Heat Shock Protein 90 Inhibitor Is Synergistic with JAK2 Inhibitor and Overcomes Resistance to JAK2-TKI in Human Myeloproliferative Neoplasm Cells

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Abstract

Purpose: We determined the activity of hsp90 inhibitor, and/or Janus-activated kinase 2 (JAK2) tyrosine kinase inhibitor (TKI), against JAK2-V617F–expressing cultured mouse (Ba/F3-JAK2-V617F) and human (HEL92.1.7 and UKE-1) or primary human CD34+ myeloproliferative neoplasm (MPN) cells.

Experimental Design: Following exposure to the hsp90 inhibitor AUY922 and/or JAK2-TKI TG101209, the levels of JAK2-V617F, its downstream signaling proteins, as well as apoptosis were determined.

Results: Treatment with AUY922 induced proteasomal degradation and depletion of JAK2-V617F as well as attenuated the signaling proteins downstream of JAK2-V617F, that is, phospho (p)-STAT5, p-AKT, and p-ERK1/2. AUY922 treatment also induced apoptosis of HEL92.1.7, UKE-1, and Ba/F3-hJAK2-V617F cells. Combined treatment with AUY922 and TG101209 caused greater depletion of the signaling proteins than either agent alone and synergistically induced apoptosis of HEL92.1.7 and UKE-1 cells. Cotreatment with AUY922 and TG101209 also induced significantly more apoptosis of human CD34+ MPN than normal hematopoietic progenitor cells. As compared with the sensitive controls, JAK2-TKI–resistant HEL/TGR and UKE-1/TGR cells exhibited significantly higher IC50 values for JAK2-TKI (P < 0.001), which was associated with higher expression of p-JAK2, p-STAT5, p-AKT, and Bcl-xl, but reduced levels of BIM. Unlike the sensitive controls, HEL/TGR and UKE/TGR cells were collaterally sensitive to the hsp90 inhibitors AUY922 and 17-AAG, accompanied by marked reduction in p-JAK2, p-STAT5, p-AKT, and Bcl-xl, with concomitant induction of BIM.

Conclusions: Findings presented here show that cotreatment with hsp90 inhibitor and JAK2-TKI exerts synergistic activity against cultured and primary MPN cells. In addition, treatment with hsp90 inhibitor may overcome resistance to JAK2-TKI in human MPN cells. Clin Cancer Res; 17(23); 7347–58. ©2011 AACR.

Introduction

Philadelphia chromosome–negative myeloproliferative neoplasms (MPN) are a group of clonal hematopoietic disorders that includes polycythemia vera, essential thrombocythemia, and primary myelofibrosis (1, 2). Recent studies have confirmed the pathogenetic involvement of an acquired, somatic, gain-of-function, activating, point mutation Janus-activated kinase (JAK2)-V617F in MPNs (2, 3). JAK2-V617F mutation disrupts the pseudokinase (JH2) domain and abolishes the autoinhibitory functions normally imposed on the JAK2 catalytic domain (JH1) by the pseudokinase JH2 domain (2, 4). This leads to an aberrant and deregulated activation of the kinase (JH1) domain, triggering progrowth and prosurvival signaling downstream of JAK2-V617F mediated by the STAT5 and STAT3, phosphoinositide-3-kinase (PI3K), and extracellular signal–regulated kinase (ERK; refs. 2, 5). JAK2-V617F mutation is present in 90% of patients with polycythemia vera and approximately 50% to 60% of patients with essential thrombocythemia or primary myelofibrosis (1, 2). In addition, mutations in exon 12 of JAK2 are present in almost all patients with polycythemia vera who are JAK2-V617F negative (6). Presence of JAK2-V617F in the various mouse models, including the retroviral bone marrow transplantation, transgenic mouse, and the knockin mouse model, has been mechanistically linked to marked polycythemia, hepatosplenomegaly, and myelofibrosis (7–11). In advanced stages, patients with MPN develop progressive bone marrow failure, extramedullary hematopoiesis, splenomegaly, and/or transformation to acute myelogenous leukemia.
Translational Relevance

The mutant JAK2-V617F tyrosine kinase is expressed in the majority of patients with BCR-ABL–negative myeloproliferative neoplasms (MPN). JAK2-V617F activates and confers progrowth and prosurvival downstream signaling through the STAT, RAS/RAF/MAPK, and PI3K/AKT pathways in MPN cells. Although treatment with JAK2 tyrosine kinase inhibitor (TKI) attenuates JAK2-V617F–mediated signaling and induces apoptosis of MPN cells, other novel agents need to be tested against JAK2-TKI–sensitive and JAK2-TKI–resistant MPN cells. Here, we show that the hsp90 inhibitor AUY922 potently depleted JAK2-V617F and inhibited JAK2-V617F–mediated downstream signaling. Cotreatment with TG101209 augmented AUY922-mediated attenuation of JAK2-V617F signaling, which was associated with synergistic in vitro activity of the combination against human MPN cells. These observations support the rationale to further evaluate the in vivo efficacy of cotreatment with AUY922 and TG101209.

Materials and Methods

Reagents and antibodies

AUY922 was kindly provided by Novartis Pharmaceuticals Inc. TG101209 was kindly provided by Sanofi-Aventis. Bortezomib was acquired from Millennium Pharmaceuticals. 17-AAG was obtained from Sigma-Aldrich. Bortezomib was acquired from Millennium Pharmaceuticals. Anti-p-JAK2 (Tyr1007/1008) and anti-p-FOXO3A were obtained from Abcam. Anti-JAK2, anti-p-STAT3 (Ser727), anti-STAT3, anti-p-AKT (Ser473), anti-AKT, anti-FOXO3A, anti-p-ERK1/2, anti-ERK1/2, anti-PARP, and BIM were obtained from Cell Signaling. Monoclonal anti-p-STAT5 (Tyr694), anti-p27, and monoclonal anti-hsp90 and anti-hsp70 antibodies were obtained from Santa Cruz Biotechnologies. Rat monoclonal anti-STAT5, anti-p-27, and monoclonal anti-RAF were obtained from BD Transduction Labs. Polyclonal anti-STAT3, anti-p-AKT, anti-FOXO3A, anti-p-ERK1/2, and monoclonal anti-hsp90 and anti-hsp70 antibodies were obtained from BioGenex Laboratories, Inc. 4,5-diarylisoxazole that binds with high affinity to hsp90 and inhibits its chaperone function, thereby promoting polyubiquitylation and degradation of the misfolded client protein by the 26S proteasome (24, 25). AUY922 has also been presented to show preclinical activity against several tumor models (26, 27). In the present studies, we determined that treatment with AUY922 or 17-AAG depletes JAK2-V617F, inhibits its downstream progrowth and prosurvival signaling, and induces apoptosis of cultured and primary MPN cells. We also determined that combined treatment with AUY922 or 17-AAG and the JAK2-TKI TG101209 exerts synergistic lethal activity against MPN cells including those transformed to AML. In addition, the hsp90 inhibitors exhibited greater activity against JAK2-TKI–resistant than JAK2-TKI–sensitive cultured MPN cells.

Cell lines and cell culture

Human erythroleukemia HEL92.1.7 (HEL) cells with homozygous expression of JAK2-V617F, as well as the murine Ba/F3-EpoR-JAK2-V617F, and Ba/F3-EpoR cells, were maintained in RPMI media with 10% FBS, 1% penicillin/streptomycin, and 1% nonessential amino acids, as

(TG; refs. 1, 11, 12, 19, 20). On the basis of these observations, the mutant JAK2 represents an excellent target for therapeutic intervention in MPNs. Several, orally bioavailable, small-molecule, ATP-competitive, JAK2-selective tyrosine kinase inhibitors (TKI) have been tested in preclinical studies and currently undergoing clinical investigation in patients with MPNs (1, 12). Preclinical studies have shown that treatment with JAK2-TKI, for example, TG101209 (TG) and TG101348 (SAR302503), attenuate p-JAK2 levels, as well as inhibit JAK2-V617F–induced p-STAT5, p-STAT3, p-AKT, and p-ERK1/2 levels in cultured and primary human MPN cells (13–15). In vivo studies in mouse models have also shown that mutant JAK2-V617F represents a novel target for therapeutic intervention with JAK2-TKI in MPNs (7, 16). Clinical trials of several of the JAK2-TKI, for example, TG101348 and INCB18424, have recently been conducted (1, 12, 17, 18). Preliminary results suggest that in the clinic, JAK2-TKI are relatively well-tolerated, ameliorate constitutive symptoms, reduce splenomegaly but neither reverse myelofibrosis nor markedly reduce the allelic burden of JAK2-V617F, have been shown to be even more dependent on the chaperone function of hsp90 than their unmutated counterparts (14, 20, 22, 23). Inhibition of ATP binding and chaperone function of hsp90 by treatment with geldanamycin analogue, for example, 17-AAG, or with AUY922 (non-geldanamycin analogue), disrupts the chaperone association of hsp90 with its client proteins (20, 21). This leads to misfolding, polyubiquitylation, and subsequent degradation of the oncoprotein by the 26S proteasome (20, 21). Many of the unmutated and mutant forms of client proteins, as noted previously, including JAK2-V617F, c-RAF, and AKT, confer progrowth and prosurvival advantage on MPN cells (14, 20, 21). AUY922 is a derivative of 4,5-diarylisoxazole that binds with high affinity to hsp90 and inhibits its chaperone function, thereby promoting polyubiquitylation and degradation of the misfolded client protein by the 26S proteasome (24, 25). AUY922 has also been presented to show preclinical activity against several tumor models (26, 27). In the present studies, we determined that treatment with AUY922 or 17-AAG depletes JAK2-V617F, inhibits its downstream progrowth and prosurvival signaling, and induces apoptosis of cultured and primary MPN cells. We also determined that combined treatment with AUY922 or 17-AAG and the JAK2-TKI TG101209 exerts synergistic lethal activity against MPN cells including those transformed to AML. In addition, the hsp90 inhibitors exhibited greater activity against JAK2-TKI–resistant than JAK2-TKI–sensitive cultured MPN cells.
previously described (14, 29). Ba/F3-hEpoR cells were supplemented with 10% WEHI preconditioned media. UKE-1 cells (kindly provided by W. Fiedler) were cultured in Iscoe’s Modified Dulbecco’s Medium containing 10% horse serum, 10% FBS, and 1 μmol/L hydrocortisone (29). Logarithmically growing cells were exposed to the designated concentrations of TG101209 and/or AUY922. Following these treatments, cells were washed free of the drug(s) prior to the performance of the studies. JAK2-TKI-resistant HEL2.1.7 (HEL/TGR) and UKE-1 (UKE-1/TGR) cells were established by continuous culturing of the cells in increasing concentrations of TG101209 starting from 0.1 μmol/L and incrementally increasing the concentration until a population of cells capable of sustained growth in 1.0 μmol/L TG101209 (HEL/TGR) or 0.5 μmol/L TG101209 (UKE/TGR) was obtained, according to a method previously described (30).

Primary myelofibrosis-MPN cells
Primary peripheral blood and/or bone marrow aspirate myelofibrosis (MF)-MPN samples were obtained with informed consent from patients with high risk (≥3) myelofibrosis (according to the International Prognostic Scoring System, IPSS; refs. 1, 31). The samples were collected in heparinized tubes, and mononuclear cells were separated using Ficoll Hypaque (Axis Shield) following the manufacturer’s protocol, washed once with complete RPMI-1640 media, resuspended in complete RPMI-1640, and counted to determine the number of cells isolated prior to their use in the various experiments, as previously described (14). Banked, delinked, and deidentified donor peripheral blood mononuclear cells procured for recipients who had since deceased, and primary MF-MPN cells were purified by immunomagnetic beads conjugated with anti-CD34 antibody prior to use in the cell viability assay (StemCell Technologies), as previously described (14).

Cell-cycle analysis
Following the designated treatments, cells were harvested and washed twice with 1× PBS and fixed in ethanol overnight. Fixed cells were washed twice with 1× PBS and stained with propidium iodide for 15 minutes at 37°C. Cell-cycle data were collected on a flow cytometer with a 488-nm laser and analyzed with ModFit 3.0, as previously described (32).

Assessment of apoptosis by Annexin-V staining
Untreated or drug-treated cells were stained with Annexin-V (Pharmingen) and TOPRO3 iodide, and the percentage of apoptotic cells was determined by flow cytometry, as previously described (14, 32). To analyze synergism between TG101209 and AUY922 or 17-AAG, cells were treated with TG101209 (200–1,000 nmol/L) and AUY922 (50–250 nmol/L) or 17-AAG (1.0–2.0 μmol/L) for 48 hours, and the percentage of apoptotic cells was determined. The combination index (CI) for each drug combination was calculated by median dose–effect analyses using 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
Following designated treatments, cells were stained with trypan blue (Sigma). The numbers of nonviable cells were determined by counting the cells that showed trypan blue uptake in a hemocytometer and reported as a percentage of untreated control cells (14, 32). Alternatively, cells were washed with 1× PBS, stained with propidium iodide, and analyzed by flow cytometry. IC50 values for the cell lines were determined using GraphPad Prism software.

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 (14, 28, 32). After centrifugation, an aliquot of each cell lysate was diluted 1:10 and protein quantitated using a bicinchoninic acid (BCA) protein quantitation kit (Pierce), according to the manufacturer’s protocol.

Immunoprecipitation of hsp90 and immunoblot analyses
Following the designated treatments, immunoprecipitation and immunoblotting of hsp90 or JAK2 were done as previously described (28, 32).

SDS-PAGE and Western blotting
Seventy-five micrograms of total cell lysate was used for SDS-PAGE. Western blot analyses of JAK2, p-STAT3 (Tyr705), STAT3, p-STAT5 (Tyr694), STAT5, p-AKT (Ser473), AKT, hsp70, p-ERK1/2, and ERK1/2 were done for SDS-PAGE. Western blot analyses of JAK2, p-STAT3 (Tyr705), STAT3, p-STAT5 (Tyr694), STAT5, p-AKT (Ser473), AKT, hsp70, p-ERK1/2, and ERK1/2 were done on total cell lysates using specific antisera or monoclonal antibodies. Blots were washed with 1× PBS with Tween (PBST) and then incubated in IRDye 680 goat anti-mouse or IRDye 800 goat anti-rabbit secondary antibodies (LI-COR) for 1 hour, washed 3 times in 1× PBST, and scanned with an Odyssey Infrared Imaging System (LI-COR), as previously described (14, 28). The expression levels of β-actin were used as the loading control for the immunoblots. Immunoblot analyses were done at least twice and representative blots were subjected to densitometric analysis. Densitometry was conducted using ImageQuant 5.2 (GE Healthcare).

Statistical analysis
Significant differences between values obtained in a population of MPN cells treated with different experimental conditions were determined using the Student t test. Values of P < 0.05 were assigned significance.

Results
Treatment with hsp90 inhibitor induces cell-cycle arrest and apoptosis of mouse HPCs and human MPN cells expressing JAK2-V617F
We first determined the effects of AUY922 on the viability of mouse pro-B Ba/F3-hEpoR and Ba/F3-hEpoR/JAK2-
V617F cells with or without the ectopic expression of JAK2-V617F. As shown in Fig. 1A, whereas treatment with 10 nmol/L was ineffective, exposure to 15 and 20 nmol/L of AUY922 induced apoptosis of Ba/F3-JAK2-V617F cells (right). AUY922 was significantly less cytotoxic against Ba/F3-hEpoR cells that lacked JAK2-V617F expression (Fig. 1A). We next determined the apoptotic and cell-cycle effects of AUY922 in the cultured human MPN HEL92.1.7 cells. Treatment with AUY922 for 24 hours dose dependently increased the percentage of cells in the G0–G1 and G2–M phases, with concomitant decline in the percentage of cells in the S-phase of the cell cycle. At this time point, we did not observe a substantial increase in sub-G1 cells (Fig. 1B and Supplementary Fig. S1). Treatment with AUY922 also induced apoptosis of HEL cells in a dose-dependent manner (Fig. 1C). As compared with HEL92.1.7, the cultured MPN UKE-1 cells were markedly more sensitive to AUY922-induced apoptosis (Fig. 1C). Similar results were obtained following treatment of the cultured MPN cells with 17-AAG (0.5–2.0 μmol/L; data not shown).

Treatment with hsp90 inhibitor reduced the levels and signaling of JAK2-V617F in the mouse HPCs and human MPN cells expressing JAK2-V617F

We next determined the effects of AUY922 on the levels and signaling of JAK2-V617F in Ba/F3-JAK2-V617F and HEL92.1.7 cells. Treatment with AUY922 dose dependently attenuated the expression of JAK2-V617F in Ba/F3-JAK2-V617F and JAK2 in Ba/F3-hEpoR cells, with more pronounced effects seen in Ba/F3-JAK2-V617F cells (Fig. 2A). This was accompanied with decline in the levels of p-JAK2, p-STAT5, p-STAT3, p-AKT, AKT, and p-ERK1/2 levels. The effect on p-STAT5 was also more pronounced in Ba/F3-JAK2-V617F than in Ba/F3-hEpoR cells (Fig. 2A). AUY922 treatment also reduced the levels of p-STAT3, p-STAT5, p-AKT, AKT, and p-ERK1/2, while simultaneously inducing the levels of hsp70, in HEL and UKE-1 cells (Fig. 2B). Treatment with 17-AAG induced similar effects in the cultured MPN cells (data not shown). Exposure to AUY922 also depleted the levels of JAK2-V617F in a time-dependent manner in HEL92.1.7 cells, with greater than 50% decline in the levels of JAK2 by 6 hours (Fig. 2C).

Treatment with AUY922 inhibits chaperone association of JAK2 with hsp90 in MPN cells

Previous reports have shown that AUY922 tightly binds to the amino-terminal, nucleotide-binding site of hsp90 and inhibits the chaperone association of hsp90 with its client proteins (25–27). Therefore, we determined the effect of AUY922 on the binding of hsp90 to JAK2-V617F in HEL92.1.7 and UKE-1 cells. Figure 3A shows that JAK2-V617F coimmunoprecipitated with hsp90 in both cell lines. Also, AUY922 treatment dose dependently inhibited the levels of JAK2-V617F in the coimmunoprecipitates with hsp90, regardless of whether the immunoprecipitates were pulled down with the anti-JAK2 or anti-hsp90 antibody (Fig. 3A). To determine whether AUY922-mediated disruption of the chaperone association between JAK2-V617F and
and human HPCs expressing JAK2-V617F. First, as previously reported (14), TG101209 dose dependently induced apoptosis of Ba/F3-JAK2-V617F but not Ba/F3-EpoR cells (Fig. 4A). Figure 4A also shows that cotreatment with a concentration of AUY922 as low as 10 nmol/L significantly increased TG101209 (200 or 500 nmol/L)-induced apoptosis of Ba/F3-JAK2-V617F but not Ba/F3-EpoR cells (P < 0.01; Fig. 4A). Treatment with 10 nmol/L AUY922 was ineffective against Ba/F3-JAK2-V617F cells. As compared with each agent alone, cotreatment with AUY922 and TG101209 also induced significantly more apoptosis of HEL92.1.7 and UKE-1 cells (Fig. 4B and C). This effect was evident at a relatively higher level of AUY922 in HEL92.1.7 than in UKE-1 cells (Fig. 4B). Cotreatment with AUY922 and TG101209 induced synergistic apoptotic effects in HEL92.1.7 and UKE-1 cells, following evaluation of the CIs through isobologram analyses (Fig. 5A). Combined treatment with 17-AAG and TG101209 also synergistically induced apoptosis of HEL92.1.7 cells (Fig. 5B). We next determined the effect of treatment with AUY922 and/or TG101209 on the levels of JAK2-V617F and the downstream signaling proteins in HEL92.1.7 cells. Figure 5C shows that as compared with each agent alone, cotreatment with AUY922 and TG101209 caused greater depletion of p-JAK2, JAK2, p-STAT5, p-STAT3, p-AKT, AKT, p-ERK1/2, ERK1/2, and hsp70 in HEL cells and greater induction of PARP cleavage. However, cotreatment with TG101209 did not increase hsp90 results in proteasomal degradation of JAK2-V617F, we determined the effect of cotreatment with a proteasomal inhibitor on AUY922-mediated decline in the levels of JAK2-V617F. As shown in Fig. 3B, cotreatment with bortezomib restored AUY922-mediated decline in the levels of JAK2-V617F. The shorter, 4-hour exposure interval for AUY922 was chosen because longer exposures caused considerable cytotoxicity in HEL92.1.7 cells. Similar restoration of levels of another hsp90 client protein, c-Raf1, was also observed, following cotreatment with AUY922 and bortezomib (Fig. 3B). We next determined the rate of recovery of expression of hsp90 client proteins in HEL cells, noted previously, following withdrawal of AUY922. As shown in Fig. 3C, levels of JAK2-V617F, p-STAT5, p-STAT3, p-AKT, AKT, and p-ERK1/2 steadily recovered between 4 and 24 hours after the withdrawal of AUY922 from the culture medium. In contrast, AUY922-induced hsp70 levels remained elevated in HEL cells for 24 hours after the withdrawal of AUY922 (Fig. 3C).

**Cotreatment with AUY922 enhances TG101209-mediated inhibition of JAK2-V617F signaling and apoptosis**

We next determined the effects of cotreatment with AUY922 on TG101209-induced apoptosis in the mouse and human HPCs expressing JAK2-V617F. First, as previously reported (14), TG101209 dose dependently induced apoptosis of Ba/F3-JAK2-V617F but not Ba/F3-EpoR cells (Fig. 4A). Figure 4A also shows that cotreatment with a concentration of AUY922 as low as 10 nmol/L significantly increased TG101209 (200 or 500 nmol/L)-induced apoptosis of Ba/F3-JAK2-V617F but not Ba/F3-EpoR cells (P < 0.01; Fig. 4A). Treatment with 10 nmol/L AUY922 was ineffective against Ba/F3-JAK2-V617F cells. As compared with each agent alone, cotreatment with AUY922 and TG101209 also induced significantly more apoptosis of HEL92.1.7 and UKE-1 cells (Fig. 4B and C). This effect was evident at a relatively higher level of AUY922 in HEL92.1.7 than in UKE-1 cells (Fig. 4B). Cotreatment with AUY922 and TG101209 induced synergistic apoptotic effects in HEL92.1.7 and UKE-1 cells, following evaluation of the CIs through isobologram analyses (Fig. 5A). Combined treatment with 17-AAG and TG101209 also synergistically induced apoptosis of HEL92.1.7 cells (Fig. 5B). We next determined the effect of treatment with AUY922 and/or TG101209 on the levels of JAK2-V617F and the downstream signaling proteins in HEL92.1.7 cells. Figure 5C shows that as compared with each agent alone, cotreatment with AUY922 and TG101209 caused greater depletion of p-JAK2, JAK2, p-STAT5, p-STAT3, p-AKT, AKT, p-ERK1/2, and Bcl-xl in HEL cells and greater induction of PARP cleavage. However, cotreatment with TG101209 did not increase

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**Figure 2.** Treatment with AUY922 depletes JAK2 and downstream signaling in MPN and Ba/F3 cells with ectopic overexpression of JAK2-V617F. A, Ba/F3-hJAK2-V617F and Ba/F3-hEpoR cells were treated with the indicated concentrations of AUY922 for 24 hours. Then, total cell lysates were prepared and immunoblot analyses were done for p-JAK2, JAK2, p-STAT5, STAT5, p-STAT3, STAT3, p-AKT, and AKT. The expression levels of β-actin in the lysates served as the loading control. B, HEL and UKE-1 cells were treated with the indicated concentrations of AUY922 for 24 hours. Following treatment, cell lysates were prepared and immunoblot analyses were done for JAK2, p-STAT5, STAT5, p-STAT3, STAT3, p-AKT, AKT, p-ERK1/2, ERK1/2, and hsp70. The expression levels of β-actin in the lysates served as the loading control. C, HEL cells were treated with 100 nmol/L of AUY922 for the indicated times. At the end of treatment, cells were harvested, cell lysates were prepared, and immunoblot analyses were done for JAK2. The expression levels of β-actin in the lysates served as the loading control.
AU922-mediated induction of hsp70 levels in HEL cells (Fig. 5C). Similar effects of the combination were observed in UKE-1 cells (data not shown).

Combined treatment with AU922 and TG101209 is selectively more active against primary MF-MPN cells expressing JAK2-V617F than normal HPCs

We next determined the effects of AU922 and/or TG101209 on the viability of primary CD34+ MF-MPN HPCs expressing JAK2-V617F harvested from the peripheral blood of patients with myelofibrosis. Treatment with TG101209 or AU922 resulted in greater loss of viability of primary MF-MPN than normal HPCs (Fig. 6A). Moreover, cotreatment with AU922 and TG101209 caused significantly more loss of cell viability of primary MF-MPN HPCs than treatment with either agent alone. In addition, the combined treatment exerted significantly greater lethality against primary MF-MPN than normal HPCs (P < 0.05; Fig. 6A). In CD34+ primary MF-MPN cells, as compared with treatment with each agent alone, cotreatment with AU922 and TG101209 caused greater decline in JAK2, p-STAT5, p-STAT3, p-AKT, and AKT levels, without significantly affecting STAT5 and STAT3 levels (Fig. 6B). In primary normal CD34+ cells, cotreatment with TG101209 and AU922 resulted in depletion of p-AKT and p-ERK1/2 but exerted minimal effects on the levels of AKT and ERK1/2 (Fig. 6C).

TKI-resistant MPN cells are collaterally sensitive to treatment with hsp90 inhibitor

We next determined the apoptotic effects of TG101209 and AU922 in the JAK2-TKI–resistant HEL/TGR versus their JAK2-TKI–sensitive parental counterparts. Figure 7A shows that treatment with 1.0 μmol/L of TG101209 for 48 hours induced apoptosis in 45% of HEL/TGR cells while inducing significantly less apoptosis in HEL/TGR cells (~15%; P < 0.0001). The IC50 values were 5.1 ± 0.2 μmol/L for the HEL/TGR cells and 1.3 ± 0.03 μmol/L for the sensitive parental HEL cell line. As compared with HEL92.1.7 cells, treatment with TG101209 also exerted minimal effects on p-JAK2 and had no effect on downstream signaling in HEL/TGR cells (Fig. 7B). HEL/TGR cells exhibited high expression levels of p-AKT and p-FOXO3A which were not altered by treatment with TG101209. Furthermore, treatment with TG101209 did not induce the nuclear localization of FOXO3A or...
Figure 4. Cotreatment with TG101209 and AUY922 induces more apoptosis than either agent alone in Ba/F3-hJAK2-V617F and MPN cells. A, Ba/F3-hJAK2-V617F and Ba/F3-hEpoR cells were treated with TG101209 and AUY922 as indicated for 48 hours. The percentages of apoptotic cells were determined by flow cytometry. Columns represent the mean of 3 experiments; bars represent the SEM. Ctrl, control.

Discussion

In the present studies, we show that treatment with the novel hsp90 inhibitor AUY922 inhibits the autophosphorylation and expression of JAK2-V617F, as well as attenuates its downstream signaling in cultured mouse and human and primary MF-MPN HPCs. Treatment with AUY922 inhibited the chaperone association of JAK2 and hsp90, which promoted the proteasomal degradation of JAK2-V617F. This degradation contributed to the overall decline in the levels of JAK2-V617F and its downstream signaling. In a previous report, we showed that treatment with pan-histone deacetylase (HDAC) inhibitor depletes HDAC6 activity, thereby inducing hyperacetylation of hsp90 and inhibiting its chaperone function, which augmented the polyubiquitylation and proteasomal degradation of hsp90 client proteins, for example, JAK2-V617F (28, 32). Disruption of JAK2-V617F binding to hsp90 by AUY922 treatment, and the restoration of the levels of JAK2-V617F by cotreatment with bortezomib and AUY922, supports the conclusion that JAK2-V617F is an hsp90 client protein. This is consistent with the reported preclinical in vitro and in vivo activity of other hsp90 inhibitors against JAK2-V617F–expressing cultured MPN cells (33, 34). It is also being recognized that several of the mutant client oncoproteins, including BCR-ABL, FLT-3, EGFR, KIT, and B-RAF, are more dependent on hsp90 chaperone support than their unmutated counterparts (22, 23, 35–37). Consequently, treatment with hsp90 inhibitor is likely to be more effective in depleting the mutant than the unmutated forms of the client oncoproteins and to exert relatively more cytotoxic activity.

AUY922 Depletes JAK2 in Myeloproliferative Disorders
selectivity of AUY922 may also be attributable to other normal CD34
V617F (all with JAK2-V617F) than in Ba/F3-hEpoR and
cannotly more apoptosis in HEL, UKE-1, and Ba/F3-JAK2-
c-RAF), as well as their progrowth and prosurvival signaling,
gation of the collateral client oncoproteins (AKT and
(21, 23, 28). This direct and JAK2-V617F–mediated abro-
c-RAF and AKT are hsp90 client proteins and, as such,
may be partly because of the direct inhibitory effect of
mediated downstream signaling, as highlighted by deple-
and TG101209 caused greater attenuation of the mutant
expressed, more ATP bound, and hyperactive as a molecular
chaperone (20, 21, 38). However, it is noteworthy that
following termination of the exposure to AUY922, the levels
of JAK2-V617F and of other progrowth and prosurvival
proteins recovered significantly over 24 hours to their
unperturbed levels. This indicates that in MPN cells,
proteins recovered significantly over 24 hours to their
unperturbed levels. This indicates that in MPN cells,
reported observations, for example, in comparison with
the untransformed cells, hsp90 in transformed cells is over-
expressed, more ATP bound, and hyperactive as a molecular
chaperone (20, 21, 38). However, it is noteworthy that
following termination of the exposure to AUY922, the levels
of JAK2-V617F and of other progrowth and prosurvival
proteins recovered significantly over 24 hours to their
unperturbed levels. This indicates that in MPN cells,
AU922-mediated in vivo growth-inhibitory and lethal
effects can be short-lived, unless active drug concentrations
are maintained for longer intervals, or more profound and
sustained effects on JAK2-V617F and other progrowth and
prosurvival signaling proteins can be achieved. In contrast,
induction of hsp70 in MPN cells by AUY922 was more
sustained. Although induction of hsp70 is known to inhibit
apoptosis due to hsp90 inhibitors (39), treatment with
AU922, despite sustained hsp70 induction, was effective
in inducing apoptosis of MPN cells.
Our findings also show that, as compared with treatment
with either agent alone, combined treatment with AUY922
and TG101209 caused greater attenuation of the mutant
effects against human HPCs that express and are "addicted"
to the mutant oncoprotein. Our findings support this by
showing that AUY922 treatment depleted JAK2-V617F
more than the wild-type JAK2 in Ba/F3-hEpoR cells, as well
as exerted greater efficacy against MF-MPN than normal
HPCs.

Treatment with AUY922 also inhibited JAK2-V617F–
meditated downstream signaling, as highlighted by deple-
tion of the levels of p-STAT5, p-AKT, and p-ERK1/2. This
may be partly because of the direct inhibitory effect of
AU922 on JAK2-V617F but may also be partly because
c-RAF and AKT are hsp90 client proteins and, as such,
directly downregulated by treatment with AU922
(21, 23, 28). This direct and JAK2-V617F–mediated abro-
gation of the collateral client oncoproteins (AKT and
c-RAF), as well as their progrowth and prosurvival signaling,
may explain why treatment with AU922 induces signifi-
cantly more apoptosis in HEL, UKE-1, and Ba/F3-JAK2-
V617F (all with JAK2-V617F) than in Ba/F3-hEpoR and
normal CD34+ human HPCs. The observed anti-MPN
selectivity of AU922 may also be attributable to other

Figure 5. Cotreatment with TG101209 and hsp90 inhibitor induces greater inhibition of JAK2 signaling and induces synergistic apoptosis of MPN cells. A, HEL and UKE-1 cells were treated with TG101209 (dose range 200–1,000 nmol/L) and AUY922 (dose range 10–250 nmol/L) for 48 hours. Following treatment, cells were stained with Annexin-V and TOPRO3 iodide, and the percentages of apoptotic cells were determined by flow cytometry. Median dose–effect and CIs for the drug combinations were obtained using CalcuSyn software using the percentage of apoptotic cells (fraction affected, Fa) resulting from the combined action of the 2 drugs compared with the effects of either drug alone. CI values less than 1.0 indicate synergism of the 2 agents. B, HEL cells were treated with TG101209 (dose range 200–1,000 nmol/L) and 17-AAG (dose range 1.0–2.0 µmol/L) for 48 hours. At the end of treatment, cells were stained with Annexin-V and TOPRO3 iodide, and the percentages of apoptotic cells were determined by flow cytometry. Median dose–effect was carried out as described previously (in A). CI values less than 1.0 indicate synergism of the 2 agents. C, HEL cells were treated with the indicated concentrations of TG101209 and/or AUY922 for 24 hours. After treatment, cell lysates were prepared and immunoblot analyses were done for p-JAK2, JAK2, p-STAT5, STAT5, p-STAT3, STAT3, p-AKT, AKT, p-ERK1/2, ERK1/2, hsp70, PARP, and Bcl-xL. The expression levels of β-actin in the lysates served as the loading control.
Figure 6. Effects of cotreatment with TG101209 and AUY922 in primary MF-MPN and normal CD34⁺ cells. A, CD34⁺ cells from patients with MF-MPN (n = 5) or normal CD34⁺ cells (n = 3) were treated with the indicated concentrations of TG101209 and AUY922 for 48 hours. Following treatment, the percentages of nonviable cells were determined by trypan blue dye uptake in a hemocytometer. Columns represent the mean of the individual samples ± SEM. †, values less significant (P < 0.05) in normal CD34⁺ cells treated with the combination of TG101209 and AUY922 than in primary MF-MPN cells. B, primary CD34⁺ MF-MPN cells were treated with the indicated concentrations of TG101209 and/or AUY922 for 24 hours. After treatment, cell lysates were prepared and immunoblot analyses were done for JAK2, p-STAT5, STAT5, p-STAT3, STAT3, p-AKT, AKT, p-ERK1/2, ERK1/2, and hsp70. The expression of β-actin in the lysates served as the loading control. C, normal CD34⁺ cells were treated with the indicated concentrations of TG101209 and AUY922 for 24 hours. Cell lysates were prepared and immunoblot analyses were done for JAK2, p-STAT5, AKT, p-ERK1/2, and ERK1/2. The expression of β-actin in the lysates served as the loading control.

JAK2-V617F levels and signaling through p-STAT3, p-STAT5, p-AKT, and p-ERK1/2 in the cultured and primary MF-MPN cells. Consistent with this, the combination also synergistically induced apoptosis in cultured MPN cells. This may be partly because of a greater effect of the combination in attenuating the JAK2-V617F–mediated pro-DNA copy number alterations—potentially able to promote AML transformation and lead to JAK2-TKI resistance in MPN (43–45). Therefore, the superior anti-JAK2-V617F activity of the combination of AUY922 and TG101209 could potentially reduce the risk of emergence of JAK2-TKI resistance and of AML transformation in advanced MPN.

Our findings, showing the collateral sensitivity of JAK2-TKI-resistant cultured MPN cells (HEL/TGR and UKE/TGR cells) to hsp90 inhibition, have important implications for resistance mechanisms that are likely to be encountered with prolonged exposures to JAK2-TKI in the clinic. These findings support the rationale to further study and characterize the mechanisms of JAK2-TKI refractoriness in MPN progenitor cells. This would assist in determining whether resistance mechanisms similar to those identified in HEL/TGR and UKE/TGR cells would also be observed clinically in
JAK2-TKI–refractory MPN progenitor cells and whether treatment with hsp90 inhibitor would overcome resistance to JAK2-TKIs. Furthermore, our observation that cotreatment with AUY922 and TG101209 exerts potent selectivity against JAK2-TKI–resistant MPN cells is similar to what has been reported with combinations of hsp90 inhibitor and anti-BCR-ABL TKIs (40). Therefore, our in vitro findings support the rationale for determining the activity of the combination of an hsp90 inhibitor and JAK2-TKI against primary MPN cells harvested from patients with JAK2-TKI–refractory MPN.

It is also noteworthy that in a JAK2-V617F knockin mouse model, the hematopoietic stem cells, but not myeloid progenitors, could initiate MPN serially, which could not be eliminated by JAK2-TKI treatment alone (11). Our findings clearly show that cotreatment with AUY922 significantly increased TG101209-induced apoptosis of primary CD34+ MF-MPN versus normal human HPCs. Therefore, the superior activity of the combination of an hsp90 inhibitor and JAK2-TKI may abrogate the leukemogenic potential of MPN HPCs. Whether this greater anti-MPN selectivity would exert superior in vivo efficacy against MPN progenitor cells remains to be established. Because treatment with JAK2-TKI alone fails to clinically achieve molecular remissions in advanced MPN, it is also important to evaluate the efficacy of the synergistic combination of an hsp90 inhibitor and JAK2-TKI in achieving molecular remissions in the clinic in advanced MPN. Phase 1 trials in patients with advanced solid malignancies have shown that hsp90 inhibitors, such as AUY922, are well tolerated (46, 47). Taken together with the findings presented here, these reports support the rationale to design and implement future clinical studies of hsp90 inhibitor and JAK2-TKI in patients with advanced MF-MPN.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

W. Fiskus, R. Rao, R. Balasu, S. Venkannagari, K. Ha, N.R. Naflouthula, J.E. Smith, and S.L. Hembrough conducted the in vitro studies with the cultured mouse, human, and primary MPN cells. S. Verstovsek, T. Manshouri, S. Abhyankar, and J. McGuirk procured and assisted in conducting the studies on primary CD5+ MP-MPN and CD34+ normal progenitor cells. K.N. Bhalla planned and supervised the in vitro studies and prepared the report.

References

31. Cervantes F, Dupirez B, Pereira A, Passamonti F, Reilly JT, Monna E, et al. New prognostic scoring system for primary myelofibrosis based...
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