

Selective Downregulation of JAK2 and JAK3 by an ATP-Competitive pan-JAK Inhibitor.

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Chemicals and reagents. PF-956980 is commercially available from Sigma Aldrich.¹ Carfilzomib was purchased from Apexbio Technology LLC (Cat. No. A1933, 50-101-3564). MG132 was purchased from CalBioChem (Cat. No. 474790). RPMI 1640 (11875093), HI FBS (Cat. No. 16140-071), D-PBS (Cat. No. 14190250), M-Per™ Mammalian Protein Extraction Reagent (Cat. No. 78501), Halt™ Protease + Phosphatase inhibitor cocktail (100X) (Cat. No. 78442), DMSO, NuPage® LDS Sample Buffer (4X) (Cat. No. NP0007), NuPage® Sample Reducing Agent (10X) (Cat. No. NP0009), NuPAGE® MOPS SDS Running Buffer (20X) (Cat. No. NP0001), SuperBlock™ T20 (TBS) Blocking Buffer (Cat. No. 37536), SuperSignal™ West Femto Maximum Sensitivity Substrate (Cat. No. 34095) were purchased from ThermoFisher Scientific (Waltham, MA, USA). PBMCs (Cat. No 70025.3, Lot# 1603210135) were purchased from STEMCELL (Vancouver, BC, Canada). JAK1 antibody (ab133666) was purchased from Abcam (Cambridge, MA, USA). JAK2 antibody (3230L), JAK3 antibody (5481S), TYK2 antibody (9312S), p38 antibody (9212S), ERK2 antibody (9108S), β -actin antibody (4967L), Anti-Rabbit IgG HRP-linked antibody (7074S), anti-Mouse IgG HRP-linked antibody (7076S) were purchased from Cell Signaling Technology (Beverly, MA, USA). Incubations were done at 37°C with 5% CO₂ in HeraCell™ incubators unless otherwise noted. For qPCR, probes for JAK1 (Hs01026983_m1), JAK2 (Hs00234567_m1), JAK3 (Hs00169663_m1), TYK2 (Hs00177464_m1) and GAPDH (Hs02758991_g1) were purchase from ThermoFisher Scientific (Waltham, MA, USA).

PF-956980 selectivity

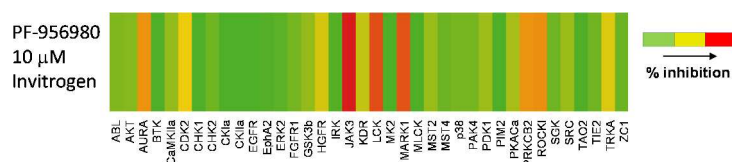


Figure S1. Kinase selectivity of PF-956980 at 10 μ M (Invitrogen).

Table S1. Kinase selectivity of PF-956980 at 1 and 10 μ M expressed as a percentage inhibition (Invitrogen)

Kinase	% Inhibition at 1 μM	% Inhibition at 10 μM
ABL	1	14.5
AKT	1	16.5
AURA	18	65.5
BTK	-3	-8
CaMKIIa	5.4	20.5
CDK2	5.6	48
CHK1	-5.4	-6.5
CHK2	-1	9.5
CKIa	2	-3.5
CKIIa	1.5	0
EGFR	-1.3	-5
EphA2	-2.9	1.5
ERK2	2.1	3.5
FGFR1	2.6	11
GSK3b	-3.8	21
HGFR	7.6	40
IRK	0.8	1.5
JAK3	98.3	99
KDR	1.3	34.5
LCK	34	85
MK2	-0.8	-1
MARK1	42.3	83
MLCK	5.3	-7
MST2	-9	22
MST4	4.6	8.5
P38	-0.4	12
PAK4	4.1	12
PDK1	-6.3	23.5

PIM2	-6.5	-7.5
PKACa	2.5	26.5
PRKCB2	30.1	65.5
ROCK1	9.9	67
SGK	-2	9.5
SRC	5.8	23
TAO2	-4.8	-4
TIE2	-4.4	10
TRKA	24.3	42.5
ZC1	3	8.5

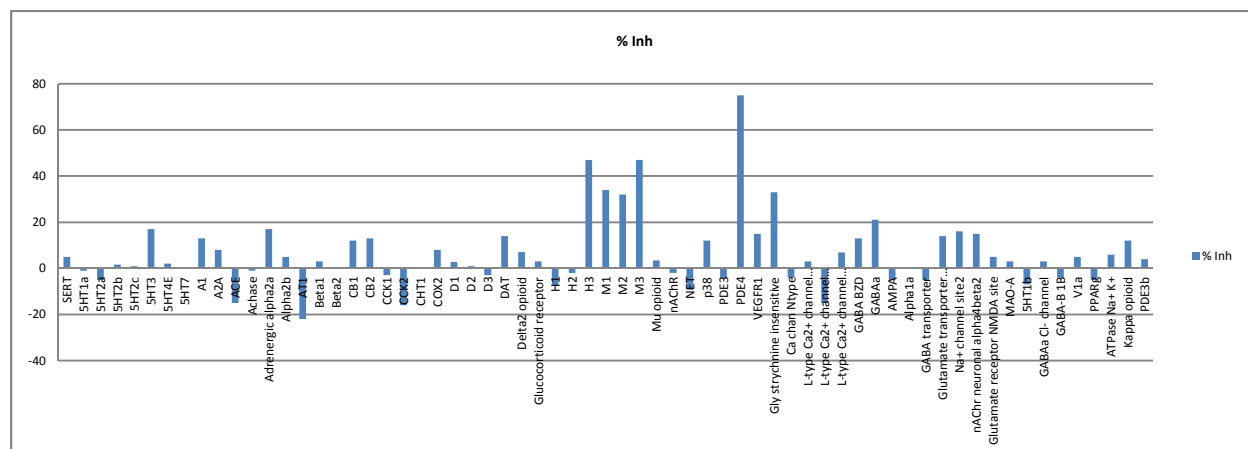


Figure S2. CEREP wide-ligand selectivity profile of PF-956980 at 10 μ M (only PDE4 is inhibited >50%).

Caliper JAK Enzyme Endpoint IC_{50} Assays. Assays to determine the IC_{50} of PF-956980 were carried out as previously described.² Specifically, the human JAK activity was determined using a microfluidic assay to monitor phosphorylation of a synthetic peptide by the recombinant human kinase domain of each of the four members of the JAK family. Each assay condition was optimized for enzyme concentration and room temperature incubation time to obtain a conversion rate of 20% - 30% phosphorylated peptide product. 250 nL of PF-956980 solubilized in 100% DMSO was added to a 384 well polypropylene plate (MatricalMP101 or Corning Costar 3676) using an non-contact acoustic dispenser. Kinase assays were carried out at room

temperature in 15 μ L reaction buffer containing 20 mM HEPES pH 7.4, 1 mM ATP, 10 mM magnesium chloride, 0.01% bovine serum albumin (BSA), 0.0005% Tween 20 and 1mM DTT. Reaction mixtures contained 1 μ M of a fluorescently-labeled synthetic peptide, a concentration less than the apparent K_m . The JAK1 and TYK2 assays contained 1 μ M of the peptide 5FAM-KKSRGDYMTMQID and the JAK2 and JAK3 assays contained 1 μ M of the peptide FITC-KGGEEEEYFELVKK. The assays were initiated by the addition of enzyme. The assays were stopped with 15 μ L of a buffer containing 180 mM HEPES, pH=7.4, 20 mM EDTA, 0.2% Coating Reagent, resulting in a final concentration of 10 mM EDTA, 0.1% Coating Reagent and 100 mM HEPES, pH=7.4. Utilizing the LabChip 3000 mobility shift technology (Caliper Life Science), each assay reaction was sampled to determine the level of phosphorylation. This technology is separation-based, allowing direct detection of fluorescently labeled substrates and products. Separations are controlled by a combination of vacuum pressure and electric field strength optimized for each peptide substrate.

PBMC treatment with PF-956980. PBMCs were thawed at 37°C and washed with warm 10% FBS in RPMI 1640. Cells were pelleted (1200 rpm, 5 minutes, room temperature) and medium aspirated. Cells were resuspended in FBS and incubated at 37°C with 5% CO₂ for 1.5-2 hours. After incubation, 47 mL of DPBS was added to the cells and they were pelleted (1200 rpm, 5 minutes, room temperature). The medium was removed and cells were resuspended in D-PBS to determine cell viability and cell count (Vi-Cell XR, Beckman Coulter). PBMCs were pelleted and resuspended in RPMI 1640 with a concentration of 16-20 x 10⁶ PBMCs/mL. The cell suspension was dispensed into 4 x 1 mL sample wells and incubated with either DMSO, 0.1 μ M, 1 μ M, or 10 μ M PF-956980 (0.1% DMSO content) at 37°C with 5% CO₂. 500 μ L aliquots were removed at 4 hours and 22 hours and cells were pelleted. The medium was aspirated, cells were washed with cold D-PBS, and again pelleted. The pellet was resuspended in 100 μ L of M-PER Lysis Buffer + 1X Halt Protease and Phosphatase inhibitor cocktail. The lysis occurred on ice for 30 minutes, the insoluble fraction was pelleted (13,000 rpm, 10 minutes, 4°C) and separated from the soluble fraction. Cell lysates were stored at -20°C.

Western blots. Cell lysate was mixed with 1X NuPage® LDS Sample Buffer and 1X NuPage® Reducing Agent then heated to 90°C for 10 minutes. Denatured samples were loaded

into a NuPage™ Novex™ 4-12% Bis-Tris Protein Gel and gel electrophoresis was performed using NuPAGE® MOPS SDS Running Buffer (1X) (200V, 1.5A, 50 minutes) and proteins transferred to iBlot2® transfer stacks PDVF membrane (P0 protocol). The membrane was blocked for 1 hour at room temperature using SuperBlock™ T20 (TBS) Blocking Buffer. Primary antibodies were diluted into blocking buffer (1:1,000) and incubated at 4°C overnight with agitation. After incubation, the primary antibody was removed and membranes washed with TBST (3 x 5 minutes). The membranes were incubated with secondary antibody (1:5,000) in TBST + 10% blocking buffer for 1 hour at room temperature with agitation. After incubation, the membrane was washed with TBST (3 x 5 minutes) and developed using SuperSignal™ West Femto Maximum Sensitivity Substrate. Membranes were visualized using a Kodak Image Station. Band intensity was normalized to DMSO for each condition.

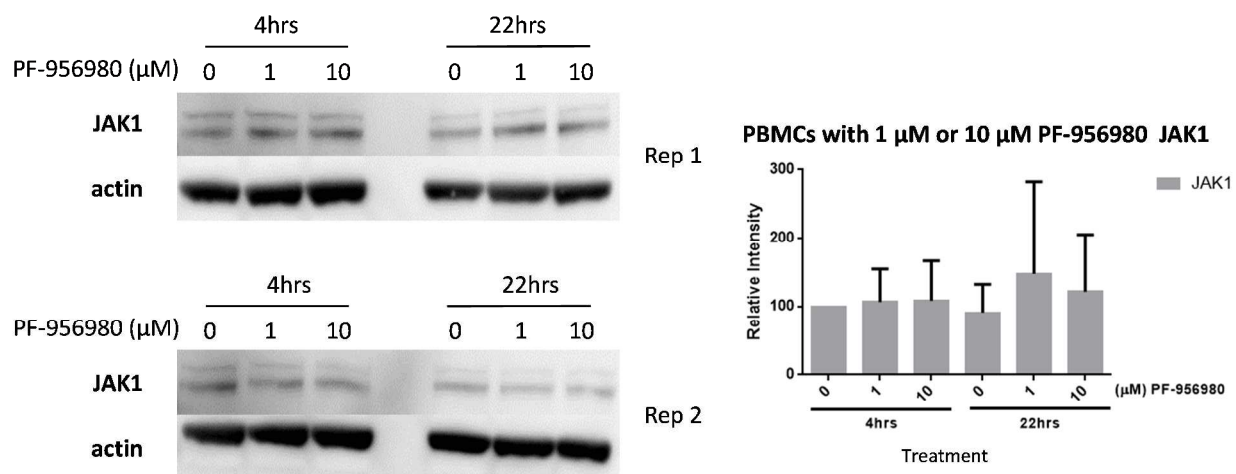


Figure S3. PF-956980 does not reduce JAK1 levels in PBMCs.

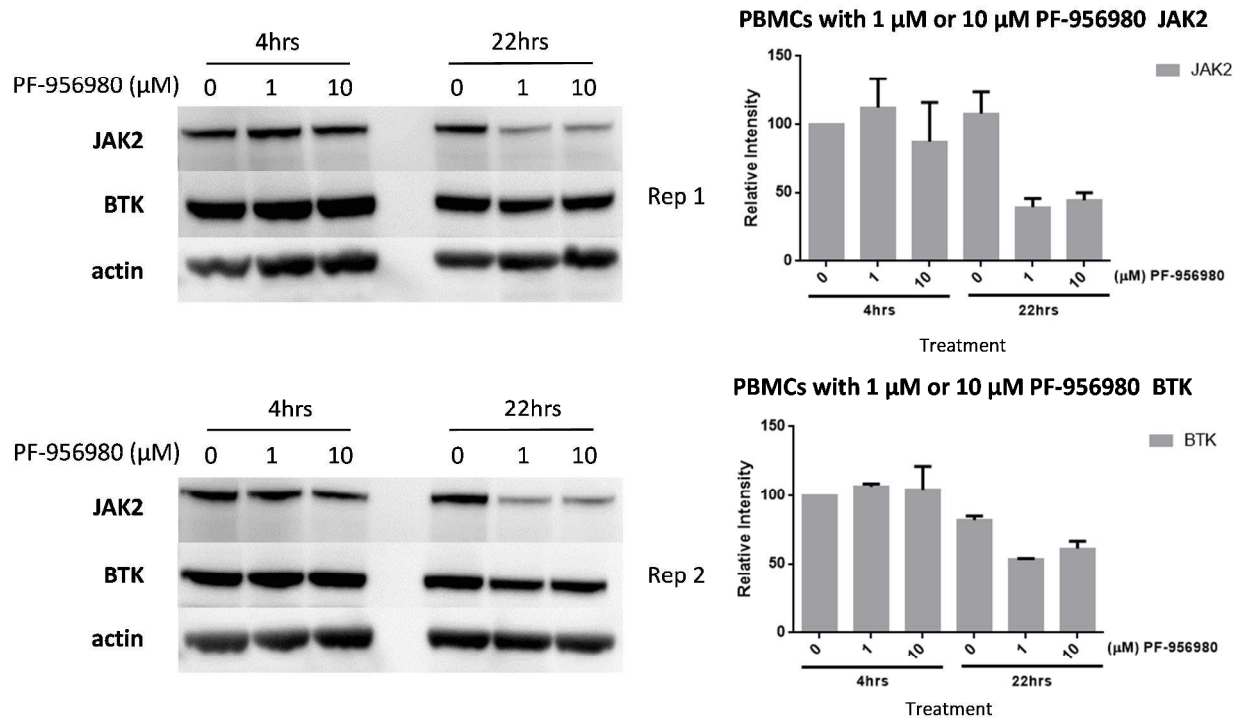


Figure S4. PF-956980 significantly reduces the levels of JAK2, but not BTK, in PBMCs.

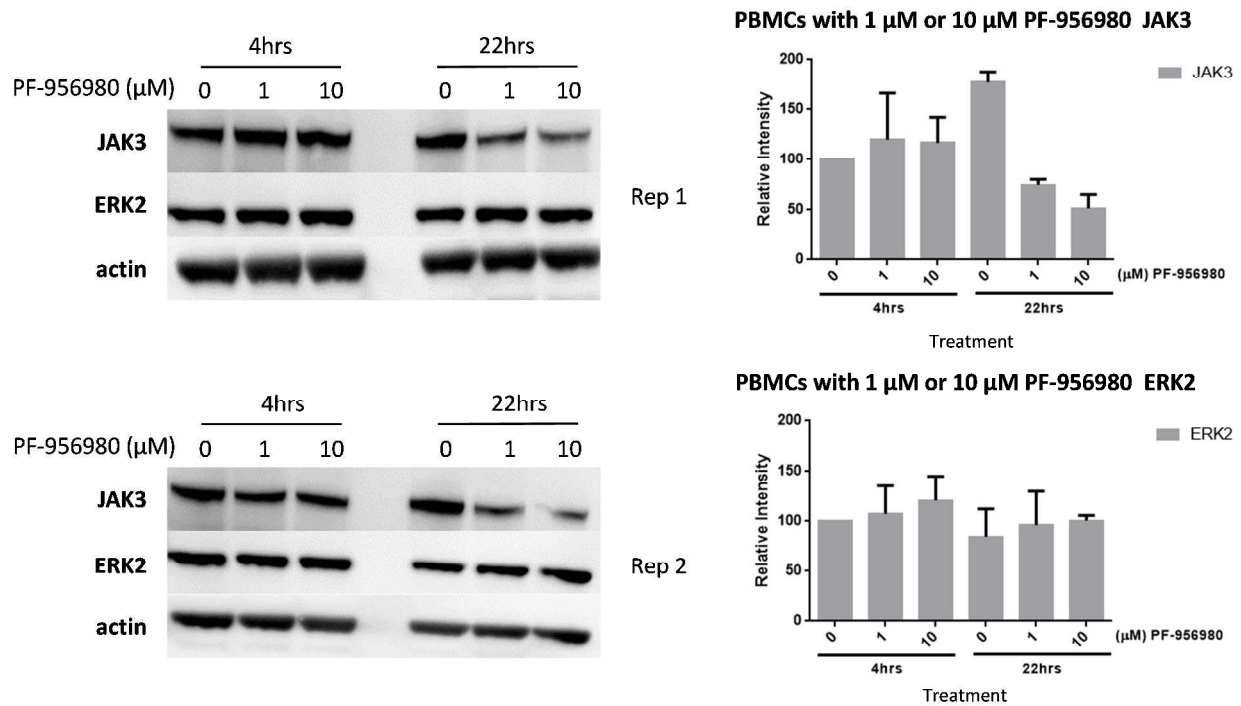


Figure S5. PF-956980 reduces the levels of JAK3, but not ERK2, in PBMCs.

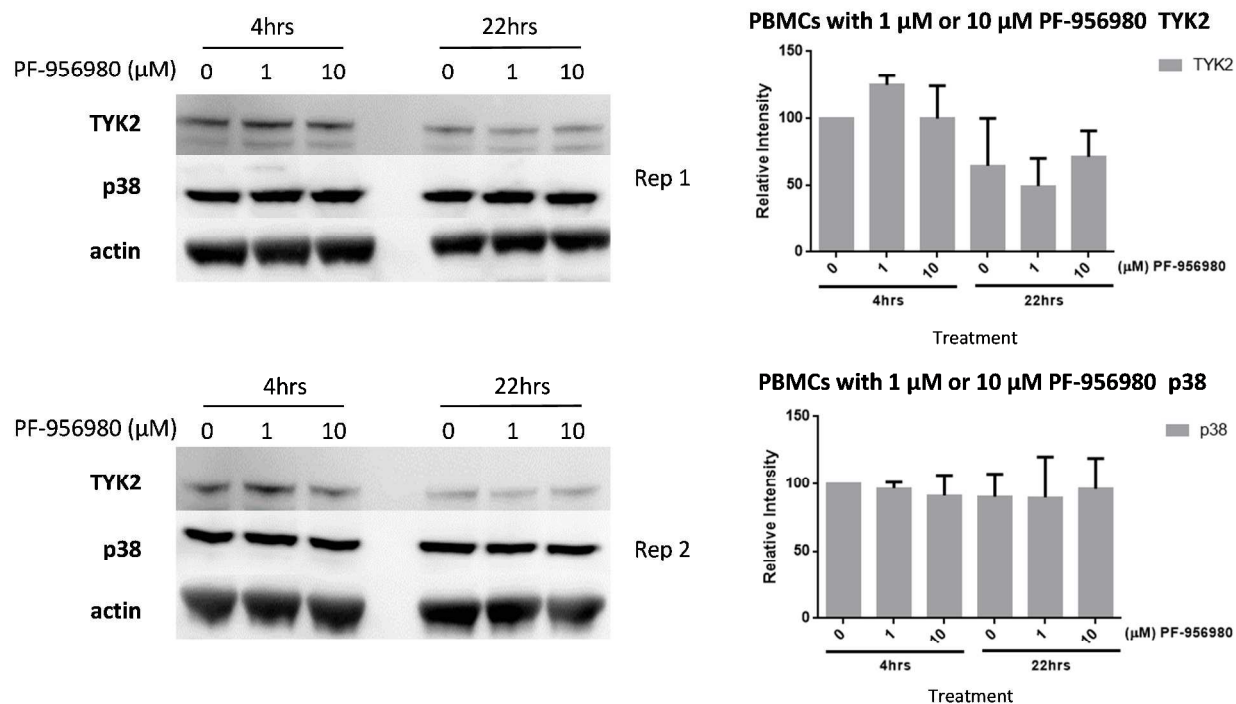


Figure S6. PF-956980 does not change the levels of TYK2 or p38 in PBMCs

PBMC treatment with MG132, PF-956980 or combination. PBMCs were thawed and counted according to the procedure described above. PBMCs were pelleted and resuspended in RPMI 1640 with a concentration of $8-10 \times 10^6$ PBMCs/mL. The cell suspension was dispensed into 4 x 1 mL sample wells and labelled with DMSO, 1 μM PF-956980, 1 μM MG132, or 1 μM PF-956980 + 1 μM MG132. Wells with MG132 were pretreated for 1hr at 37°C before addition of 1 μM PF-956980 and incubated at 37°C with 5% CO₂. 500 μL aliquots of cells were pelleted at 4 hrs and 22 hrs. The medium was aspirated, cells were washed with cold D-PBS, and again pelleted. The pellet was resuspended in 100 μL of M-PER Lysis Buffer + 1X Halt Protease and Phosphatase inhibitor cocktail. The lysis occurred on ice for 30 minutes, the insoluble fraction was pelleted (13,000 rpm, 10 minutes, 4°C) and separated from the soluble fraction. Cell lysates were stored at -20°C. Western blots for proteins were conducted as stated above.

PBMC treatment with Carfilzomib, PF-956980 or combination. PBMCs were thawed and counted according to the procedure described above. PBMCs were pelleted and resuspended in RPMI 1640 with a concentration of $8-10 \times 10^6$ PBMCs/mL. The cell suspension was dispensed

into 5 x 1 mL sample wells and incubated with either DMSO, 1 μ M of PF-956980, 5 μ M Carfilzomib, 1 μ M PF-956980 + 5 μ M Carfilzomib, or 10 μ M PF-956980 + 5 μ M Carfilzomib (0.2% DMSO content) at 37°C with 5% CO₂. Cells were pelleted after 22 hrs. The medium was aspirated, cells were washed with cold D-PBS, and again pelleted. The pellet was resuspended in 100 μ L of M-PER Lysis Buffer + 1X Halt Protease and Phosphatase inhibitor cocktail. The lysis occurred on ice for 30 minutes, the insoluble fraction was pelleted (13,000 rpm, 10 minutes, 4°C) and separated from the soluble fraction. Cell lysates were stored at -20°C. Western blots for proteins were conducted as stated above.

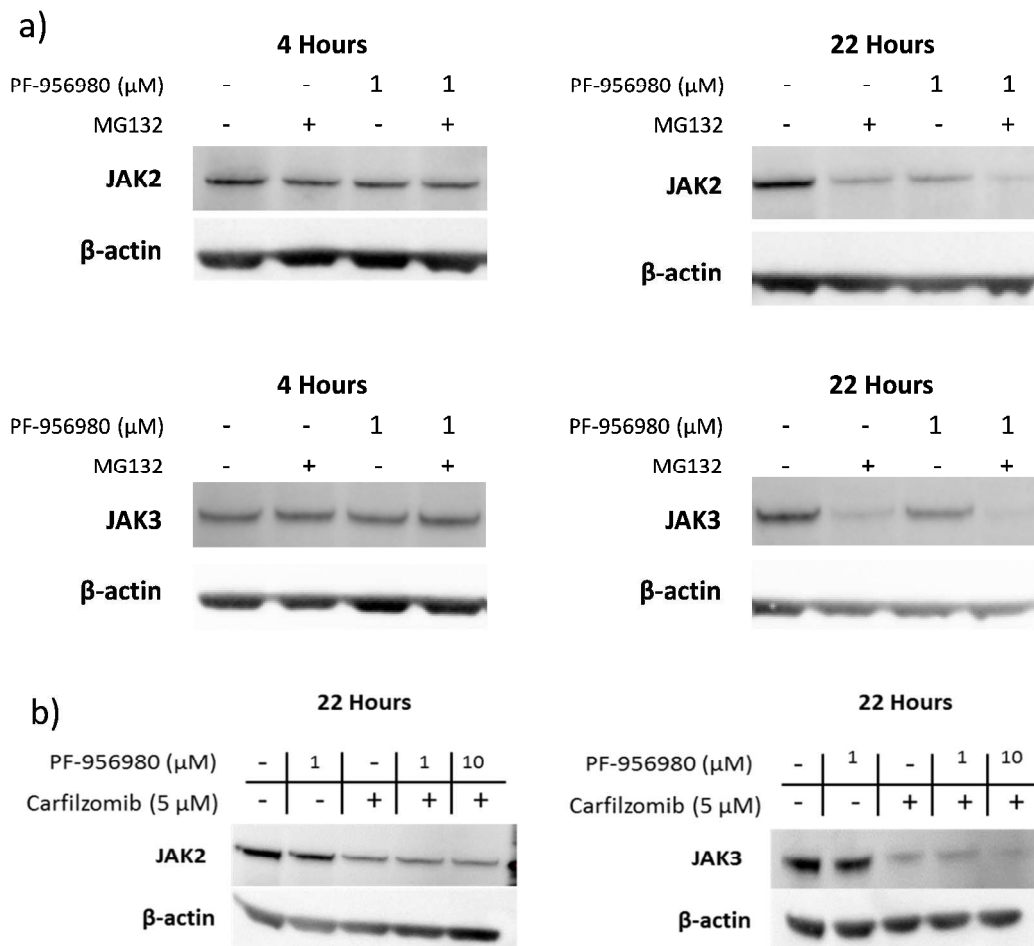


Figure S7. a) PBMCs were treated with DMSO, 1 μ M PF-956980 alone or pretreated with 1 μ M proteasome inhibitor MG132 followed by 1 μ M PF-956980 for 4 and 22 hours at 37°C. MG132 reduces JAK2 and JAK3 levels in PBMCs at 22 hours. b) PBMCs were treated with DMSO, 5 μ M Carfilzomib, 1 μ M PF-956980, a combination of 5 μ M Carfilzomib + 1 μ M PF-956980, or 5 μ M Carfilzomib + 10 μ M PF-956980 for 22 hours at 37°C. Carfilzomib also reduces JAK2 and JAK3 levels in PBMCs at 22 hours.

Treatment of PBMCs with cycloheximide. PBMCs were thawed and counted according to the procedure above. PBMCs were pelleted and resuspended in RPMI 1640 with a concentration of $8-10 \times 10^6$ PBMCs/mL. The cell suspension was dispensed into 10 x 1.5 mL sample wells and incubated with either DMSO or 1 μ M of PF-956980 for 1hr. PBMCs were then treated with 100 μ g/mL cycloheximide and incubated at 37°C with 5% CO₂. Aliquots were taken at 0 hr, 2hr, 4hr, 8hr, and 24hr and the cells were pelleted. The medium was aspirated, cells were washed with cold D-PBS, and again pelleted. The pellet was resuspended in 100 μ L of M-PER Lysis Buffer + 1X Halt Protease and Phosphatase inhibitor cocktail. The lysis occurred on ice for 30 minutes, the insoluble fraction was pelleted (13,000 rpm, 10 minutes, 4°C) and separated from the soluble fraction. Cell lysates were stored at -20°C. Western blots for proteins were conducted as stated above.

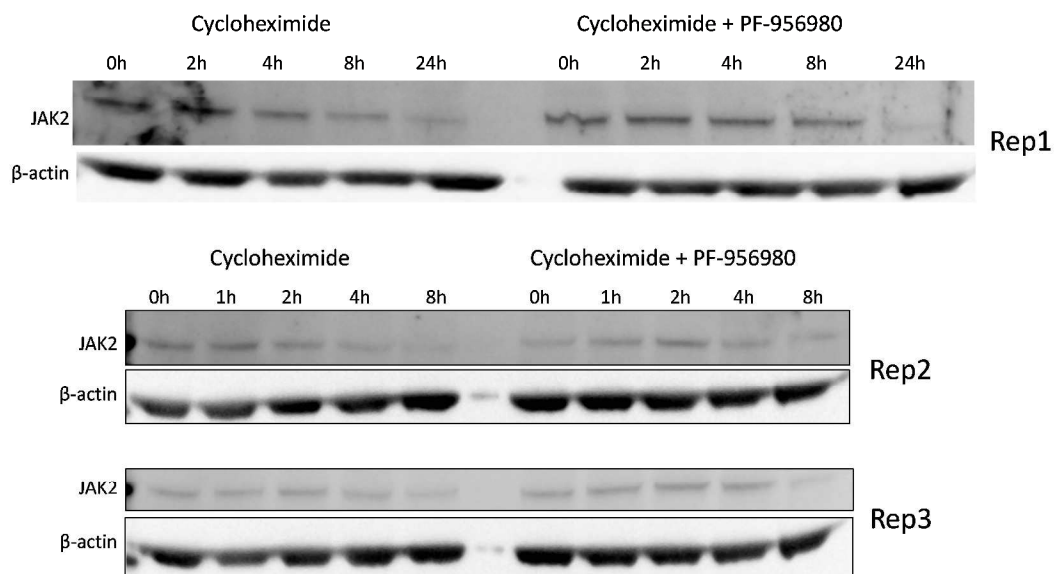


Figure S8. PBMCs were treated with 100 μ g/mL cycloheximide alone or 100 μ g/mL cycloheximide followed by 1 μ M PF-956980 at 37°C, and JAK2 levels were assessed in 3 separate experiments.

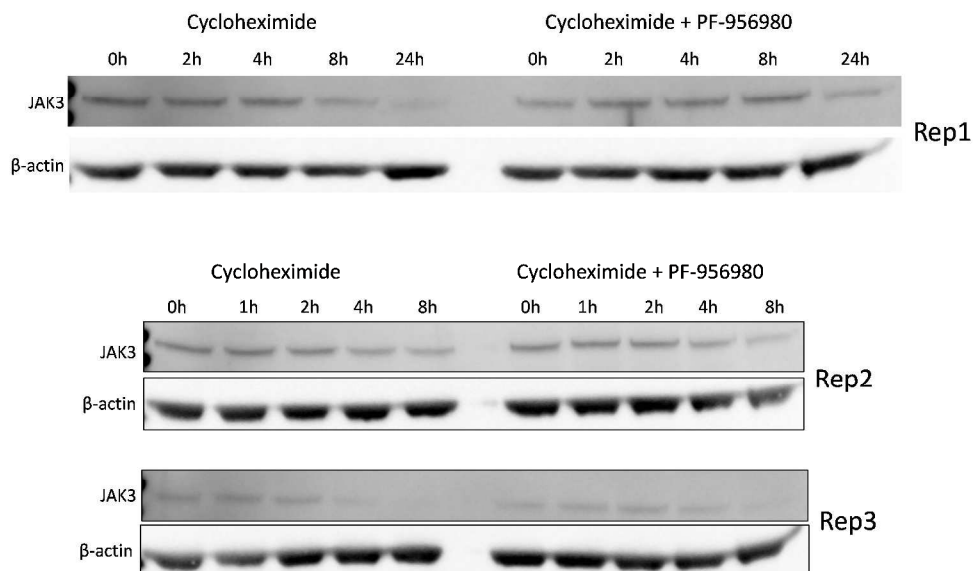


Figure S9. PBMCs were treated with 100 $\mu\text{g/mL}$ cycloheximide alone or 100 $\mu\text{g/mL}$ cycloheximide followed by 1 μM PF-956980 at 37°C, and JAK3 levels were assessed in 3 separate experiments.

qPCR to measure JAK family RNA levels. 5×10^6 PBMCs were treated with PF-956980 as reported above and pelleted after incubating for 22 hours (37°C, 5% CO_2). RNA was extracted using Qiagen RNeasy Mini Kit (Cat. No. 74106) following their Quick-Start Protocol using On-column DNase digestion (Qiagen RNase-free DNase set Cat. No. 79254). RNA concentration was measured using a Nanodrop and qPCR was conducted using TaqMan® RNA-to-Ct™ 1-Step kit (Cat. No. 4392938) following the Component (Applied Biosystems Gene Expression Assays) protocol (50 ng of template RNA in 10 μL reaction volume) using a QuantStudio 12K Flex Real-Time PCR instrument. RNA levels were normalized to GAPDH and fold change relative to DMSO was calculated using $\Delta\Delta\text{Ct}$.

1. The structure of PF-956980 (CAS 1262750-62-8) provided by Sigma Aldrich is correct:

<http://www.sigmaaldrich.com/catalog/product/sigma/pz0151>.

2. Telliez, J.-B.; Dowty, M. E.; Wang, L.; Jussif, J.; Lin, T.; Moy, E.; Balbo, P.; Li, W.; Zhao, Y.; Crouse, K.; Dickinson, C.; Symanowicz, P.; Hegen, M.; Banker, M. E.; Vincent, F.; Unwalla, R.; Liang, S.; Gilbert, A. M.; Brown, M. F.; Hayward, M.; Montgomery, J.; Yang, X.; Bauman, J.; Trujillo, J. I.; Casimiro-Garcia, A.; Vajdos, F. F.; Leung, L.; Geoghegan, K. F.; Quazi, A.; Xuan, D.; Jones, L. H.; Hett, E.; Wright, K.; Clark, J. D.; Thorarensen, A., Discovery of a novel JAK3-selective inhibitor: Functional differentiation of JAK3-selective inhibition over pan-JAK or JAK1-selective inhibition. *ACS Chem. Biol.* **2016**, *11*, 3442-3451.