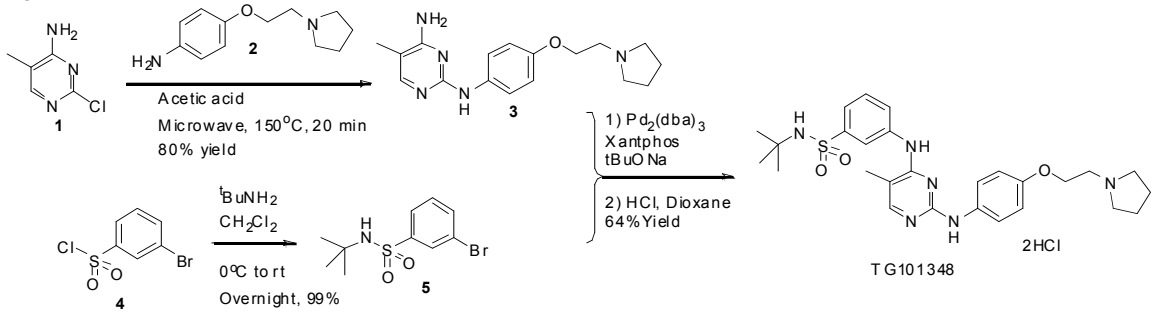
HSP90 as a therapeutic target for JAK2 dependent myeloproliferative neoplasms in mice and humans

Supplemental Information

The Synthesis of TG101348

The patent protocol (Noronha et al, 2007) calls for reacting bromide **5** with chloropyrimidine **1** and condensation of the resulting product with amine **2**. This gave lower yields and complex mixtures. Upon re-examination of the procedure, we reasoned that reversing the sequence of chemical steps might be more efficient and less prone . We were pleased to find out that indeed the modified process gave cleaner intermediates and better yields (Synthesis Scheme below). We have started the synthesis by reacting commercial amine **2** with chloropyrimidine **1** in acetic acid under microwave irradiation which provided compound **3** in 80% yield. Subsequently, bromosulfonamide **5,** which we have prepared by reacting tertiobutylamine with sulfonylchloride **4**, was condensed with amine **3** under palladium catalysis to give the free base of TG101348 in 64% yield. We have found that the bis-hydrochloride salt of TG101348 provides a more soluble form of the drug. The latter was obtained by treating a dioxane solution of the free base with 2.0 equivalents of hydrochloric acid.

Synthesis Scheme:



Chemistry Experimental Section:

5-Methyl-N^2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)pyrimidine-2,4-diamine **(3)**

At room temperature, a 50 ml microwave (MW) vessel was charged with chloropyrimidine **1** (1.6 g, 11.1 mmol), and amine **2** (2.5 g, 12.1 mmol) in 20 ml of glacial acetic acid. The vessel was then sealed and submitted to MW heating at 150°C for 20 minutes. Upon cooling, the vessel was unsealed and acetic acid was removed under reduced pressure. The residual oily solid was diluted with ethyl acetate and the resulting solution was washed with 1.0 N NaOH until pH reached 12.0. This was then dried over Na_2SO_4 and evaporated under reduced pressure to give 2.8 g of yellow-green solid. This was pure enough for use in the next step.

^{1}H NMR ($CDCl_3$, 500MHz), δ 7.79 (s, 1 H), 7.43 (d, 2 H, J = 9.7 Hz), 6.87 (d, 2 H, J = 8.7 Hz), 6.63 (bs, 1 H), 4.65 (bs, 1 H), 4.09 (t, 2 H, J = 6.2 Hz), 2.89 (t, 2 H, J = 6.2 H), 2.64-2.61 (m, 4 H), 1.99 (s, 3 H), 1.82-1.78 (m, 4 H). Calculated for $C_{17}H_{24}N_5O$ ($M+H^+$) 314.2, found 314.4.

3-Bromo-N-(tert-butyl)benzenesulfonamide (**5**)

At 0°C, under argon, a solution of bromosulfonyl chloride **4** (25 g, 98 mmol) in 700 ml of anhydrous dichloromethane was treated with tertiobutylamine (60 ml, 0.57 mol), and temperature was allowed to warm up to the ambient overnight. The reaction mixture

was then washed with water, dried and concentrated under vacuum to give a white crystalline solid of high purity.
^{1}H NMR ($CDCl_3$, 500MHz), δ 8.03 (t, 1 H, J = 1.0 Hz), 7.82 (dd, 1 H, J = 7.8 Hz, J = 1.0 Hz), 7.67 (dt, 1 H, J = 8.0 Hz, J = 1.0 Hz), 7.37 (t, 1 H, J = 8.0 Hz), 4.48 (bs, 1 H), 1.26 (s, 9H).

N-(tert-butyl)-3-((5-methyl-2-((4-(2-(pyrrolidin-1-yl)ethoxy)penyl)amino)pyrimidin-4-yl)amino)benzenesulfonamide
A mixture of 5-methyl-N^2-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)pyrimidine-2,4-diamine **3** (9.6 g, 30.6 mmol), 3-bromo-benzenesulfonamide **5** (10.3 g, 35.3 mmol), $Pd_2(dba)_3$ (1.0 g, 1.1 mmol), Xantphos (1.2 g, 2.1 mmol) and sodium tert-butoxide (5 g, 52 mmol) in dioxane (300 mL) was refluxed for 3 h. After cooling to room temperature, the resulting mixture was filtrated and the solid was washed with EtOAc. The filtrate was concentrated, and the solid residue was dissolved in EtOAc, then washed with aq. $NaHCO_3$ and brine, dried over Na_2SO_4 and filtered. The filtrate was concentrated and purified by flash chromatograph using the elution gradient 3% to 10 % 3.5 M ammonia /MeOH in dichloromethane to afford the desired product 11.3 g (64%) as a free base. ^{1}H NMR ($CDCl_3$, 500MHz), δ 8.07 (t, 1 H, J = 1.8 Hz), 7.92-7.91 (m, 2 H), 7.56 (dt, 1 H, J = 1.5 Hz, J = 0.9 Hz), 7.42-7.38 (m, 3 H), 6.91 (d, 2 H, J = 8.9 Hz), 6.71 (bs, 1 H), 6.41 (bs, 1 H), 4.44 (bs, 1 H), 4.13 (t, 2 H, J = 6.0 Hz), 2,91 (t, 2 H, J = 6.0 Hz), 2.64 (bs, 4 H), 2.13 (s, 3 H), 1.83-1.80 (m, 4 H), 1.22 (s 9 H). LRMS(ESI): calculated for $C_{27}H_{37}N_6O_3S$ ($M+H^+$) 525.3, found 525.3.

N-(tert-butyl)-3-((5-methyl-2-((4-(2-(pyrrolidin-1-yl)ethoxy)penyl)amino)pyrimidin-4-yl)amino)benzenesulfonamide dihydrogen chloride salt

N-(tert-butyl)-3-((5-methyl-2-((4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)amino)pyrimidin-4-yl)amino)benzenesulfonamide (9.84 g, 18.8 mmol) in 300 ml dioxane was slowly treated with hydrogen chloride in dioxane (4 M, 9.9 ml). After 3 hours, the mixture was filtered and the solid was washed with ether. The solid was dried under high vacuum to afford the bis-hydrogen chloride salt 11.2 g in quantitative yield. ^{1}H NMR (D_2O, 500MHz), δ 7.76 (bs, 1 H), 7.56-7.54 (M, 1 H), 7.43-7.38 (m, 3 H), 7.02, (d, 2 H, J = 8.9 Hz,) 6.73 (d, 1 H, J = 8.9 Hz), 4.10 (t, 2 H, J = 4.6 Hz), 3.52-3.50 (m, 2 H), 3.42-3.40 (m, 2 H), 1,97-1.94 (m, 2H), 1.94 (s, 3 H), 1.93-1.81 (m, 2 H), 0.90 (s, 9 H). LRMS(ESI): calculated for $C_{27}H_{37}N_6O_3S$ ($M+H^+$) 525.3, found 525.3; calculated for $C_{27}H_{35}N_6O_3S$ ($M-H^-$) 523.3, found 523.3, calcd for $C_{27}H_{36}ClN_6O_3S$ ($M+Cl^-$) 559.2, found 559.4.

TG 101348 Patent: Noronha *et al*: Application No.: US 2007/0259904

A

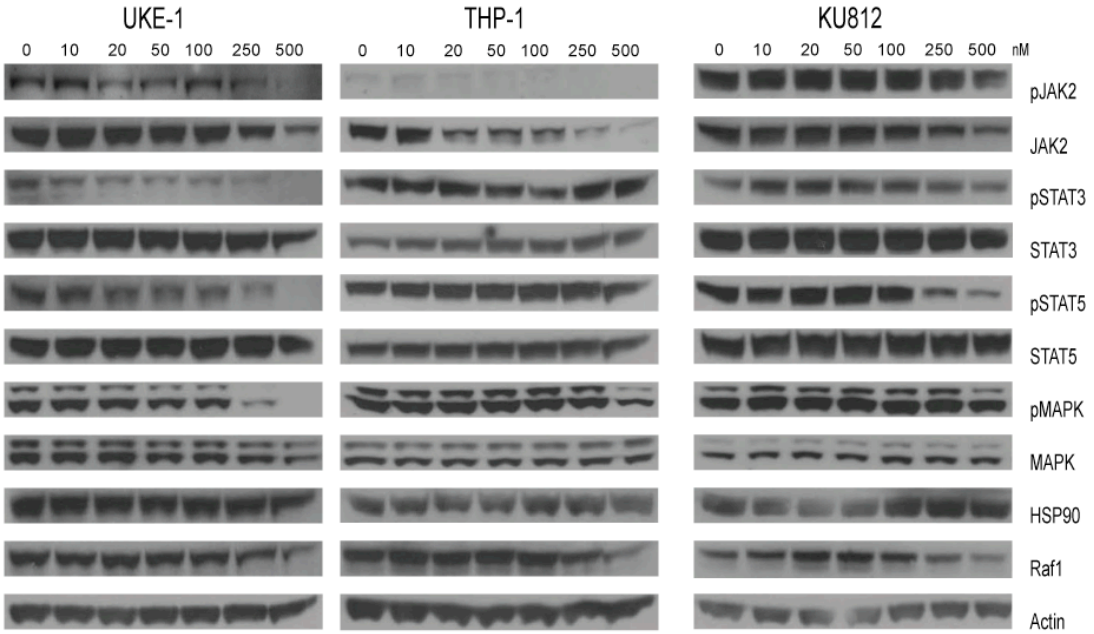
Isogenic

Cell Line	IC_{50} (nM)	SEM
Ba/F3 BCR-ABL	110.1	0.8
Ba/F3 EPOR K539L	52.3	9.3
Ba/F3 EPOR mJAK2V617F	29.0	3.0
Ba/F3 hMPLW515L	66.7	3.5
Ba/F3 hMPLW515L + mJAK2 WT	100.5	17.7

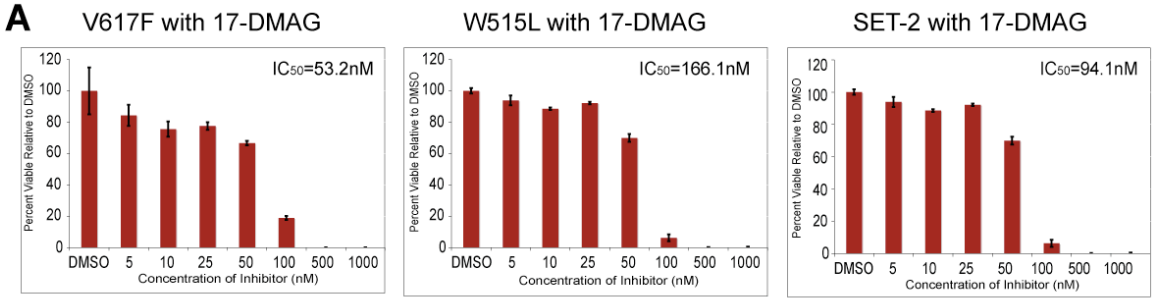
Leukemic

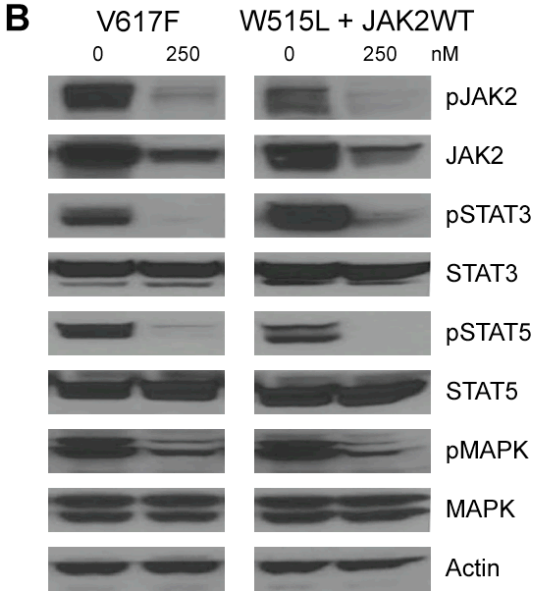
Cell Line	IC_{50} (nM)	SEM
KU812	312.8	29.3
MUTZ-5	123.8	2.5
SET-2	94.1	9.1
THP-1	152.1	52.1
UKE-1	116.7	70.0

B

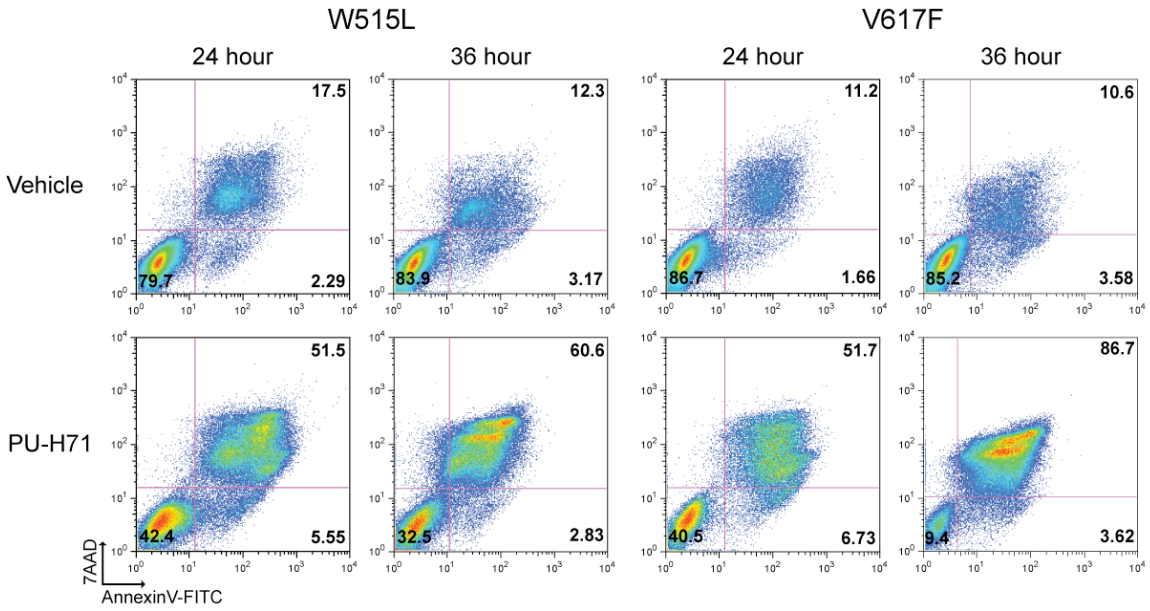


Supplemental Figure 1: Effects of PU-H71 on viability and signaling in isogenic and human leukemic cells. A) Table showing IC_{50} values of various JAK2 dependent and independent isogenic and human leukemic cell lines in 48 hour viability assays. All experiments were done in three biological replicates and data is indicated as value ± SEM. B) Western blot analyses of various human leukemic cell lines with increasing concentrations of PU-H71 shows dose dependent degradation of JAK2 and downmodulation of signaling intermediates. HSP90 and Actin are loading controls.

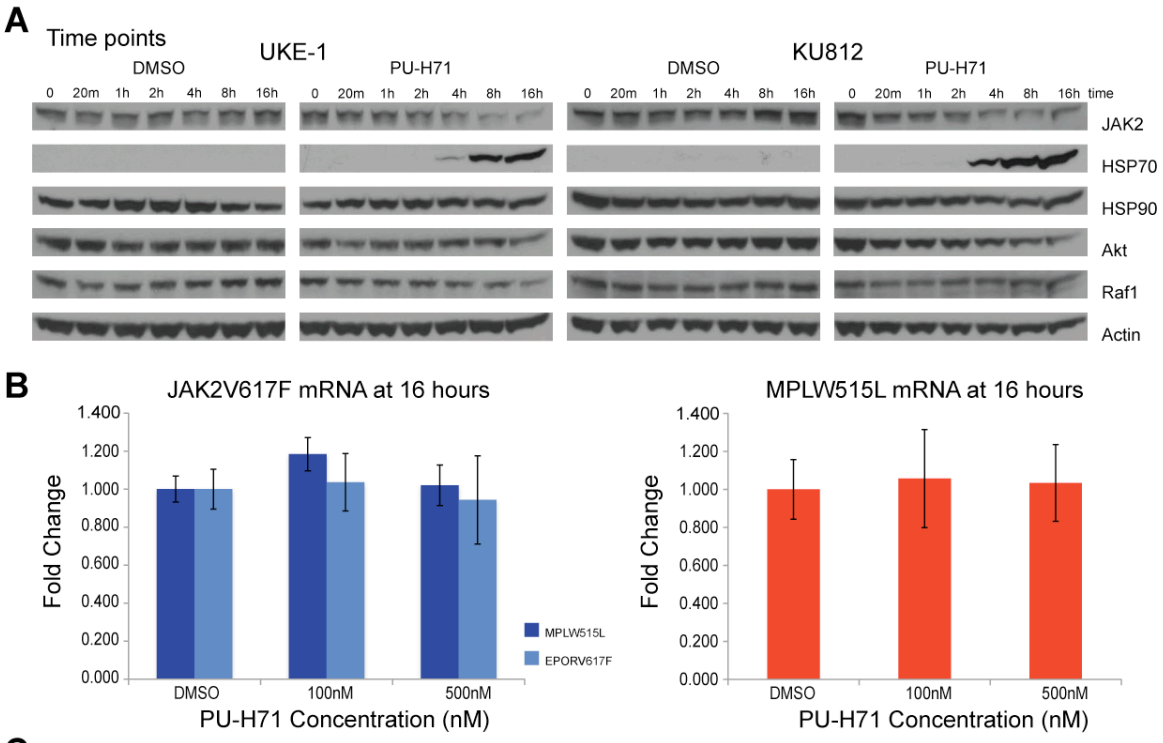




Supplemental Figure 2: Evidence for on-target mechanism of action of HSP90 inhibitors using 17-DMAG. Growth inhibition and biochemistry of isogenic cells and human leukemic cells treated with 17-DMAG, another HSP90 inhibitor. A) Inhibitor assays with isogenic and human leukemic cell lines with constitutively activated JAK-STAT pathway show inhibition with 17-DMAG, similar to PU-H71. B) Western blots of isogenic cells lines Ba/F3 EPOR-V617F and Ba/F3 MPLW515L overexpressing the wild type JAK2 shows a decrease in JAK2 levels after treatment with 17-DMAG.



Supplemental Figure 3: PU-H71 induces apoptosis in MPN cells. Ba/F3 isogenic cell lines bearing MPLW515L or JAK2V617F mutations show rapid induction of apoptosis and necrosis upon PU-H71 treatment in comparison with vehicle treated samples. Apoptosis was measured by Annexin V FITC staining.



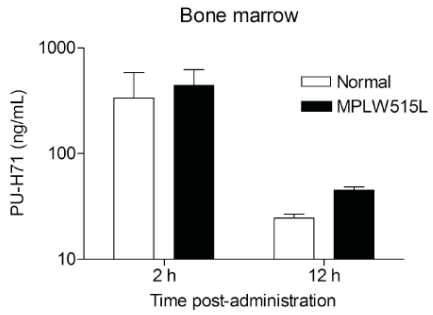
Supplemental Figure 4: JAK2 is rapidly degraded by PU-H71. A) Cells were incubated with either DMSO or PU-H71 (250nM) for different time points and then were harvested for western blotting analyses. JAK2 degradation following PU-H71 treatment begins at 4 hours. B) mRNA levels of JAK2 or MPL remain unchanged upon incubation with PU-H71. Cells were incubated with either DMSO or different concentrations of PU-H71 for 16 hours. C) Ba/F3-EPOR-V617F show a shift in viability from 16 to 48 hours. At shorter time points, higher concentrations of PU-H71 were required to inhibit proliferation of JAK2 mutant cells indicating that the effects of PU-H71 on viability and signaling were comparable at shorter time points.

A

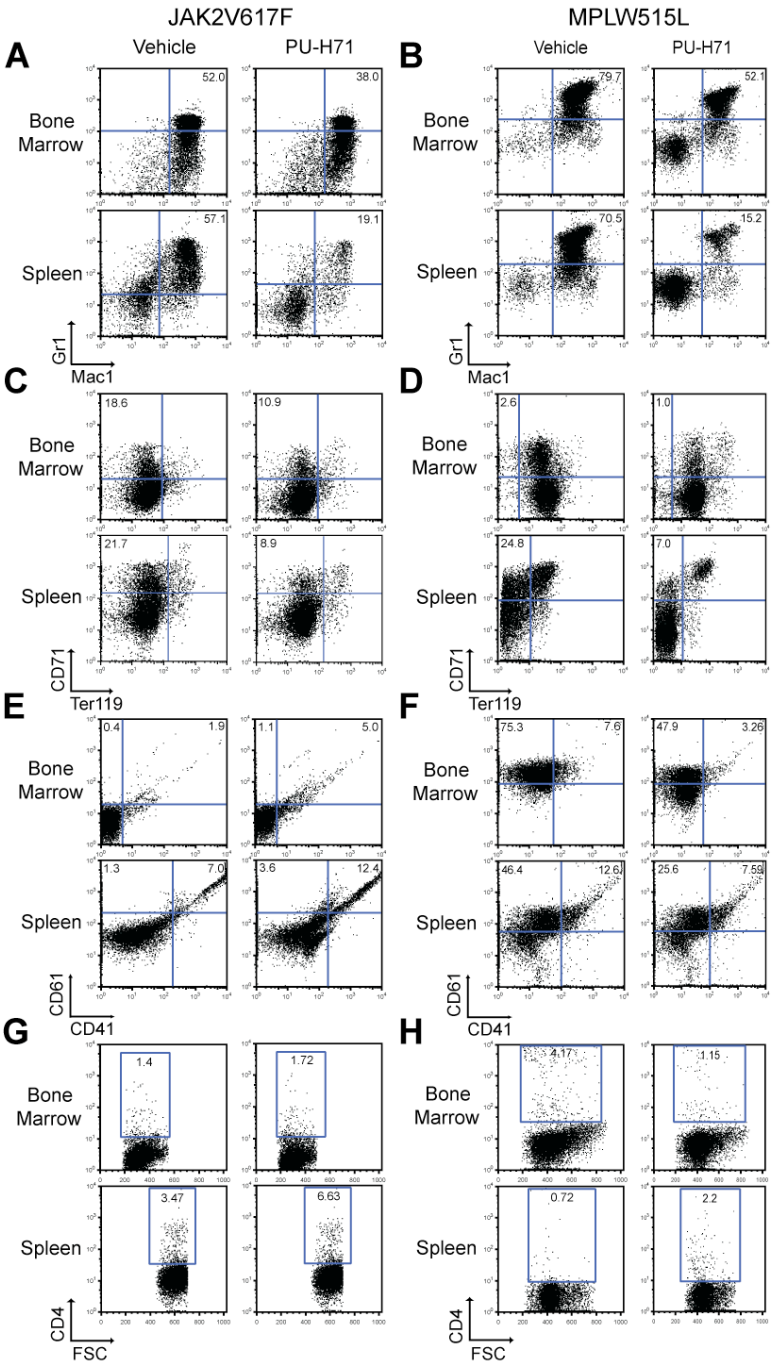
	Day 1 JAK2V617F		Day 15 JAK2V617F		Day 29 JAK2V617F	
	Vehicle	75mg/kg PU-H71	Vehicle	75mg/kg PU-H71	Vehicle	75mg/kg PU-H71
WBC K/μL	39 ± 7	44 ± 10	69 ± 17	16 ± 1	73 ± 13	21 ± 2
Hematocrit %	73 ± 3	75 ± 2	74 ± 3	60 ± 3	80 ± 2	63 ± 2
Platelet K/μL	741 ± 56	724 ± 35	722 ± 61	539 ± 93	660 ± 55	783 ± 46

	Day 1 MPLW515L		Day 15 MPLW515L		Day 29 MPLW515L	
	Vehicle	75mg/kg PU-H71	Vehicle	75mg/kg PU-H71	Vehicle	75mg/kg PU-H71
WBC K/μL	157 ± 7	152 ± 6	570 ± 129	60 ± 10	N/A	51 ± 9
Hematocrit %	55 ± 2	57 ± 1	59 ± 1	59 ± 2	N/A	54 ± 3
Platelet K/μL	2345 ± 122	2386 ± 57	2996 ± 333	1194 ± 85	N/A	1455 ± 248

B



Supplemental Figure 5: Vehicle and PU-H71 treated mouse blood counts over time, and retention of PU-H71 in bone marrow of MPLW515L mice. A) Blood counts of mice treated with either vehicle or PU-H71 over the course of treatment. Peripheral blood counts of MPLW515L and JAK2V617F mice treated with either vehicle or PU-H71 at randomization (Day1), Day 15 and at end of trial (Day 29 following treatment). B) PU-H71 levels in bone marrow tissue at 2 and 12 hours after a single 75 mg/kg dose of PU-H71 shows a higher level of PU-H71 retained in the MPLW515L mice at 12 hours post administration compared to normal mice.



Supplemental Figure 6: Flow cytometry analyses of PU-H71 and vehicle treated bone marrow and spleen. Flow analyses showed decreased number of Gr1/ Mac1 positive neutrophils with PU-H71 treatment in both JAK2V617F and MPLW515L bone marrow and spleen (A and B). Further, PU-H71 treated JAK2V617F bone marrow and spleen cells and MPLW515L spleen cells showed a significant decrease in CD71+ erythroid progenitor cells (C and D), while there was no marked change in CD71+ MPLW515L bone marrow with PU-H71 treatment (D). MPLW515L CD61+ and CD41+ CD61+ double positive megakaryocyte population also decreased with PU-H71 treatment (F) while JAK2V617F bone marrow showed no significant difference between CD61+ and CD41+CD61+ double positive populations with treatment (E). No difference was seen in either JAK2V617F or MPLW515L CD4+ levels with vehicle or PU-H71 treatment (G and H).