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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Running title: AUY922 depletes JAK2 in myeloproliferative disorders

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ABSTRACT

**Purpose:** We determined the activity of heat shock protein (hsp) 90 inhibitor (HI), and/or JAK2 tyrosine kinase inhibitor (TKI) against JAK2-V617F-expressing cultured mouse (Ba/F3-JAK2-V617F) and human (HEL92.1.7 and UKE1) or primary human CD34+ myeloproliferative neoplasm (MPN) cells.

**Experimental Design:** Following exposure to the HI 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, i.e., 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. Co-treatment with AUY922 and TG101209 also induced significantly more apoptosis of human CD34+ MPN versus normal hematopoietic progenitor cells. As compared to the sensitive controls, JAK2-TKI-resistant HEL/TGR and UKE1/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 HIs 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 demonstrate that co-treatment with HI and JAK2-TKI exerts synergistic activity against cultured and primary MPN cells. Additionally, treatment with HI may overcome resistance to JAK2-TKI in human MPN cells.

**Statement of Translational Relevance:** The mutant JAK2-V617F tyrosine kinase (TK) is expressed in the majority of patients with BCR-ABL negative myeloproliferative neoplasms (MPNs). JAK2-V617F activates and confers pro-growth and pro-survival downstream signaling through the STAT, RAS/RAF/MAPK and PI3K/AKT pathways in MPN cells. Although treatment with JAK2 TK 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 -resistant MPN cells. Here, we demonstrate that the heat shock protein 90 inhibitor AUY922 proteasomally depleted JAK2-V617F and inhibited JAK2-V617F mediated downstream signaling. Co-treatment 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 co-treatment with AUY922 and TG101209.

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 JAK2-V617F in MPNs (2,3). JAK2-V617F mutation disrupts the pseudokinase (JH2) domain, and abolishes the auto-inhibitory functions normally imposed on the JAK2 catalytic domain (JH1) by the pseudokinase JH2 domain (2,4). This leads to an aberrant and de-regulated activation of the kinase (JH1) domain, triggering pro-growth and pro-survival signaling downstream of JAK2-V617F mediated by the signal transducers and activators of transcription 5 and 3 (STAT5 and STAT3), phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) (2,5). JAK2-V617F mutation is present in 90% of patients with PV and approximately 50-60% of patients with ET or PMF (1,2). In addition, mutations in exon 12 of JAK2 are present in almost all patients with PV 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 knock-in 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 myeloid leukemia (AML) (1,2). Based on these observations, the mutant JAK2 represents an excellent target for therapeutic intervention in MPNs. Several, orally bio-available, small molecule, ATP-competitive, JAK2-selective
tyrosine kinase inhibitors (TKIs) have been tested in pre-clinical studies and currently undergoing clinical investigation in patients with MPNs (1,12). Pre-clinical studies have shown that treatment with JAK2-TKI, e.g., 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, e.g., TG101348 and INC18424, have recently been performed (1,12,17,18). Preliminary results suggest that in the clinic JAK2-TKI are relatively well-tolerated, ameliorate constitutional symptoms, reduce splenomegaly, but neither reverse myelofibrosis nor markedly reduce the allelic burden of JAK2-V617F mutant clone in advanced MPN (1,12,19). Also, similar to first and second generation anti-BCR-ABL TKIs, JAK2-TKIs may also be less active against MPN-initiating stem cells or the AML-transformed MPN HPCs (1,11,12,19). These observations create a strong rationale for evaluating potential mechanisms of resistance to JAK2-TKIs and developing and testing additional novel, JAK2-V617F targeted combinations against MPN cells.

Hsp90 is a highly conserved, homo-dimeric, ATP-dependent molecular chaperone, which helps fold and maintain its client proteins, e.g., c-RAF, AKT and JAK2, in a functionally active conformation, thus preserving their pro-growth and pro-survival activity in MPN cells (20,21). In transformed myeloid cells, several mutant oncoprotein kinases, e.g., BCR-ABL, FLT3-ITD and JAK2-V617F have been shown to be even more dependent on the chaperone function of hsp90 than their un-mutated counterparts (14,20,22,23). Inhibition of ATP binding and chaperone function of hsp90 by treatment with geldanamycin analogue (GAA), e.g., 17-AAG, or with AUY922 (non-GAA), disrupts the chaperone association of hsp90 with its client proteins (20,21). This leads to misfolding, polyubiquitylation and subsequent degradation of the onco-client proteins by the 26S proteasome (20,21). Many of the un-mutated and mutant forms of client proteins, as noted above, including JAK2-V617F, c-RAF and AKT, confer pro-growth and pro-survival advantage on MPN cells (14,20,21). AUY922 is a derivative of 4,5-diarylisoazole 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 shown to demonstrate pre-clinical 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 pro-growth and pro-survival 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. Additionally, the hsp90 inhibitors exhibited greater activity against JAK2-TKI-resistant versus –sensitive cultured MPN cells.

Methods and Materials
Reagents and antibodies: AUY922 was kindly provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ). TG101209 (TG) was kindly provided by Sanofi-Aventis (Bridgewater, NJ). Bortezomib was acquired from Millennium Pharmaceuticals (Cambridge, MA). 17-AAG was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-pJAK2 (Tyr1007/1008) and anti-pFOXO3A were obtained from Abcam (Cambridge, MA) Anti-JAK2, anti-pSTAT3 (Tyr705), anti-pSTAT3 (Ser727), anti-STAT3, anti-pAKT (Ser473), anti-AKT, anti-FOXO3A, anti-pERK1/2, anti-ERK1/2, anti-PARP and BIM were obtained from Cell Signaling (Beverly, MA). Monoclonal anti-pSTAT5 (Tyr694), anti-p27 and monoclonal anti-c-RAF were obtained from BD Transduction Labs (San Jose, CA). Polyclonal anti-STAT5 and anti-Bcl-xL antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Rat monoclonal anti-hsp90 and monoclonal anti-hsp70 antibodies were obtained from StressGen Biotechnologies (Vancouver, British Columbia) (28). Monoclonal anti-β-actin was obtained from Sigma-Aldrich (St. Louis, MO).

Cell lines and cell culture. Human erythroleukemia HEL 92.1.7 (HEL) cells with homozygous expression of JAK2-V617F, as well as the murine Ba/F3-EpoR-JAK2V617F, and Ba/F3-EpoR cells, were maintained in RPMI media with 10% FBS, 1% penicillin/streptomycin and 1% non-essential amino acids, as previously described (14,29). Ba/F3-hEpoR cells were supplemented with 10% WEHI pre-conditioned media. UKE1 cells (kindly provided by W. Fiedler) were cultured in IMDM media containing 10% horse
serum, 10% FBS and 1 µM 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 HEL92.1.7 (HEL/TGR) and UKE1 (UKE1/TGR) cells were established by continuous culturing of the cells in increasing concentrations of TG101209 starting from 0.1 µM and incrementally increasing the concentration until a population of cells capable of sustained growth in 1.0 µM TG101209 (HEL/TGR) or 0.5 µM TG101209 (UKE/TGR) was obtained, according to a method previously described (30).

**Primary MF-MPN cells:** Primary peripheral blood and/or bone marrow aspirate MF-MPN samples were obtained with informed consent from patients with high risk (≥ 3) MF (according to the International Prognostic Scoring System, IPSS) (1,31). The samples were collected in heparinized tubes, and mononuclear cells were separated using Ficoll Hypaque (Axis Shield, Oslo, Norway) 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, de-linked and de-identified, donor peripheral blood CD34+ mononuclear cells procured for recipients who had since deceased and primary MF-MPN cells were purified by immuno-magnetic beads conjugated with anti-CD34 antibody prior to utilization in the cell viability assay (StemCell Technologies, Vancouver, British Columbia), as previously described (14).

**Cell cycle analysis.** Following the designated treatments, cells were harvested and washed twice with 1X PBS and fixed in ethanol overnight. Fixed cells were washed twice with 1X 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, San Diego, CA) and TOPRO3 iodide and the percentage of apoptotic cells was determined by flow cytometry, as previously described
To analyze synergism between TG101209 (TG) and AUY922 or 17-AAG, cells were treated with TG (200-1000 nM) and AUY922 (50-250 nM) or 17-AAG (1.0-2.0 µM) for 48 hours and the percentage of apoptotic cells were determined. The combination index (CI) for each drug combination was calculated by median dose effect analyses utilizing the commercially available software CalcuSyn (Biosoft, Ferguson, MO). CI values of less than 1.0 represent synergism of the two drugs in the combination.

Assessment of percentage non-viable cells: Following designated treatments, cells were stained with trypan blue (Sigma, St. Louis, MO). The numbers of non-viable 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 1X PBS, stained with propidium iodide and analyzed by flow cytometry. IC₅₀ values for the cell lines were determined utilizing GraphPad Prism software (La Jolla, CA).

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 BCA protein quantitation kit (Pierce, Rockford, IL), according to the manufacturer’s protocol.

Immunoprecipitation of hsp90 and immunoblot analyses: Following the designated treatments, immunoprecipitation and immunoblotting of hsp90 or JAK2 was performed 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 (Tyr 694), STAT5, p-AKT (Ser473), AKT, hsp70, p-ERK1/2 and ERK1/2 were performed on total cell lysates using specific antisera or monoclonal antibodies. Blots were washed with 1X PBST then incubated in IRDye 680 Goat anti-mouse or IRDye 800 Goat anti-rabbit secondary antibodies (LI-COR, Lincoln, NE) for 1 hour, washed 3 times in 1X PBST and scanned with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE), as
previously described (14,28). The expression levels of β-actin were used as the loading control for the immunoblots. Immunoblot analyses were performed at least twice and representative blots were subjected to densitometric analysis. Densitometry was performed using ImageQuant 5.2 (GE Healthcare, Piscataway, NJ).

**Statistical Analysis:** Significant differences between values obtained in a population of MPN cells treated with different experimental conditions were determined using the Student’s t-test. P values of less than 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 demonstrated in Figure 1A, while treatment with 10 nM was ineffective, exposure to 15 and 20 nM of AUY922 induced apoptosis of Ba/F3-JAK2-V617F cells (right panel). AUY922 was significantly less cytotoxic against Ba/F3-hEpoR cells that lacked JAK2-V617F expression (Figure 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 % of cells in the G0/G1 and G2/M phases, with concomitant decline in the % 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 (Figure 1B and Supplemental Figure 1). Treatment with AUY922 also induced apoptosis of HEL cells in a dose dependent manner (Figure 1C). As compared to HEL92.1.7, the cultured MPN UKE1 cells were markedly more sensitive to AUY922-induced apoptosis (Figure 1C). Similar results were obtained following treatment of the cultured MPN cells with 17-AAG (0.5 to 2.0 µM) (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 (Figure 2A). This was accompanied with decline in the levels of pJAK2, 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 versus Ba/F3-hEpoR cells (Figure 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 UKE1 cells (Figure 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 (Figure 2C).

**Treatment with AUY922 inhibits chaperone association of JAK2 with hsp90 in MPN cells.** Previous reports have demonstrated 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 UKE1 cells. Figure 3A demonstrates that JAK2-V617F co-immunoprecipitated with hsp90 in both cell lines. Also, AUY922 treatment dose-dependently inhibited the levels of JAK2-V617F in the co-immunoprecipitates with hsp90, regardless of whether the immunoprecipitates were pulled down with the anti-JAK2 or anti-hsp90 antibody (Figure 3A). To determine whether AUY922-mediated disruption of the chaperone association between JAK2-V617F and hsp90 results in proteasomal degradation of JAK2-V617F, we determined the effect of co-treatment with a proteasomal inhibitor on AUY922-mediated decline in the levels of JAK2-V617F. As shown in Figure 3B, co-treatment with bortezomib (BZ) 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 co-treatment with AUY922 and BZ (Figure 3B). We next determined the rate of recovery of expression of hsp90 client
proteins in HEL cells, noted above, following withdrawal of AUY922. As shown in Figure 3C, levels of JAK2-V617F, p-STAT5, p-STAT3, p-AKT, AKT and p-ERK1/2 steadily recovered between 4 to 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 (Figure 3C).

**Co-treatment with AUY922 enhances TG101209-mediated inhibition of JAK2-V617F signaling and apoptosis.** We next determined the effects of co-treatment with AUY922 on TG101209-induced apoptosis in the mouse and human hematopoietic progenitor cells expressing JAK2-V617F. First, as previously reported (14), TG101209 dose-dependently induced apoptosis of Ba/F3-JAK2-V617F but not Ba/F3-EpoR cells (Figure 4A). Figure 4A also demonstrates that co-treatment with a concentration of AUY922 as low as 10 nM significantly increased TG101209 (200 or 500 nM)-induced apoptosis of Ba/F3-JAK2-V617F but not Ba/F3-hEpoR cells (p < 0.01) (Figure 4A). Treatment with 10 nM AUY922 was ineffective against Ba/F3-JAK2-V617F cells. As compared to each agent alone, co-treatment with AUY922 and TG101209 also induced significantly more apoptosis of HEL92.1.7 and UKE1 cells (Figure 4B and 4C). This effect was evident at a relatively higher level of AUY922 in HEL92.1.7 versus UKE1 cells (Figure 4B). Co-treatment with AUY922 and TG101209 induced synergistic apoptotic effects in HEL92.1.7 and UKE1 cells, following evaluation of the combination indices through isobologram analyses (Figure 5A). Combined treatment with 17-AAG and TG101209 also synergistically induced apoptosis of HEL92.1.7 cells (Figure 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 demonstrates that as compared to each agent alone, co-treatment with AUY922 and TG101209 caused greater depletion of pJAK2, JAK2, p-STAT5, p-STAT3, p-AKT, AKT p-ERK1/2 and Bcl-xL in HEL cells and greater induction of PARP cleavage. However, co-treatment with TG101209 did not increase AUY922-mediated induction of hsp70 levels in HEL cells (Figure 5C). Similar effects of the combination were observed in UKE1 cells (data not shown).
Combined treatment with AUY922 and TG101209 is selectively more active against primary MF-MPN cells expressing JAK2V617F versus normal HPCs. We next determined the effects of AUY922 and/or TG101209 on the viability of primary CD34+ MF-MPN HPCs expressing JAK2-V617F harvested from the peripheral blood of patients with MF. Treatment with TG101209 or AUY922 resulted in greater loss of viability of primary MF-MPN than normal HPCs (Figure 6A). Moreover, co-treatment with AUY922 and TG101209 caused significantly more loss of cell viability of primary MF-MPN HPCs than treatment with either agent alone. Additionally, the combined treatment exerted significantly greater lethality against primary MF-MPN versus normal HPCs (p < 0.05) (Figure 6A). In CD34+ primary MF-MPN cells, as compared to treatment with each agent alone, co-treatment with AUY922 and TG101209 caused greater decline in JAK2, p-STAT5, p-STAT3, p-AKT and AKT levels, without significantly affecting STAT5 and STAT3 levels (Figure 6B). In primary normal CD34+ cells, co-treatment with TG101209 and AUY922 resulted in depletion of p-AKT and p-ERK1/2 but exerted minimal effects on the levels of AKT and ERK1/2 (Figure 6C).

TKI resistant MPN cells are collaterally sensitive to treatment with hsp90 inhibitor. We next determined the apoptotic effects of TG101209 and AUY922 in the JAK2-TKI-resistant HEL/TGR and UKE1/TGR versus their JAK2-TKI sensitive parental counterparts. Figure 7A demonstrates that treatment with 1.0 μM of TG101209 for 48 hours induced apoptosis in 45% of HEL92.1.7 cells, while inducing significantly less apoptosis in HEL/TGR cells (~15%) (p< 0.0001). The IC50 values were 5.1 ± 0.2 μM for the HEL/TGR cells and 1.3 ± 0.03 for the sensitive parental HEL cell line. As compared to 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 (Figure 7B). HEL/TGR cells exhibited high expression levels of p-AKT and p-FOXO3A which were not altered by treatment with TG101209. Further, treatment with TG101209 did not induce the nuclear localization of FOXO3A or upregulate BIM expression in HEL/TGR cells, as compared to HEL cells (Supplemental Figure 2). We next determined the effects of treatment with AUY922 against HEL92.1.7 and HEL/TGR cells. As shown in Figure 7C, while displaying resistance to JAK2–TKI, HEL/TGR cells exhibited collateral sensitivity...
to AUY922. Exposure to 50 nM AUY922 induced apoptosis in >80% of HEL/TGR (IC\textsubscript{50} value 9.5 ± 1.2 nM) versus 40% of the parental HEL92.1.7 cells (IC\textsubscript{50} value 963.3 ± 188.7) (Figure 7C). Combined treatment of HEL/TGR cells with AUY922 (20 and 50 nM) and TG101209 (1.0 µM) exhibited significantly more apoptosis of HEL/TGR versus HEL92.1.7 cells (p< 0.05), thus overcoming resistance of HEL/TGR cells to TG101209.

We also determined the sensitivity of TG101209-resistant UKE1 cells (UKE/TGR) to TG101209 or AUY922. The IC\textsubscript{50} values for TG101209 were 1.7 ± 0.03 µM for the UKE/TGR cells and 0.63 ± 0.02 for the sensitive parental UKE1 cell line. Similar to the observations in HEL/TGR cells, TG101209-resistant UKE1 cells demonstrated collateral sensitivity to AUY922 (IC\textsubscript{50} value 13.9 ± 0.2 nM) compared to parental UKE1 cells (IC\textsubscript{50} value 26.4 ± 1.9 nM) (Supplemental Figure 3). The enhanced sensitivity of HEL/TGR cells to hsp90 inhibitors was not restricted to AUY922. HEL/TGR cells also demonstrated greater sensitivity to the geldanamycin analogue, 17-AAG (Supplemental Figure 4). Enhanced sensitivity of HEL/TGR cells to AUY922 was associated with significantly greater decline in the levels of p-JAK2, JAK2, p-STAT5, p-AKT, AKT, p-FOXO3A, FOXO3A and Bcl-x\textsubscript{L} as well as induction of BIM in the HEL/TGR cells versus HEL92.1.7 cells (Figure 7D).

**Discussion**

In the present studies, we demonstrate that treatment with the novel hsp90 inhibitor AUY922 inhibits the auto-phosphorylation 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 demonstrated that treatment with pan-histone deacetylase (HDAC) inhibitor depletes HDAC6 activity, thereby inducing hyper-acetylation of hsp90 and inhibiting its chaperone function, which augmented the polyubiquitylation and proteasomal degradation of hsp90 client proteins, e.g., JAK2-V617F (28,32). Disruption of JAK2-V617F binding to hsp90 by AUY922 treatment, and the restoration of the levels of JAK2-V617F by co-treatment with bortezomib and
AUY922, supports the conclusion that JAK2-V617F is an hsp90 client protein. This is consistent with the reported pre-clinical 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 un-mutated counterparts (22,23,35-37). Consequently, treatment with hsp90 inhibitor is likely to be more effective in depleting the mutant as compared to the un-mutated forms of the client oncoproteins, and to exert relatively more cytotoxic effects against human HPCs that express and are ‘addicted’ to the mutant oncoprotein. Our findings support this by demonstrating 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 versus normal HPCs.

Treatment with AUY922 also inhibited JAK2-V617F-mediated downstream signaling, as highlighted by depletion of the levels of p-STAT5, p-AKT, and p-ERK1/2. This may be partly due to the direct inhibitory effect of AUY922 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 AUY922 (21,23,28). This direct and JAK2-V617F-mediated abrogation of the collateral client oncoproteins (AKT and c-RAF), as well as their pro-growth and pro-survival signaling, may explain why treatment with AUY922 induces significantly more apoptosis in HEL, UKE1 and Ba/F3-JAK2-V617F (all with JAK2-V617F) versus Ba/F3-hEpoR and normal CD34+ human HPCs. The observed anti-MPN selectivity of AUY922 may also be attributable to other reported observations, e.g., in comparison to the untransformed cells, hsp90 in transformed cells is overexpressed, more ATP bound and hyper-active 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 pro-growth and pro-survival proteins recovered significantly over 24 hours to their unperturbed levels. This indicates that, in MPN cells, AUY922-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 pro-growth and pro-survival 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 AUY922, in spite of sustained hsp70 induction, was effective in inducing apoptosis of MPN cells.

Our findings also demonstrate that, as compared to treatment with either agent alone, combined treatment with AUY922 and TG101209 caused greater attenuation of the mutant JAK2-V617F levels and signaling through p-STAT5, p-STAT3, 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 due to a greater effect of the combination in attenuating the JAK2-V617F-mediated pro-growth and pro-survival signaling through STAT5 and STAT3, as well as partly due to greater activity of the combination in abrogating AKT and c-RAF mediated collateral pro-growth and pro-survival signaling. This is similar to the report where combined treatment with hsp90 inhibitor and an inhibitor of BCR-ABL and FLT-3 exerted greater toxicity in CML and AML cells, respectively, than treatment with each agent alone (40,41). Although we did not directly evaluate the effect of co-treatment with AUY922 and TG101209 in HPCs containing exon 12 mutations of JAK2, because of its downstream signaling similar to JAK2-V617F, it is likely that the combination would be similarly effective against HPCs containing exon 12 mutations of JAK2. High levels of expression and deregulated activity of JAK2-V617F in HPCs has been shown to stimulate increased centrosome and ploidy abnormalities, genomic instability and homologous recombination (42). Presence of activating mutations in tyrosine kinases (TKs) has also been shown to induce the intracellular levels of reactive oxygen species (ROS) in myeloid leukemia cells, which may contribute to the emergence of DNA damage, genomic instability and DNA copy number alterations—all 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 demonstrating the collateral sensitivity of JAK2-TKI-resistant cultured MPN cells (HEL/TGR and UKE/TGR cells) to hsp90 inhibition has 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 co-treatment 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 hsp90 inhibitor and JAK2-TKI combination against primary MPN cells harvested from patients with JAK2-TKI refractory MPN.

It is also noteworthy that in a JAK2-V617F knock-in 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 co-treatment 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. Since 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 I trials in patients with advanced solid malignancies have demonstrated 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.
Acknowledgements:
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Authorship:

References


Figure Legends

Figure 1. Treatment with AUY922 induces G0/G1 and G2/M accumulation and apoptosis of MPN cells and Ba/F3 cells with ectopic over-expression of JAK2 V617F. A. Ba/F3 hJAK2V617F and Ba/F3 hEpoR cells were treated with the indicated concentrations of AUY922 for 48 hours. After treatment, cells were stained with annexin V and TOPRO3 iodide and the percentages of apoptotic cells were determined by flow cytometry. Columns represent the mean of three experiments; Bars represent the standard error of the mean. B. HEL cells were treated with the indicated concentrations of AUY922 for 24 hours. Cells were fixed in ethanol and stained with TOPRO3 iodide. Cell cycle status was determined by flow cytometry. Values represent the mean of three experiments ± the standard error of the mean. C. HEL and UKE1 cells were treated with the indicated concentrations of AUY922 for 48 hours. After treatment, cells were stained with annexin V and TOPRO3 iodide and the percentages of apoptotic cells were determined by flow cytometry. Columns represent the mean of three experiments; Bars represent the standard error of the mean.
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 performed for pJAK2, 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 UKE1 cells were treated with the indicated concentrations of AUY922 for 24 hours. Following treatment, cell lysates were prepared and immunoblot analyses were performed 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 nM of AUY922 for the indicated times. At the end of treatment, cells were harvested, 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.

Figure 3. Treatment with AUY922 inhibits chaperone association of JAK2 with hsp90 in MPN cells. A. HEL and UKE1 cells were treated with the indicated concentrations of AUY922 for 8 hours. Following treatment, hsp90 or JAK2 immunoprecipitates were prepared from total cell lysates. Normal rat or normal rabbit IgG served as an immunoprecipitation control. Immunoblot analyses were performed for JAK2 and hsp90 in the immunoprecipitates. Total cell lysate was used as an immunoblot (input) control. B. HEL cells were treated with AUY922 and bortezomib (BZ) as indicated for 4 hours. Total cell lysates were prepared and immunoblot analyses were performed for JAK2 and c-RAF. The expression levels of β-actin in the lysates served as the loading control. C. HEL cells were treated with 100 nM of AUY922 for 16 hours. Following treatment, the cells were washed 3X with complete media to remove the drug and re-plated for the indicated times. Immunoblot analyses were performed for JAK2, p-STAT5, STAT5, p-STAT3, STAT3, p-AKT, AKT, p-ERK1/2, ERK1/2 and hsp70 on the total cell lysates. The expression levels of β-actin in the cell lysates served as the loading control.

Figure 4. Co-treatment with TG101209 and AUY922 induces more apoptosis than either agent alone in Ba/F3-JAK2 V617F and MPN cells. A. Ba/F3 hJAK2-V617F and Ba/F3 hEpoR cells were treated with TG101209 (TG) and AUY922 as indicated 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. Columns represent the mean of three experiments; Bars represent the standard error of the mean. B-C. HEL and UKE1 cells were treated with the indicated concentrations of TG101209 and/or AUY922 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. Columns represent the mean of three experiments; Bars represent the standard error of the mean.

Figure 5. Co-treatment with TG and hsp90 inhibitor induces greater inhibition of JAK2 signaling and induces synergistic apoptosis of MPN cells. A. HEL and UKE1 cells were treated TG101209 (dose range 200-1000 nM) and AUY922 (dose range 10 nM
-250 nM) 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 combination indices for the drug combinations were obtained using CalcuSyn software utilizing the percentage of apoptotic cells (fraction affected) resulting from the combined action of the two drugs compared to the effects of either drug alone. CI values less than 1.0 indicate synergism of the two agents. B. HEL cells were treated with TG101209 (dose range 200-1000 nM) and 17-AAG (dose range 1.0 -2.0 µM) 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 performed as described above in (A). CI values less than 1.0 indicate synergism of the two 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 performed for pJAK2, 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 co-treatment with TG101209 and AUY922 in primary MF-MPN and normal CD34+ cells. A. CD34+ cells from MF-MPN patients (n=5) or normal CD34+ cells (n=3) were treated with the indicated concentrations of TG and AUY922 for 48 hours. Following treatment, the percentages of non-viable cells were determined by trypan blue dye uptake in a hemocytometer. Columns represent the mean of the individual samples ± the standard error of the mean. † indicates values significantly less (p < 0.05) in normal CD34+ cells treated with the combination of TG101209 and AUY922 compared to primary MF-MPN cells. B. Primary CD34+ MF-MPN cells were treated with the indicated concentrations of TG and/or AUY922 for 24 hours. After treatment, cell lysates were prepared and immunoblot analyses were performed 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 TG and AUY for 24 hours. Cell lysates were prepared and immunoblot analyses were performed for JAK2, p-STAT5, p-AKT, AKT, p-ERK1/2 and ERK1/2. The expression of β-actin in the lysates served as the loading control.

Figure 7. JAK2 TKI-resistant MPN cells exhibit collateral sensitivity to AUY922. A. HEL92.1.7 and HEL/TGR cells were treated with the indicated concentrations of TG101209 for 48 hours. Following treatment, the cells were stained with annexin V and TOPRO3 iodide and the percentages of apoptotic cells were determined by flow cytometry. Columns represent the mean of three experiments; Bars represent the standard error of the mean. B. HEL and HEL/TGR cells were treated with the indicated concentrations of TG101209 for 24 hours. At the end of treatment, total cell lysates were prepared and immunoblot analyses were performed for pJAK2, JAK2, p-STAT5, STAT5, p-STAT3, STAT3, p-AKT, AKT, p-FOXO3A, FOXO3A, p-ERK1/2, ERK1/2, Bcl-xL, BIM, and PARP. The expression of β-actin in the lysates served as the loading control. C. HEL and HEL/TGR cells were treated with the indicated concentrations of AUY922 and/or TG101209 for 48 hours. Then, cells were stained with annexin V and TOPRO3
iodide and the percentages of apoptotic cells were determined by flow cytometry. Columns represent the mean of three experiments; Bars represent the standard error of the mean. (*) indicates values significantly greater (p< 0.05) in the combination treatment of HEL/TGR as compared to combination treatment in HEL92.1.7 cells. D. HEL and HEL/TGR cells were treated with the indicated concentrations of AUY922 for 24 hours. At the end of treatment, total cell lysates were prepared and immunoblot analyses were performed for pJAK2, JAK2, p-STAT5, STAT5, p-AKT, AKT, p-FOXO3A, FOXO3A, p-ERK1/2, ERK1/2, Bcl-xL, and BIM. The expression of β actin in the lysates served as the loading control.
Figure 1

A

Ba/F3-JAK2V617F
Ba/F3-hEpoR

% apoptosis

nM, AUY922, 48 hours

B

HEL 92.1.7

% of cells in cycle

G0/G1  S  G2/M

Control  39.6 ± 0.9  45.1 ± 0.5  15.3 ± 0.4

10 nM AUY922  41.0 ± 2.3  43.0 ± 2.4  16.0 ± 0.2

20 nM AUY922  35.6 ± 1.8  46.2 ± 2.9  18.2 ± 1.1

50 nM AUY922  59.2 ± 0.9  18.0 ± 2.9  22.8 ± 2.1

100 nM AUY922  53.0 ± 3.1  11.3 ± 1.9  35.7 ± 3.2

250 nM AUY922  50.5 ± 4.1  15.8 ± 1.2  33.7 ± 3.1

C

HEL92.1.7
UKE1

% apoptosis

Control  20  50  250
nM, AUY922, 48 hours

Research. on April 20, 2017. © 2011 American Association for Cancer Research.
Figure 2

A  
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Figure 3

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Figure 4

A

% apoptosis

Ba/F3-hJAK2V617F
Ba/F3-hEpoR

Ctrl 0.2 µM TG 0.5 µM TG 10 nM AUY922 0.2 µM TG + 10 nM AUY922 0.5 µM TG + 10 nM AUY922

B

HEL92.1.7

% apoptosis

0.2 µM TG 1.0 µM TG 50 nM AUY922 1.0 µM TG + 50 nM AUY922

C

UKE1

% apoptosis

0.2 µM TG 0.5 µM TG 20 nM AUY922 0.2 µM TG + 20 nM AUY922 0.5 µM TG + 20 nM AUY922
Figure 5

A

Fractional Effect

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Fractional Effect

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HEL 92.1.7

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- p-JAK2 (Tyr1007/1008)
- JAK2
- p-STAT5 (Tyr694)
- STAT5
- p-STAT3 α/β (Tyr705)
- STAT3
- p-AKT (Ser473)
- AKT
- p-ERK1/2
- ERK1/2
- hsp70
- PARP
- cleaved PARP
- Bcl-xL
- β-actin
Figure 6

A

[Graph showing percentage of non-viable cells for different treatments.]

- Primary CD34+ MF-MPN (n=5)
- Normal CD34+ (n=3)

B

CD34+ MF-MPN cells
- + - + 1.0 µM TG101209
- - + + 25 nM AUY922

C

Normal CD34+ cells

+ 25 nM AUY922

0 0.2 1.0 0 0.2 1.0 µM TG101209

- JAK2
- p-STAT5 (Tyr694)
- STAT5
- p-STAT3 (Tyr705)
- STAT3
- p-AKT (Ser473)
- AKT
- p-ERK1/2
- ERK1/2
- hsp70
- β-actin

Original caption in text: Figure 6: A) Graph showing percentage of non-viable cells for different treatments. B) CD34+ MF-MPN cells treated with different concentrations of TG101209 and AUY922. C) Normal CD34+ cells treated with TG101209 and AUY922.
Figure 7

A

% apoptosis

HEL92.1.7
HEL/TGR

Control 0.5 0.75 1 µM, TG101209, 48 hours

p<0.01

p<0.0001

B

HEL92.1.7  HEL/TGR

0 0.5 1.0 0 0.5 1.0 µM, TG101209, 24 hours

p-JAK2

JAK2

p-STAT5

STAT5

p-AKT

AKT

p-FOXO3a (Ser253)

FOXO3a

Bcl-xL

BIMEL

BIM

α-actin

p-FOXO3a (Ser253)

FOXO3a

p-JAK2

JAK2

p-STAT5

STAT5

p-AKT

AKT

p-FOXO3a (Ser253)

FOXO3a

Bcl-xL

BIMEL

BIM

α-actin

C

% apoptosis

HEL92.1.7  HEL/TGR

Control 20 nM AUY922 50 nM AUY922 1 µM TG 20 nM AUY922 + TG 50 nM AUY922 + TG

D

HEL92.1.7  HEL/TGR

0 10 20 0 10 20 nM, AUY922, 24 hours

p-JAK2

JAK2

p-STAT5

STAT5

p-AKT

AKT

p-FOXO3a (Ser253)

FOXO3a

Bcl-xL

BIMEL

BIM

α-actin

p-FOXO3a (Ser253)

FOXO3a

Bcl-xL

BIMEL

BIM

α-actin
Heat shock protein 90 inhibitor is synergistic with JAK2 inhibitor and overcomes resistance to JAK2-TKI in human myeloproliferative neoplasm cells

Warren Fiskus, Srdan Verstovsek, Taghi Manshouri, et al.

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