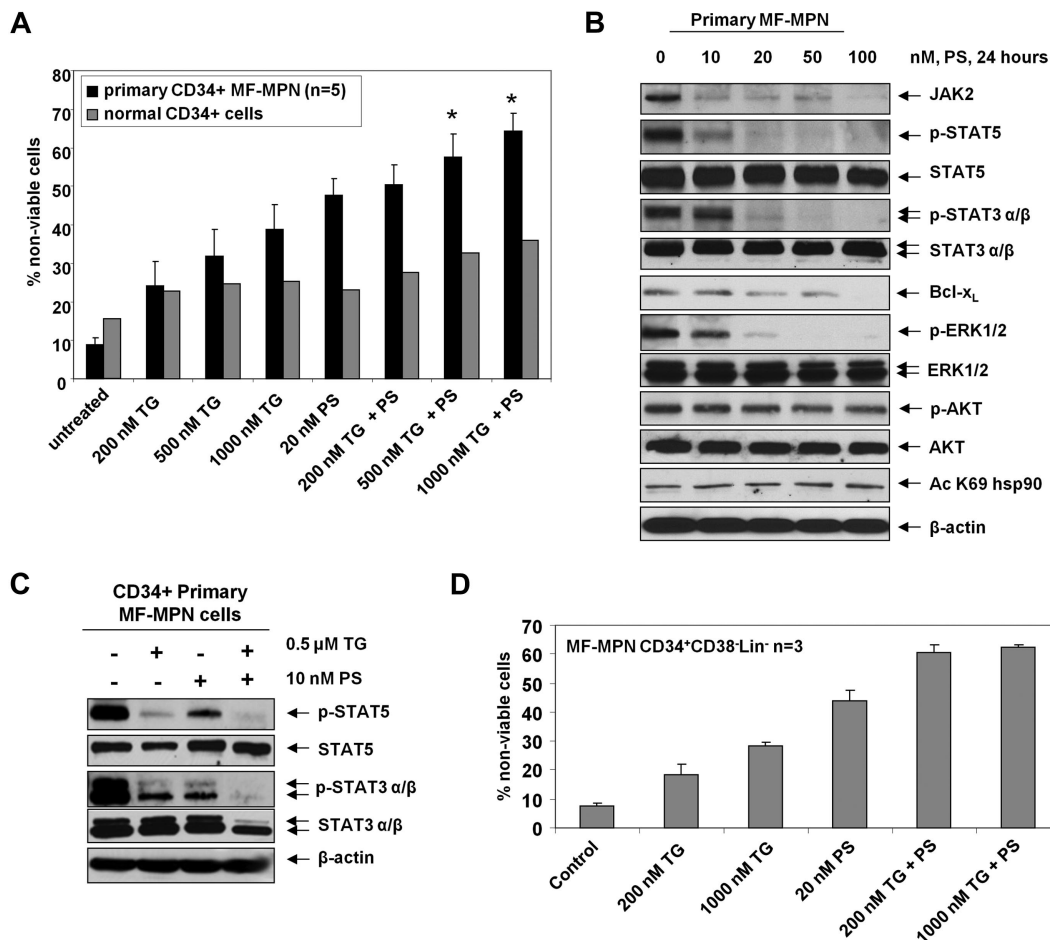
Figure 6. Cotreatment with PS and/or TG exerts greater down-regulation of JAK2 and downstream signaling, as well as greater cytotoxicity than either agent alone in primary MF-MPN cells. (A) CD34+ progenitor cells isolated from primary MF-MPN peripheral blood samples and normal CD34+ bone marrow progenitor cells were treated with the indicated concentrations of TG and/or panobinostat for 48 hours. After treatment, the percentages of nonviable cells were determined by trypan blue dye uptake in a hemocytometer. * indicates values significantly greater ($P < .05$) than treatment with either agent alone. (B) Primary MF-MPN cells were treated with the indicated concentrations of PS for 24 hours. Cell lysates were prepared and immunoblot analyses were performed for JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), STAT3, Bcl-x_L, pAKT (Ser473), AKT, pERK1/2, ERK1/2, and acetylated K69 hsp90. The expression levels of β-actin in the lysates served as the loading control. (C) Primary MF-MPN cells were treated with the indicated concentrations of TG and/or panobinostat for 24 hours. Then, cell lysates were prepared and immunoblot analyses were performed for JAK2, pSTAT5 (Tyr694), STAT5, pSTAT3 (Tyr705), and STAT3. The expression levels of β-actin in the lysates served as the loading control. (D) Primary CD34+CD38−Lin− stem cells from MF-MPN patients were treated with the indicated concentrations of TG and PS for 48 hours. After treatment, the percentages of nonviable cells were determined by trypan blue dye uptake in a hemocytometer.

degradation of hsp90 client proteins, including BCR-ABL, FLT-3, AKT, RAF1, and CDK4.[27,31] The PS-mediated down-regulation of JAK2 protein is relatively modest, even though PS inhibits transcription of JAK2 and promotes its degradation by the proteasome. This is mainly due to the relatively long half-life (~18 hours) of the protein. Disruption of JAK2V617F binding to hsp90 due to PS treatment and restoration of the levels of JAK2V617F by cotreatment with bortezomib support the conclusion that JAK2V617F is also an hsp90 client protein. This was also supported by the observations that the geldenamycin analog hsp90 inhibitor or AUY922 also partially deplete JAK2V617F in the MF-MPN HPCs (data not shown). It is also increasingly being recognized that the mutant oncoprotein kinases are more dependent on the chaperone association with hsp90 than their unmutated counterparts, for example as noted for BCR-ABL, FLT-3, EGFR, and B-RAF.[27,29,38,39] Consequently, treatment with pan-HDAC or hsp90 inhibitors has been shown to be more effective in depleting the mutant versus the unmutated forms of these oncoprotein kinases. Our finding that PS treatment depletes JAK2V617F more than unmutated JAK2, as observed in Ba/F3-hEpoR cells, is consistent with these reports. Treatment with PS was also noted to

inhibit JAK2V617F-mediated downstream signaling, as highlighted by PS-mediated depletion of the levels of p-STAT5, p-AKT, p-GATA1, and pERK1/2. This may be partly due to not only the direct inhibitory effects of PS on JAK2V617F, but also to the known PS-mediated depletion of p-AKT and p-ERK1/2, or through the effects of PS on other upstream signaling kinases. It should also be noted that although down-regulation of JAK2 protein by PS is relatively modest, the inhibition of phosphorylation of JAK2 and its downstream targets (eg, STAT3 and STAT5, AKT, and ERK1/2) is more prominent. There are 2 potential reasons for this finding. One could be a technical reason based on the relative difference in the epitope detection by the specific antibodies for the unphosphorylated versus phosphorylated forms of the protein. The other, more likely reason could be that panobinostat (PS)–induced misfolding of JAK2 could have a greater, and earlier, affect on the detection of the phosphorylated epitope than the lowering of JAK2 by proteasomal degradation, which ensues later and is regulated by other factors. Regardless, the likely net effect of PS was to attenuate the progrowth and prosurvival signaling more in HEL and Ba/F3-JAK2V617F than in Ba/F3-hEpoR cells. In addition, because HEL and Ba/F3-JAK2V617F cells are more dependent on this signaling,

PS also induced significantly more apoptosis of HEL and Ba/F3-JAK2V617F than of Ba/F3-hEpoR and normal CD34$^+$ human HPCs. A similarly selective effect of the panhistone deacetylase inhibitor ITF2357 was also reported against JAK2V617F and HEL cells.[33] Importantly, in the present studies the in vitro inhibitory effects of PS on p-JAK2V617F, p-STAT3, and p-STAT5 were also confirmed in patient-derived CD34$^+$ MF-MPN cells.

Our findings also demonstrate that, compared with treatment with either agent alone, combined treatment with PS and TG is more effective in attenuating not only the mutant JAK2V617F, p-STAT3, and p-STAT5 but also p-AKT and p-GATA1 levels, especially when PS was combined with lower levels of TG (200 nM). This was associated with a significant increase in apoptosis, which suggests that down-regulation of multiple survival mechanisms contributes to the lethal effects of the combination in cells that have endogenous expression of JAK2V617F. Combined treatment also induces significantly more apoptosis of HEL, Ba/F3-JAK2V617F, and primary CD34$^+$ MF-MPN cells than of Ba/F3-hEpoR and CD34$^+$ normal human HPCs with unmutated JAK2. This observation mimics what was also noted when combination of pan-HDAC inhibitors such as PS or vorinostat, or the hsp90 inhibitor 17-AAG, was used with BCR-ABL or FLT-3 TK inhibitor in chronic myeloid leukemia (CML) and acute myeloid leukemia cells that expressed the mutant forms of BCR-ABL or FLT-3.[27,29] Previous reports have described the individual activity of TG101209 (TG) and TG101348 against JAK2V617F-expressing human MPN cells and Ba/F3 cells.[21-24] Based on the more pronounced inhibitory effects of the combination of PS and TG on the levels and signaling downstream of JAK2V617F, there is clearly the potential for accruing additional in vivo therapeutic advantages due to treatment with the combination versus treatment with JAK2 TK inhibitors alone. High level of expression and deregulated activity of JAK2V617F in HPCs can stimulate homologous recombination, genomic instability, and increased centrosome and ploidy abnormalities.[40] High intracellular levels of reactive oxygen species that may contribute to genomic instability and disease progression have also been noted.[41,42] In this context, it is noteworthy that compared with treatment with each of the agents alone combined treatment with PS and TG exhibited higher lethal activity against the CD34$^+$, CD38$^-$, Lin$^-$ primary MF-MPN stem cells with mutant JAK2V617F. Although not directly investigated here, it is also likely that PS-mediated anti-hsp90 activity and superior activity of the combination of PS and TG would exert greater anti-PIM/BAD/Bcl-xL effect downstream of JAK2V617F, since PIM kinase is also known to be an hsp90 client protein.[19,43-45] This would also attenuate the resulting PIM-mediated survival signaling and MYC function in MF-MPN cells.[19,44,45] It is also important to note that anti-hsp90 effects of cotreatment with PS and TG may retard the emergence of any other mutant JAK2 clones that could potentially

confer resistance against treatment with a JAK2 TK inhibitor alone, as has been observed with BCR-ABL and FLT-3 TK inhibitors in CML and acute myeloid leukemia cells.[46-48]

Early clinical trials with TG101348 and other JAK2 TK inhibitors suggest a promising clinical benefit of these agents in patients with MF-MPN.[22,25] However, complete remissions similar to those seen in CML with BCR-ABL TK inhibitors have not as yet been observed. Recently, in a phase 1 clinical trial of PS in a wide spectrum of hematologic malignancies, clinical benefit was observed in patients with MF-MPN[49] (C. Paley, oral communication, March 3, 2009) Based on this, a phase 2 multi-institution clinical trial of PS is being implemented in patients with advanced MF-MPN (C. Paley, oral communication, March 3, 2009). Taken together with the findings presented here, these encouraging developments support the rationale to design and implement future clinical studies of PS and JAK2 TK inhibitors in patients with advanced MF-MPN.

Acknowledgments

Authorship

Contribution: Y.W., W.F., D.G.C., K.M.B., R.R., A.J., R.B., S.K., J.C., and A.S. performed the in vitro studies with the cultured mouse, human, and primary MPN cells; K.N., C.U., and A.P.J. procured and assisted in performing the studies on primary CD34$^+$ MF-MPN and CD34$^+$ normal progenitor cells; P.A. and R.L.L. provided reagents for the study; and K.N.B. planned and supervised the in vitro and in vivo studies and prepared the report.

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