Supporting Information

Discovery and Optimization of C-2 Methyl Imidazo- pyrrolopyridines as Potent and Orally Bioavailable JAK1 Inhibitors with Selectivity over JAK2

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Table of Contents:

Page(s)	Information
S3	<i>In vitro</i> hepatocyte metabolic stability: experimental details
S3	<i>In vitro</i> microsome metabolic stability experiments: references
S3	Reversible CYP inhibition experiments: references
S3 – S4	Time-dependent CYP inhibition experiments: references
S4	MDCK permeability experiments: references
S4 – S5	<i>In vivo</i> PK: experimental details
S5 – S6	JAK enzyme assays: experimental details
S6	JAK pathway cellular assays: experimental details
S6 – S7	Human whole blood assays: experimental details
S7	IL-6 induced pSTAT3 PK/PD study of compound 20 in the mouse: experimental details
S8	hERG assay: experimental details
S8 – S9	Quantum mechanical computational methods
S9 – S12	X-ray crystallography: experimental methods, data collection, and refinement summary
S12 – S13	Measured LogD _{7.2} : experimental procedures
S13	Measured thermodynamic solubility (pH _{7.4} and pH _{2.0}): experimental procedures
S14	Analytical LCMS and SFC conditions
S15	Biochemical inhibition of JAK1, JAK2, JAK3, and TYK2 by compounds 3 – 24 .

In vitro hepatocyte metabolic stability experiments were carried out as follows:

The metabolic stability of test compounds (1 μ M test concentration) was determined in human, Sprague–Dawley rat, CD-1 mouse, beagle dog, and cynomolgus monkey, cryopreserved primary hepatocytes (CellzDirect Invitrogen Corporation, Durham, NC, USA, pooled donors). Incubations (final incubation volume = 250 μ L) were performed at 37 °C under an atmosphere of 95%/5% CO₂/O₂ using 0.5 million cells mL⁻¹ in hepatocyte incubation media (In Vitro Technologies, Baltimore, MD, USA). 50- μ L aliquots (cells and media) were taken at zero, 1, 2 and 3 h following the start of the incubation and quenched using 100 μ L of ice-cold acetonitrile. The resulting samples were centrifuged at 3000g for 10 min. Supernatant (90 μ L) was diluted in water (180 μ L) and the percent remaining test compound was assessed by LC/MS/MS. All incubations were performed in quadruplicate.

In vitro microsome metabolic stability experiments were carried out as described in:

Halladay, J. S.; Wong, S.; Jaffer, S. M.; Sinhababu, A. K.; Khojasteh-Bakht, S. C. Metabolic stability screen for drug discovery using cassette analysis and column switching. *Drug Metab. Lett.* **2007**, *1*, 67–72.

Reversible CYP inhibition experiments were carried out as described in:

Halladay, J. S.; Delarosa, E. M.; Tran, D.; Wang, L.; Wong, S.; Khojasteh-Bakht, S. C. High-throughput, 384-well, LC-MS/MS CYP inhibition assay using automation, cassette-analysis technique, and streamlined data analysis. *Drug Metab. Lett.* **2011**, *5*, 220–230.

Time-dependent CYP inhibition experiments were carried out as described in:

(a) Mukadam, S.; Tay, S.; Tran, D.; Wang, L.; Delarosa, E. M.; Khojasteh, S. C.; Halladay, J. S.; Kenny, J. R. Evaluation of Time-Dependent Cytochrome P450 Inhibition in a High-Throughput, Automated Assay: Introducing a Novel Area Under the Curve Shift Approach. *Drug Metab. Lett.* **2012**, *6*, 43–53.

(b) Obach, R. S.; Walsky, R. L.; Venkatakrishnan, K. Mechanism-based inactivation of human cytochrome p450 enzymes and the prediction of drug-drug interactions. *Drug Metab. Dispos.* **2007**, *35*, 246–255.

MDCK permeability experiments were carried out as described in:

(a) Plise, E. G.; Sindelar, E.; Duck, B.; Cheong, J.; Sodhi, J.; Salphati, L. Fully Automated 24-Well Madin-Darby Canine Kidney (MDCK) Permeability Assay Utilizing the Hamilton Microlab Star Liquid handling Platform. AAPS Annual Meeting 2009.

AAPS Abstract: http://www.aapsj.org/abstracts/AM_2009/AAPS2009-001769.PDF

Hamilton Robotics website:

http://www.hamiltonrobotics.com/fileadmin/user_upload/prodb/app_notes/Metabolomics/Genentech2.pdf

(b) Irvine, J. D.; Takahashi, L.; Lockhart, K.; Cheong, J.; Tolan, J. W.; Selick, H. E.; Grove, J. R. MDCK (Madin-Darby canine kidney) cells: a tool for membrane permeability screening. *J. Pharm. Sci.* **1999**, *88*, 28–33.

In vivo PK experiments were carried out as follows:

The pharmacokinetics of test compounds were evaluated following a single intravenous bolus (IV) of solution at a dose of 0.2 – 1 mg/kg and oral administration (PO) of solution/suspension at doses of 1 – 5 mg/kg in male cynomolgus monkey, beagle dogs, or sprague dawley rats using a parallel study design. Blood samples for the IV dose group were collected prior to administration (predose) and at 0.033, 0.083, 0.25, 0.5, 1, 3, 6, 9 and 24 hours post dose. Blood samples for PO dose groups were collected prior to administration (predose) and at 0.083, 0.25, 0.5, 1, 3, 6, 9 and 24 hours post dose. For the IV group, urine was collected from each animal at predose and from 0 – 6 and 6 – 24 hours post dose. The vehicle used for IV dose groups was a combination of PEG400 with citrate buffer (pH_{5.0}) or PEG400 with DMSO/H₂O, and for PO groups was MCT. Plasma and urine concentrations were quantitated at Genentech Inc. using a non-validated LC/MS/MS method. The lower limit of quantitation (LLOQ) of the plasma and urine assays was 0.005 μ M. PK parameters were determined by non-compartmental methods using WinNonlin (version 5.2, Pharsight Corporation, Mountain View, CA).

JAK Enzyme Assays were carried out as follows:

The activity of the isolated JAK1, JAK2 or TYK2 kinase domain was measured by monitoring phosphorylation of a peptide derived from JAK3 (Val-Ala-Leu-Val-Asp-Gly-Tyr-Phe-Arg-Leu-Thr-Thr) fluorescently labeled on the N-terminus with 5-carboxyfluorescein using the Caliper LabChip technology (Caliper Life Sciences, Hopkinton, MA). Alternatively, the activity of the isolated JAK3 kinase domain was measured by monitoring phosphorylation of a different peptide derived from JAK3 (Leu-Pro-Leu-Asp-Lys-Asp-Tyr-Tyr-Val-Val-Arg) fluorescently labeled on the N-terminus with 5-carboxyfluorescein using the same Caliper LabChip technology. To determine inhibition constants (K_i), compounds were diluted serially in DMSO and added to 50 μ L kinase reactions containing purified enzyme (1.5 nM JAK1, 0.2 nM JAK2, 5nM JAK3 or 1 nM TYK2), 100 mM HEPES buffer (pH 7.2), 0.015% Brij-35, 1.5 μ M peptide substrate, ATP (5 μ M for JAK3, 25 μ M for JAK1, JAK2, and TYK2), 10 mM MgCl₂, 4 mM DTT at a final DMSO concentration of 2%. Reactions were incubated at 22 °C in

384-well polypropylene microtiter plates for 30 minutes and then stopped by addition of 25 μ L of an EDTA containing solution (100 mM HEPES buffer (pH 7.2), 0.015% Brij-35, 150 mM EDTA), resulting in a final EDTA concentration of 50 mM. After termination of the kinase reaction, the proportion of phosphorylated product was determined as a fraction of total peptide substrate using the Caliper LabChip 3000 according to the manufacturer's specifications. K_i values were then determined using the Morrison tight binding model (Morrison, J.F., Biochim. Biophys. Acta. 185:269-296 (1969); William, J.W. and Morrison, J.F., Meth. Enzymol., 63:437-467 (1979)).

JAK Pathway Assay in Cell Lines were carried out as follows:

The activities of compounds were determined in cell-based assays that are designed to measure JAK-dependent STAT phosphorylation. TF-1 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). TF-1 cells were starved overnight in OptiMEM medium with 0.5% Charcoal/Dextran stripped fetal bovine serum (FBS), 0.1 mM non-essential amino acids (NEAA), 1 mM sodium pyruvate and without phenol red at 37 °C. Compounds were serially diluted in DMSO and incubated for 20 minutes at 37 °C with TF-1 cells in 384-well microtiter plates in RPMI-1640 at a final cell density of 100,000 cells per well and a final DMSO concentration of 0.2%. Human recombinant cytokines, IL-6 (30 ng/mL) or EPO (10 U/mL) were then added at the final concentrations indicated to the microtiter plates containing the TF-1 cells and compound and the plates were incubated for 30-45 min at 37 °C. Compound-mediated effects on STAT3 (IL-6), or STAT5 (EPO) phosphorylation were then measured in the lysates of cells using the Meso Scale Discovery (MSD) technology (Gaithersburg, Maryland) according to the manufacturer's protocol. EC_{50} values were determined as the concentration of compound required for 50% inhibition of STAT phosphorylation relative to that measured for the DMSO control.

Human whole blood assays were carried out as follows:

The activities of compounds were determined in whole blood assays that are designed to measure JAK1- dependent signaling. Compounds were serially diluted in DMSO and incubated for 20 minutes at 37 °C with whole human blood collected in either citrate or EDTA. Blood was dispensed into 384-well microtiter plates at a final DMSO concentration of 0.2%. Human recombinant IL-6 (Invitrogen Corp.; Carlsbad, CA) was then added at a final concentration of 300 ng/mL to the microtiter plates containing the whole blood and compound and the plates were incubated for 45 minutes at 37 °C. Compound-mediated effects on STAT3 phosphorylation were then measured in the lysates of the blood incubated in the presence of IL-6 using the Meso Scale Discovery (MSD) technology (Gaithersburg, Maryland) according to the manufacturer's protocol and EC50 values were determined.

The IL-6 induced pSTAT3 PK/PD study of compound **20** in the mouse was carried out as follows:

8-10 weeks old female DBA/2 mice were orally administered saline, vehicle (MCT) or ascending doses (3 – 100 mg/kg) of **20** formulated in MCT in 200 µL volume at t = 0. 30 minutes later, mice were stimulated intraperitoneally with 1µg recombinant mouse IL-6 in 200 µL saline. 30 min after IL-6 administration, animals were sacrificed and plasma samples and snap frozen spleen samples were collected for analysis of concentrations of compound **20** and pSTAT3, respectively.

The pSTAT3 levels in mouse spleen were determined as follows:

Spleen homogenates were thawed on ice, and their total protein concentration was measured by the BCA method (Thermo Scientific/Pierce) per the manufacturer's protocol. Samples were diluted to 1 µg/mL in water, and 30 µL per well (30 µg/well) was transferred to a MesoScale Discovery (Gaithersburg, Maryland) pSTAT3 plate exposed to a 3% BSA solution to minimize nonspecific antibody binding. The pSTAT3 assay was run according to the manufacturer's protocol. Induction of pSTAT3 by cytokine was defined as the difference between the vehicle and PBS (no compound) controls, and the ratio of these was the fold induction. Inhibition by compound was the difference

between the raw signals of the vehicle control and the compound-containing wells divided by the induction.

hERG assays were carried out as follows:

The in vitro potential for hERG (human ether-a-go-go-related gene) potassium channel current inhibition was assessed according to the study site standard procedures (ChanTest, Cleveland, OH). In brief, hERG-expressing HEK-293 cells (n=2/concentration) were evaluated at 1 and 10 mM in the automated PatchXpress 7000A system (Molecular Devices, Sunnyvale, CA) for 5 minutes after adding the test article. Data are expressed as percent of maximal current.

Quantum mechanical computational methods:

Initial conformers for both **3** and **4** were calculated using OMEGA (Version 2.4.3 OpenEye Scientific Software, Santa Fe, NM). As there were some differences between the rotamers generated for **3** versus **4**, additional conformations of **3** were created by demethylating the conformations generated for **4**. These conformations were used as the starting point for quantum mechanical minimizations using the B3LYP density functional theory method along with the 6-311G**++(d,f) basis set and a solvation model using Gaussian09 (Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, Jr., J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, N. J.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.;

Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. Gaussian 09, Rev. A.1; Gaussian, Inc.: Wallingford, CT, 2009). Two solvation models were used, a polarizable continuum method (PCM) (Scalmani, G.; Frisch, M. J.; *J. Chem. Phys.* **2010**, *132*, 113110/1–113110/15) and a solvation model density (SMD) method (Marenich, A. V.; Cramer, C. J.; Truhlar, D. G. *J. Phys. Chem. B* **2009**, *113*, 6378–6396). The keywords opt=tight and Int=UltraFine were used in order to ensure convergence and the reliability of frequencies to confirm these minima. Most structures converged to two low energy conformers, either an **in** or **out** minimum, for both **3** and **4**. Frequency calculations were performed on the **in** and **out** conformations of both **3** and **4** to confirm that each possess no imaginary frequencies and that each structure is a true minimum. The relative electronic energies of the **in** and **out** conformations for both **3** and **4** are shown in Figure 8 of the manuscript.

Protein expression and purification for X-Ray crystallography were carried out as follows:

Recombinant baculoviruses containing the JAK1 kinase domain (854-1154) in frame with an N-terminal, thrombin cleavable His6-GST tag or the JAK2 kinase domain (amino acids 833-1132) in frame with a thrombin cleavable N-terminal 6xHis tag were generated using a slightly modified version of pAcgfp67 (BD Biosciences). A 22L Wave bioreactor was inoculated with *T ni* pro cells (Expression Systems LLC) at 10^6 cells/mL in serum-free ESF921 (Expression Systems LLC). The cells were grown to 2×10^6 cells/mL and infected with the appropriate virus at a ratio of virus/culture of 5.0 mL/L (vol/vol) for *T ni* pro cells. The Wave reactor was maintained at 27 °C and 25 R.P.M. with a fixed angle of 9° and 0.3 L/m of 30% oxygen. At 48 to 72 h post infection, the cells were harvested by centrifugation at 4000 R.C.F. for 15 min and frozen at -80 °C.

JAK1 was purified from clarified lysate generated by homogenization in phosphate buffered saline (pH 7.3) containing 10% glycerol, 0.1% Thesit, 5 mM β -mercaptoethanol, and protease inhibitors and

ultracentrifugation for 30 min at 51000 g. JAK1 was captured on GSTPrep FF resin (GE Healthcare), washed extensively with lysis buffer and eluted in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% Thesit, 2 mM DTT and 10 mM reduced glutathione. Peak fractions were pooled, digested with Thrombin overnight, dialyzed into phosphate buffered saline (pH 7.3) containing 10% glycerol, 0.1% Thesit, 5mM β -mercaptoethanol and re-run on GSTPrep FF resin to remove free GST. The flow-through was collected, concentrated, and further purified by Superdex 75 size exclusion chromatography in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol and 5 mM DTT. The late-running peak, containing only doubly-phosphorylated JAK1 as determined using mass spectrometry, was concentrated to ~10 mg/mL and stored at -80 °C for crystallization. JAK2 cell paste was lysed by microfluidization in 20 mM imidazole pH 7, 250 mM NaCl, 10% glycerol, 0.5% Brij35, 1 mM PMSF, and 5 mM β -mercaptoethanol and clarified by centrifugation for 1 h at 40000 g. JAK2 was captured on a Ni affinity column (GE Healthcare), washed with lysis buffer and step eluted with elution buffer plus 250 mM imidazole pH 7.5. Eluted protein was loaded directly onto a Superdex 75 size exclusion column equilibrated with 25 mM Bicine pH 8.5, 250 mM NaCl, 10% glycerol and 1 mM TCEP. The peak fraction containing JAK2 was cleaved overnight with Thrombin in the presence of the JAK2 inhibitor of interest at a final concentration of 50 μ M. JAK2 was further purified by SP fast flow cation exchange chromatography after dilution to a final concentration of 50mM NaCl using 25 mM Bicine pH 8.5, 1 mM TCEP and passing the flow-through (after concentration) over a Superdex 75 size exclusion column. JAK2 was concentrated to ~8 mg/mL for crystallization in a buffer containing 250mM NaCl, 25mM Bicine pH 8.5, 2% glycerol, 1mM TCEP.

Crystallization and X-Ray structure determination were carried out as follows:

JAK1 (~10 mg/mL) was treated with fresh DTT to bring the concentration to 20 mM and ligand added to a concentration of 1 mM from a 100 mM DMSO solution. Diffraction quality crystals grew in 2 days in 1 μ L drops containing protein/ligand complex mixed with an equal volume of seed stock diluted in

reservoir (0.1 M MES pH 5-6 and 25-35% PEG 6000). Crystals were cryoprotected in reservoir augmented with ~25% ethylene glycol or PEG 400, flash frozen in liquid nitrogen, and data collected under cryo-cooled conditions at 100 K at a synchrotron beamline (compound **4** at ALS 5.0.1 and compound **20** at ALS 5.0.2).

JAK2 (~8 mg/mL) was brought to nominal 2 mM of inhibitor by addition from 50 mM DMSO stock, and diffraction quality crystals grew in 3 days from 4 uL drops containing protein mixed with an equal volume of seed stock diluted in reservoir (0.2 M ammonium acetate, 0.1M sodium citrate pH6, 18-35% PEG 8000). Crystals were cryoprotected in reservoir augmented with ~30% glycerol, flash frozen in liquid nitrogen, and data collected under cryo-cooled conditions at 100 K at a synchrotron beamline (compound **3** at ALS 5.0.1 and compound **4** at SSRL 7-1).

The JAK1 and JAK2 structures were solved by molecular replacement with Phaser (McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. Phaser crystallographic software. *J. Appl. Crystallogr.* **2007**, *40*, 658–674). Refinement was performed using Refmac5 (Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. Refinement of macromolecular structures by the maximum-likelihood method. *Acta. Crystallogr. D Biol. Crystallogr.* **1997**, *53*, 240–255) or Phenix (Adams, P.D.; Mustyakimov, M.; Afonine, P.V.; Langan, P. Generalized X-ray and neutron crystallographic analysis: more accurate and complete structures for biological macromolecules *Acta. Crystallogr. D Biol. Crystallogr.*, **2009**, *D65*, 567–573) and model manipulations employed Coot (Emsley, P.; Cowtan, K. Coot: model-building tools for molecular graphics. *Acta. Crystallogr. D Biol. Crystallogr.* **2004**, *D60*, 2126–2132). Data reduction and final refinement statistics appear in Table S1.

Table S1: Data Collection and Refinement for JAK1 and JAK2 Complexes

	JAK1/Cmpd4	JAK1/Cmpd20	JAK2/Cmpd3	JAK2/Cmpd4
<u>Data collection</u>	ALS 5.0.1	ALS 5.0.2	ALS 5.0.1	SSRL 7-1
space group	P2 ₁	P2 ₁	P4 ₁	P2 ₁
unit cell (Å, °)	<i>a</i> = 42.71, <i>b</i> = 172.2, <i>c</i> = 88.03 β = 92.3	<i>a</i> = 42.76, <i>b</i> = 173.3, <i>c</i> = 44.60 β = 94.3	<i>a</i> = <i>b</i> =110.9, <i>c</i> = 70.73	<i>a</i> = 44.44, <i>b</i> = 69.49 <i>c</i> = 50.23 β = 101.17
V _M (Å ³ /Dalton)	2.5	2.5	3.3	2.3
Resolution (Å)	30-2.17 (2.25-2.17)	50-2.22 (2.30-2.22)	50-2.82 (2.92-2.82)	50-2.40 (2.52-2.39)
R _{sym} ^{a,b}	0.100 (0.373)	0.069 (0.383)	0.073 (0.413)	0.112 (0.255)
Number of observations	229452	102998	103910	42269
Unique reflections	63591	32187	20873	11920
Completeness (%) ^b	95.7 (87.6)	99.7 (99.8)	99.8 (98.3)	99.7 (99.8)
I/ σ I ^b	14 (3.2)	15 (2.7)	15 (2.4)	8.3 (4.1)
Wilson B (Å ²)	23.8	40.0	73.9	24.9
<u>Refinement</u>				
Resolution (Å)	30 – 2.17	50 – 2.22	50 – 2.82	50 – 2.40
No. reflections (F>0 σ (F))	60351	30234	20852	11788
Final R ^c , R _{FREE}	0.171, 0.229	0.200, 0.239	0.211, 0.243	0.201, 0.259
Molecules/asymmetric unit	4	2	2	1
protein residues	1136	573	583	290
solvent molecules	561	118	0	107
atoms	9938	4818	4820	2495
Mean B-factor (Å ²)	30.6	32	58	25
Rmsd bonds (Å)	0.009	0.010	0.011	0.013
Rmsd angles (°)	1.09	1.10	1.40	1.75
Number of TLS groups	9	4	4	1
Ramachandran (%)	98/0	96/0	98/0	96/0
(favored/outliers)				

^a R_{sym} = $\Sigma ||I| - \langle I \rangle| / \Sigma \langle I \rangle$, where I is the intensity of a single observation and $\langle I \rangle$ the average intensity for symmetry equivalent observations.

^b In parenthesis, for the highest resolution shell.

^c R = $\Sigma |F_o - F_c| / \Sigma |F_o|$, where F_o and F_c are observed and calculated structure factor amplitudes, respectively. R_{FREE} is calculated as R for reflections sequestered from refinement.

Measured LogD_{7.2} experimental procedures:

Experimental LogD values at pH 7.2 (LogD_{7.2}) were determined as follows: 10mM DMSO stock solutions of test compounds were prepared. Each DMSO stock was then added to a 1:1 mixture of octanol / aqueous phosphate buffered saline (PBS, pH = 7.2) to give final DMSO concentrations of 1%. The samples were shaken for 1 hour, then the phases were separated and transferred to separate microtiter plates. The concentrations of test compounds in each phase were monitored by a MRM (multiple reaction monitoring) response with a 2 min LC/MS/MS method on the Sciex API-4000. The

LogD_{7.2} values were calculated as the log of the signal ratio in the two phases, Log[octanol-MRM response/PBS-MRM response]. 16 commercial compounds were included in every assay run as standard controls. The dynamic range encompassed LogD_{7.2} values between -0.5 to 5.5.

Measured thermodynamic solubility (pH_{7.4} and pH_{2.0}) experimental procedures:

A standard curve for each compound tested was prepared as follows: A 10 mM DMSO stock solution was prepared. The DMSO stock was then serially diluted with a 1:1 mixture of acetonitrile/water to concentrations of 200 µM, 20 µM, and 2 µM to provide standard samples. The UV absorbance at a wavelength of 254nm was then measured using a LC/UV method for each of the standard samples to make a calibration curve. The testing sample was then prepared as follows: 0.45 mL of phosphate buffered saline (PBS, pH = 7.4), or phosphate buffered saline containing HCl (PBS/HCl, pH = 2.0) was added to 2 mg of solid test sample in a Whatman UniPrep filter vial. The vial was shaken for 24 hours at room temperature, then the aqueous mixture was filtered. The filtrate was diluted 10-fold with PBS or PBS/HCl buffer and an aliquot was then injected onto a LC/UV instrument. The UV absorbance of the test sample at 254nm was measured and compared with its calibration curve to determine the concentration and, hence, the aqueous solubility of the test sample.

Table S2: Analytical LCMS and SFC conditions

Method	Instrument	Column brand	Column diameter (mm)	Column length (mm)	Column particle size (µm)	Column temperature (°C)	Mobile phase: A	Mobile phase: B	Gradient	Flow rate (mL/min)	UV Detection Wavelength (nm)	run length (min)	Mass spectrometer	Ionization	Scan range (AMU)
LCMS1	Hewlett Packard 1050 HPLC	Phenomenex Luna C18(2)	4.6	30	3.0	25	water with 0.1% formic acid	acetonitrile with 0.1% formic acid	A:B = 95:5 (0.5 min); 95:5 - 5:95 (over 4.0 min); 5:95 (1.0 min); 5:95 - 95:5 (over 0.5 min)	2.0	220 and 254	6.0	Finnigan AQA single quadrupole	ESI ⁺	110 - 800
LCMS2	Waters Acquity UPLC	Acquity BEH C18 or Acquity BEH Shield RP18	2.1	100	1.7	40	water with 0.1% formic acid	acetonitrile with 0.1% formic acid	A:B = 95:5 (0.4 min); 95:5 - 5:95 (over 5.6 min); 5:95 (0.8 min); 5:95 - 95:5 (over 1.2 min)	0.4	220 and 254	8.0	Waters Micromass ZQ2000 quadrupole	ESI ⁺	110 - 800
LCMS3	Waters Acquity UPLC	Acquity UPLC BEH C18	2.1	30	1.7	80	water with 0.1% formic acid	acetonitrile with 0.1% formic acid	A:B = 95:5 - 5:95 (over 1.4 min); 5:95 (0.3 min)	0.8	220 and 254	1.7	Waters SQ	ESI ⁺	100 - 800
LCMS4	Agilent 1200 HPLC	Agilent SD-C18	2.1	30	1.8	40	water with 0.05% trifluoroacetic acid	acetonitrile with 0.05% trifluoroacetic acid	A:B = 97:3 - 5:95 (over 8.5 min); 5:95 (2.5 min)	0.7	220 and 254	11.0	Agilent quadrupole 6140	ESI ⁺	110 - 800
LCMS5	Waters Acquity UPLC	Acquity UPLC BEH C18	2.1	30	1.7	40	water with 0.1% formic acid	acetonitrile with 0.1% formic acid	A:B = 95:5 - 5:95 (over 1.4 min); 5:95 (0.3 min)	0.8	220 and 254	1.7	Waters SQ	ESI ⁺	100 - 800
LCMS6	Agilent 1200 HPLC	Agilent SD-C18	3.0	100	3.5	40	water with 0.05% trifluoroacetic acid	acetonitrile with 0.05% trifluoroacetic acid	A:B = 98:2 - 2:98 (over 25.5 min); 2:98 (4.5 min)	0.7	220 and 254	30.0	Agilent quadrupole 6140	ESI ⁺	110 - 800
LCMS7	Hewlett Packard HP1100 HPLC	Phenomenex Luna C18(2)	4.6	30	3.0		water with 0.1% formic acid	acetonitrile with 0.1% formic acid	A:B = 95:5 (0.5 min); 95:5 - 5:95 (over 4.0 min); 5:95 (1.0 min); 5:95 - 95:5 (over 0.5 min)	2.0	220 and 254	6.0	Waters Platform LC quadrupole	ESI ⁺	100 - 800
LCMS8	Agilent 1200 HPLC	Onyx-C18	2.0	50			water with 0.05% trifluoroacetic acid	acetonitrile with 0.05% trifluoroacetic acid	A:B = 95:5 - 65:35 (over 4.0 min)	0.8	220 and 254	4.0	Agilent quadrupole 6140	ESI ⁺	60 - 1000
LCMS9	Agilent 1200 Series HPLC	Xtimate	2.1	30.0	3.0	50	water with 0.375% trifluoroacetic acid	acetonitrile with 0.187% trifluoroacetic acid	A:B = 100:0 - 60:40 (over 2.0 min); 60:40 (0.9 min)	1.2	220 and 254	2.9	Agilent MSD VL	ESI ⁺	100 - 1000
SFC1	Berger Analytical SFC/MS	Chiralpak AD	4.6	100	5.0	40	Carbon dioxide	Methanol	isocratic, A:B = 75:25	5.0	220 and 254	3.0	Waters ZQ quadrupole	ESI ⁺	140 - 800
SFC2	Berger Analytical SFC/MS	Chiralpak IC	4.6	100	5.0	40	Carbon dioxide	methanol with 0.1% diethylamine	isocratic, A:B = 70:30	5.0	220 and 254	3.0	Waters ZQ quadrupole	ESI ⁺	140 - 800
SFC3	Berger Analytical SFC/MS	Chiralpak AD	4.6	100	5.0	40	Carbon dioxide	ethanol with 0.1% diethylamine	isocratic, A:B = 65:35	5.0	220 and 254	3.0	Waters ZQ quadrupole	ESI ⁺	140 - 800
SFC4	Berger Analytical SFC/MS	Chiralpak AD	4.6	100	5.0	40	Carbon dioxide	ethanol with 0.1% diethylamine	isocratic, A:B = 70:30	5.0	220 and 254	3.0	Waters ZQ quadrupole	ESI ⁺	140 - 800
SFC5	Berger Analytical SFC/MS	Chiralpak AD	4.6	100	5.0	40	Carbon dioxide	Methanol	isocratic, A:B = 60:40	5.0	220 and 254	3.0	Waters ZQ quadrupole	ESI ⁺	140-800
SFC6	Thar SFC	Chiralpak AD ₃	4.6	150	3.0	35	Carbon dioxide	methanol with 0.05% diethylamine	A:B = 95:5 - 60:40 (over 7.8 min); 60:40 (3.0 min); 60:40 - 95:5 (over 4.2 min)	2.5	220	15.0			

Table S3: Biochemical inhibition of JAK1, JAK2, JAK3, and TYK2 by compounds **3 – 24**.

Ex	JAK1 K_i (nM)	JAK2 K_i (nM)	JAK3 K_i (nM)	TYK2 K_i (nM)
3	46	250	540	380
4	10	200	250	290
5	1.3	2.9	5.8	70
6	0.9	2.1	6.0	6.8
7	1.3	5.0	4.9	3.4
8	18	66	21	50
9	2.8	12	39	20
10	150	580	520	720
11	1.2	6.4	2.2	6.2
12	9.3	74	80	98
13	2.0	11	3.5	14
14	1.5	9.3	14	19
15	4.5	11	56	34
16	6.1	17	24	40
17	2.6	12	48	31
18	5.8	24	31	26
19	6.7	18	14	21
20	1.8	12	22	6.2
21	7.3	21	33	22
22	90	520	300	220
23	4.8	26	16	44
24	5.4	28	15	48