Comparison of the Enzymatic and Cellular Profiles of Clinical JAK2 Inhibitors for the Treatment of Myelofibrosis

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Introduction

- 4 Janus kinase family inhibitors (JAKinibs) have been approved by the US Food and Drug Administration for the treatment of myelofibrosis (MF): ruxolitinib, fedratinib, pacritinib, and momelotinib
- In addition to Janus kinase 2 (JAK2), these drugs inhibit other kinases, with potential implications for their observed pharmacology
- Published data on kinase inhibitory profiles of each drug were determined under differing assay conditions,[1-4] which limits head-to-head comparisons and understanding of their effects unrelated to JAK2 inhibition
- These JAKinibs are orthosteric adenosine triphosphate (ATP) competitors and as such, the half-maximal inhibitory concentration (IC_{50}) in enzymatic assays will vary significantly with ATP concentration[5]
- Here, we present full kinome profiling data for the 4 approved JAKinibs. Additionally, we have compared their effects on signaling and proliferation in myeloid and other human cells

Materials and Methods

- **Compounds:** Ruxolitinib (HY-50856), fedratinib (HY-10409), pacritinib (HY-16379), and momelotinib (HY-10961) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Based on vendor-supplied Certificate of Analysis, compounds were >98% pure. Momelotinib metabolite M21[6] was synthesized in house and purity was >99% by HPLC analysis
- Drug structures and concentrations were verified by NMR spectroscopy
- More details of Materials and Methods can be accessed via the QR code

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JAK Family Profiling

Inhibition of JAK Family Kinases in Biochemical Assays

- JAK1, JAK2, JAK3, and TYK2 enzymatic assays were performed at 1 mM ATP, within the range of its physiological concentration
- Ruxolitinib is the most potent and selective JAK2 inhibitor, with an IC_{50} of 2.9 nM (Table 1)
- Varying degrees of inhibition of JAK1 and TYK2 biochemical activity were observed with JAKinibs
- Enzymatic activities were consistent with cellular inhibition of signal transducer and activator of transcription (STAT) signaling in response to different cytokines

Table 1. Inhibition of JAK Family Kinases

Activity Assay	Ruxolitinib IC50 (nM) GeoMean	GeoSD	Fedratinib IC50 (nM) GeoMean	GeoSD	Pacritinib IC50 (nM) GeoMean	GeoSD	Momelotinib IC50 (nM) GeoMean	GeoSD	M21* IC50 (nM) GeoMean	GeoSD
JAK2	2.9	1.1	17	1.1	39	1.1	29	1.1	118	1.1
JAK1	4.3	1.1	99	1.0	921	1.1	130	1.2	271	1.0
JAK3	540	1.1	4291	1.3	1551	1.1	1277	1.2	2941	1.0
TYK2	22	1.2	761	1.1	380	1.2	53	1.2	70	1.2

*M21 is a morpholino lactam metabolite of momelotinib.[6] GeoMean, geometric mean; GeoSD, geometric standard deviation.

On-Target Assessment of Phosphorylated STAT5 Using JAK2-Dependent Cell Lines

- Quantification of phosphorylated STAT5 in response to individual JAKinibs using Baf3_JAK2WT, Baf3_JAK2V617F, and SET2 (JAK2V617F) cells. Cells were treated with compounds for 2 hours (Figure 1)
- The observed ranking of on-target potency in these cellular assays is in line with IC50 values of JAK2 inhibition at 1 mM ATP

Figure 1. Inhibition of JAK2/STAT5 Signaling in JAK2-Dependent Cell Lines by JAKinibs

JAKinib	Baf3_JAK2 WT IC50 (nM) GeoMean	GeoSD	Baf3_JAK2 V617F IC50 (nM) GeoMean	GeoSD	SET2 (JAK2 V617F) IC50 (nM) GeoMean	GeoSD	Published JAK2 Potency IC50 (nM)	[ATP] μM	JAK2 Potency at 1 mM ATP IC50 (nM)
Ruxolitinib	58	2.2	63	2.0	14	1.3	2.8†	1000†	2.9
Fedratinib	1161	1.2	1097	1.7	669	1.1	3²	Km² (~30)	17
Pacritinib	1417	1.3	1338	1.3	421	1.2	1.3³	150³	39
Momelotinib	811	1.3	889	1.1	201	1.3	18⁴	NA⁴	29

n>3. 1. Quintás-Cardama A, et al. Blood. 2010;115:3109-17. 2. Wernig G, et al. Cancer Cell. 2008;13:311-20. 3. William AD, et al. J Med Chem. 2011;54:4638-58. 4. Pardanani A, et al. Leukemia. 2009;23:1441-5. GeoMean, geometric mean; GeoSD, geometric standard deviation; NA, not available.

Comprehensive Kinase Profiling

Full Kinome Profiling

- 356 kinases were tested at 100 nM per drug (at 10 μM ATP); all kinases showing ≥50% inhibition by ≥1 drug were then tested for dose response against all drugs (at 100 μM ATP) (Figure 2)

Figure 2. Dendrogram Representation of the Selectivity Profiles for Indicated JAKinibs

IC50: ● <10 nM, ● <100 nM, ○ <1000 nM, ○ <10,000 nM, ○ ≥10,000 nM. Size by IC50 value at 100 μM ATP

Group names: AGC, containing PKA, PKG, PKC families; CAMK, calcium/calmodulin-dependent protein kinase; CK1, casein kinase 1; CMGC, containing CDK, MAPK, GSK3, CLK families; STE, homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases; TK, tyrosine kinase; TKL, tyrosine kinase-like.

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Inhibition of Kinases in Biochemical Assays

- Fedratinib, momelotinib, and pacritinib inhibited many kinases outside of the JAK family with similar potency to JAK2 inhibition, whereas ruxolitinib was relatively selective for JAK2 inhibition (Figure 3)
- In contrast to published reports on the inhibitory activities of pacritinib and momelotinib against ALK2 (aka ACVR1), the inhibition of ALK2 observed was modest as to ascertain pharmacology through this kinase at clinical doses (see poster 1789)
- Fedratinib, pacritinib, and momelotinib, but not ruxolitinib, exhibited high inhibitory potencies against FLT3 and/or KIT kinases, both of which are critical for the self-renewal and differentiation of normal hematopoietic stem cells

Figure 3. Kinases Inhibited by JAKinibs With Potential Pharmacological Effects

Only IC50 values within 100-fold of JAK2 inhibition are shown; labels mark kinases in the JAK family, other kinases with strong biology in hematopoiesis, and, on the right side of every panel, kinases essential for cell proliferation according to DepMap.[7]

c-KIT and FLT3 Kinase Profiling

Inhibition of c-KIT and FLT3 Kinases in Cell-Based Assays

- Consistent with the biochemical assays as shown in Figure 3, ruxolitinib exhibited the weakest activity against c-KIT or FLT3 kinases compared with other JAKinibs in Baf3_cKIT and Baf3_FLT3 reporter cells (Figure 4)

Figure 4. Profiling of JAKinibs in c-KIT and FLT3 Baf3 Reporter Cells

	Phosphorylated c-KIT (Tyr719) IC50 (nM) GeoMean	GeoSD	Phosphorylated STAT5 (FLT3 Readout) IC50 (nM) GeoMean	GeoSD
JAKinib				
Ruxolitinib	>10,000	N/A	5980	1.4
Fedratinib	758	1.0	44	1.4
Pacritinib	331	1.1	47	1.5
Momelotinib	6391	1.1	206	1.4

n=3. GeoMean, geometric mean; GeoSD, geometric standard deviation; N/A, not assessable.

Functional Profiling in HEK-Blue Reporter Cells

- Human embryonic kidney (HEK)-Blue reporter cell lines were used to investigate the on- and off-target activity of JAKinibs for JAK1, JAK2, JAK3, and TYK2
- Each cell line was pretreated with JAKinibs for 1 hour, followed by respective cytokine treatment overnight. Secreted embryonic alkaline phosphatase (SEAP) activity was measured using QUANTI-Blue (Figure 5)

Figure 5. Inhibition of JAK/STAT Signaling Pathways in HEK-Blue Reporter Cells in Response to TPO, IL-2, or INF-α

JAKinib	TPO (JAK2/JAK2) IC50 (nM) GeoMean	GeoSD	IL-2 (JAK1/JAK3) IC50 (nM) GeoMean	GeoSD	IFN-α (JAK1/TYK2) IC50 (nM) GeoMean	GeoSD
Ruxolitinib	97	1.4	87	1.4	435	2.0
Fedratinib	672	1.3	1135	1.2	2219	1.9
Pacritinib	666	1.8	1767	1.5	982	1.7
Momelotinib	598	1.5	1620	1.7	5621	1.8

n≥3. GeoMean, geometric mean; GeoSD, geometric standard deviation.

Cytotoxicity Assessment on Non–JAK2-Dependent Cell Lines

- Quantification of viability in SET2 (on target), HEK293, HepG2, human umbilical vein endothelial cells (HUVEC), and human lung fibroblasts using a CellTiter-Glo assay. Cells were treated for 72 hours
- HEK293 cells are used to evaluate general cellular toxicity. HepG2 cells are commonly used to screen for hepatotoxicity studies. Human lung fibroblasts and HUVEC were used to assess acute toxicity in a more physiologically relevant cell type
- While ruxolitinib showed a clear separation between on-target inhibition signaling and growth of SET2 cells and off-target antiproliferative effects, all other JAKinibs inhibited the proliferation of non–JAK2-dependent cell lines (HEK293, HepG2, human lung fibroblasts, and HUVEC), with potencies very similar to their inhibitory effect on JAK2-dependent cell line (Figure 6)

Figure 6. On-Target Inhibition Compared With Cytotoxicity in Several Human Cell Lines Not Dependent on JAK2/STAT5

	SET2 IC50 (nM) GeoMean	GeoSD	n	HepG2 IC50 (nM) GeoMean	GeoSD	n	Human Lung Fibroblasts IC50 (nM) GeoMean	GeoSD	n	HUVEC IC50 (nM) GeoMean	GeoSD	n
JAKinib												
Ruxolitinib	33	1.2	4	>20,000	N/A	4	>5000	N/A	4	>5000	N/A	2
Fedratinib	301	1.1	4	4633	1.3	4	2716	1.3	4	2032	1.0	2
Pacritinib	105	1.1	4	831	1.1	4	2144	1.6	4	2662	1.2	2
Momelotinib	191	1.2	4	9785	1.3	4	1974	1.5	4	3000	1.1	2

GeoMean, geometric mean; GeoSD, geometric standard deviation; HEK, human embryonic kidney; HUVEC, human umbilical vein endothelial cells; N/A, not assessable.

Cytotoxicity Assessment on Normal Hematopoietic Cell Differentiation

- Normal CD34+ cells were induced to differentiate with hematopoietic cytokines in the presence of clinically relevant doses of ruxolitinib, fedratinib, pacritinib, and momelotinib for 6 days (Figure 7A)
- 6 days post differentiation, overall viability, CD34+ hematopoietic stem and progenitor cells as well as CD41+ CD42b+ mature megakaryocytes were quantified (Figure 7B)
- Unlike fedratinib, pacritinib, and momelotinib, ruxolitinib exhibits minimal toxicity toward the differentiation of normal human CD34+ cells into mature megakaryocytes at concentrations of drugs used in this study, selected based on available clinical data (Figure 7C)

Figure 7. Cytotoxicity Assessment Using Primary Human CD34+ Cells

JAKinib	Cavg,ss (μM)	Cmax,ss (μM)	2× Cmax,ss (μM)
Ruxolitinib	0.011	0.048	0.096
Fedratinib	0.267	0.425	0.850
Pacritinib	0.084	0.089	0.178
Momelotinib	0.135	0.280	0.560

*Free drug concentrations in plasma were estimated from total concentrations in plasma of each drug at steady state (reported from clinical trials) and human protein binding (96.4% for ruxolitinib, 87.5% for fedratinib, 99.5% for pacritinib, and 86.4% for momelotinib) (data generated at Incyte Corporation, using standard methodology). Cavg,ss, average steady-state plasma drug concentration during multiple-dose administration; Cmax,ss, maximum steady-state plasma drug concentration during a dosage interval; HSPC, hematopoietic stem and progenitor cell; MgK, megakaryocyte.

Conclusions

- JAKinibs clinically approved for the treatment of MF exhibit distinct kinase inhibition profiles and cellular activities
- Among the agents tested in this study, ruxolitinib was the most potent and selective JAK2 inhibitor
- At concentrations several fold above clinically relevant concentrations, ruxolitinib had no observable effects on the health of cells not reliant on JAK-/STAT-mediated signaling

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