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Discovery of a JAK1/3 Inhibitor and Use of a Prodrug To Demonstrate Efficacy in a Model of Rheumatoid Arthritis

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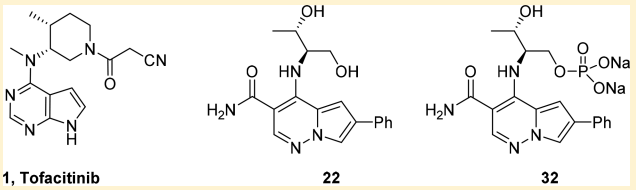
Supporting Information

ABSTRACT: The four members of the Janus family of nonreceptor tyrosine kinases play a significant role in immune function. The JAK family kinase inhibitor, tofacitinib **1**, has been approved in the United States for use in rheumatoid arthritis (RA) patients. A number of JAK inhibitors with a variety of JAK family selectivity profiles are currently in clinical trials. Our goal was to identify inhibitors that were functionally selective for JAK1 and JAK3. Compound **22** was prepared with the desired functional selectivity profile, but it suffered from poor absorption related to physical properties. Use of the phosphate prodrug **32** enabled progression to a murine collagen induced arthritis (CIA) model. The demonstration of a robust efficacy in the CIA model suggests that use of phosphate prodrugs may resolve issues with progressing this chemotype for the treatment of autoimmune diseases such as RA.



KEYWORDS: *JAK1, JAK2, JAK3, TYK2, prodrug, mouse CIA model*

The Janus Kinase (JAK) family is made up of four structurally related kinases, JAK1, JAK2, JAK3, and TYK2 (Tyrosine Kinase-2). The JAK family kinases, through the actions of specific cytokines binding to their receptors, are key drivers of immune system and inflammatory responses.[1] Once these receptors are activated, the kinases phosphorylate cytokine receptors and subsequently activate signal transducers and activators of transcription (STATs). The STATs dimerize and are translocated to the nucleus where they bind to regions of genes that promote cytokine production leading to a propagation of inflammatory and immune signals. As a result of their function, the JAK family kinases have been of significant interest to the pharmaceutical industry, and various inhibitor profiles have been explored as possible treatments for autoimmune disease. Tofacitinib is a pan-JAK family inhibitor that has been approved for the treatment of rheumatoid arthritis (RA),[2] psoriatic arthritis,[3] and ulcerative colitis.[4] Baricitinib is a selective JAK1/2 inhibitor that has been recently approved for the treatment of RA.[5] Selectively targeting JAK1 would be expected to not only inhibit γc chain cytokine signaling (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21), but also block signaling from other pro-inflammatory cytokines such as IL-6 and Type I interferon. Selective inhibitors of JAK1 such as figlotinib,[6] upadicitnib,[7] and abrocitinib[8] are currently in clinical trials for the treatment of RA and other autoimmune or inflammatory diseases. Early attempts to identify JAK3 selective inhibitors suggested that JAK1 inhibition was also required to obtain potent inhibitors of γc chain cytokine signaling.[9] A detailed analysis of the JAK family members K_m for ATP helped explain this observation.[10] This limitation toward selectively targeting JAK3 has recently been overcome by pursuing a covalent inhibition strategy, which has identified inhibitors that selectively block γc chain cytokine signaling.[11−16] PF-06651600 is one such covalent inhibitor that has advanced to clinical trials. As a result of these considerations and our own difficulty in identifying potent selective noncovalent JAK3 inhibitors, we chose to pursue a JAK1/3 selective strategy in the hope of accessing a 'JAK1 selective profile'.

Our initial report described the discovery of pyrrolopyridazine-3-carboxamides (PPZ) as potent inhibitors of the JAK kinase family.[17] Subsequently, we discovered that addition of an aryl or heteroaryl group at the C-6 position significantly increased JAK family potency. Compound **2** (Figure 1) demonstrated efficacy in a pseudoestablished mouse collagen-induced arthritis (mouse CIA) model; however, a dose response was not obtained.[18] Further progression of **2** was also hampered by multiple cardiovascular liabilities, which included hemodynamic effects attributed to inhibition of Rho kinase (ROCK1 IC_{50} = 50 nM) and the potential for QTc

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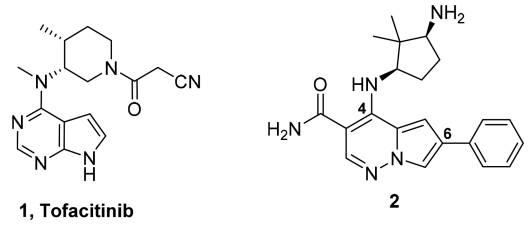
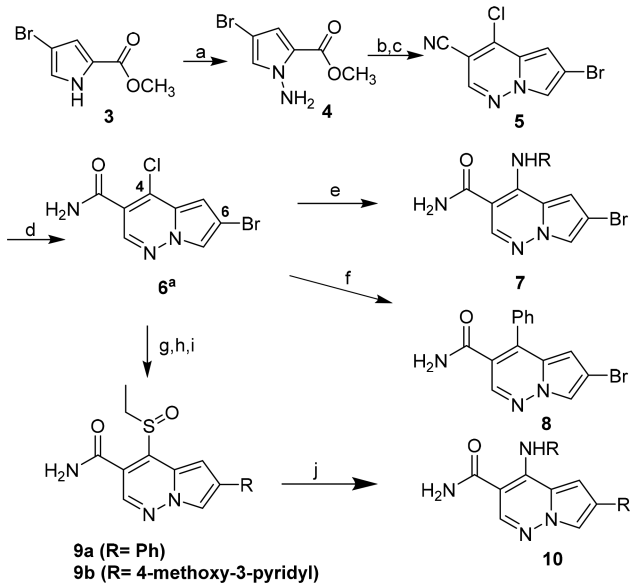


Figure 1.

prolongation due to significant inhibition of the hERG ion channel.

Our chemistry library plan centered on utilizing intermediate **6**, 6-bromo-4-chloro-pyrrolopyridazine-3-carboxamide, to rapidly expand SAR at the 4 and 6 position in this series (Scheme 1). Reaction of **6** with amines provided **7**, which could be

Scheme 1. Synthetic Strategy for C-4 Diversity[1]



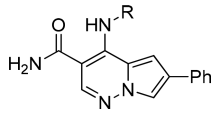
[1]Experimental details described in the Supporting Information. Scheme conditions: (a) (i) NaH, DMF; (ii) Chloramine solution in MTBE (90%); (b) (i) 3,3-diethyoxypropionitrile, IPA, 85 °C; (ii) DBU, DCE, 85 °C (c) $POCl_3$, 75 °C. 29%, 2 steps); (d) Conc. H_2SO_4, 55 °C (89%); (e) R-NH_2; (f) Ar−$B(OH)_2$/Suzuki conditions; (g) EtSH, K_2CO_3, DMF, rt, 18 h (65%); (h) $ArB(OH)_2$, X-Phos, $Pd(OAc)_2$, 2 M K_3PO_4, dioxane 125 °C ; (i) oxone, acetone, water; (j) R-NH_2, DMF, 85 °C.

further elaborated at C-6 via a Suzuki-Miyaura coupling procedure. However, when we attempted to conduct the Suzuki-Miyaura coupling as the first step on **6**, we obtained undesired **8** as the major product. To circumvent this regiochemical outcome, **6** was reacted with ethanethiol to provide the more versatile C-6 ethylsulfide intermediate, which enabled regio-controlled installation of the aryl group at C-6 followed by oxidation of the sulfide with Oxone to afford **9a** and **9b**. Reaction of **9a** and **9b** with amines then provided the desired analog **10**.

Compounds were evaluated in JAK1, JAK2, JAK3, and TYK2 enzymatic assays and an IL-2 dependent T-cell proliferation assay.[19] Compounds of interest were also evaluated in a human whole blood IL-2 driven IFNγ production assay (IL-2 IFNγ hWB), which provided an indication of the level of protein binding and suggested a minimal concentration to target for PK/PD and *in vivo* efficacy studies. Selected compounds were further profiled for cellular JAK2 activity (SET2 proliferation assay[20] and an EPO phosphoSTAT5 assay) and cellular TYK2 activity (IL23 KIT225 STAT reporter assay).

Initial efforts involved fixing the C-6 substituent as phenyl while varying the C-4 position using aliphatic amines as represented by compounds **11**−**15** (Table 1). These analogs

Table 1. Secondary Alkyl Amines 11−15[a,b]



Cmpd[c]	R	JAK3 IC_{50}, nM	JAK1 IC_{50}, nM	TYK2 IC_{50}, nM	JAK2 IC_{50}, nM
11		15	40	150	190
12		4	30	63	160
13		11	110	160	220
14		8	33	70	110
15		5	13	20	50

[a]Assay protocols are described in the Supporting Information. [b]Values in table represent n = 1. [c]Synthesized in library format.

were potent nanomolar inhibitors of the JAK family, particularly JAK3. However, **11**−**15** showed poor inhibition in the IL2 IFNγ hWB assay (IC_{50} > 4 μM), poor metabolic stability (<50% remaining after 10 min incubation with mouse microsomes), and poor aqueous solubility (<1 μg/mL). The overall lipophilicity of the molecules (cLogP 2.9−3.7, LogP **12** = 3.94, **13** = 5.08) was suspected to be a contributing factor to the observed liabilities.

Subsequent efforts focused on reducing lipophilicity and improving solubility within the series,[21,22] with the hope of improving whole blood potency, by incorporating a more polar 4-methoxy-3-pyridyl group at C-6 and exploring amino alcohol side chains at C-4 (Table 2). Given the potent hERG activity of **2**, we avoided incorporation of basic amines as part of this SAR effort. Compounds **16**, **17**, **19**, **21**, and **22** were potent inhibitors of JAK3 and gratifyingly showed submicromolar potency in the IL-2 driven T-cell proliferation assay (IL-2 T-cell) with the more potent *c*-propyldiol diastereomer **19** displaying submicromolar potency in IL2 INFγ hWB (IC_{50} = 780 nM). Truncating the alkyl side chain in **20** resulted in a significant decrease in JAK family potency. We postulated that the cyclopropyl group might be projecting into the tofacitinib "methyl pocket".[23]

Proceeding on this assumption we decided to explore a change of the cyclopropyl group to a methyl group to provide **21** and **22**, both of which were in the same potency range as the cyclopropyl analog **19**, and significantly more potent than **20**. All four possible diol isomers were prepared and **22**,

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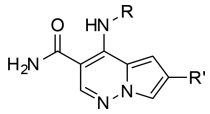
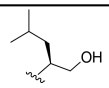
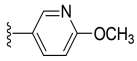
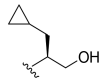
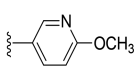
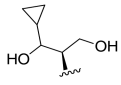
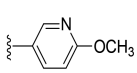
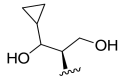
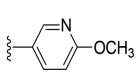
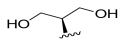
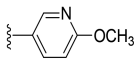
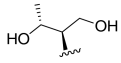
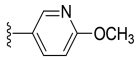
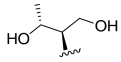
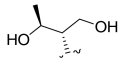
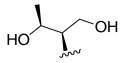
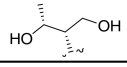
Table 2. C-4 Amino Alcohol Derivatives[a,c]



Cmpd	R	R'	JAK3 IC_{50}, nM	JAK1 IC_{50}, nM	TYK2 IC_{50}, nM	JAK2 IC_{50}, nM	IL2 T Cell[d] IC_{50}, nM	IL2 IFNγ hWB IC_{50}, nM	ClogP (LogP)[e]
16[b]			3±0.2 n=3	23±7 n=4	180±40 n=4	30±16 n=4	190	3900	2.3
17[b]			1	32	120	25	290	>4000	2.3
18			100	620	2000	1400	4800	ND	1.8
19 Isomer A			2	21	130	45	500	730	1.8
20[b] Isomer B			38±3 n=2	200	330	240	11,000	ND	0.1
21[b]			2	6	27	57	370	ND	0.4 (2.2)
22			3±1 n=2	5±2 n=3	34±13 n=2	70±18 n=2	70±30 n=2	500±210 n=2	1.2 (2.42)
23			160	180	1300	1800	2000	ND	1.2
24			13	38	210	370	870	ND	1.2
25			180	110	510	1700	1800	ND	1.2

[a]Assay protocols are described in the Supporting Information. [b]Synthesized in library format. [c]Values in table represent $n = 1$ unless otherwise noted. [d]T-cell proliferation assay. [e]Octanol–water (pH 6.5) HPLC determination.

derived from the natural amino acid, threonine, proved to be the most potent against JAK1/3, and more importantly the most potent in the human whole blood assay. On examination of the JAK3 inhibitor complex with **22**, we were somewhat surprised to see that the methyl group of the secondary alcohol did not fully occupy the "methyl pocket" observed in the tofacitinib JAK3 crystal structure and the pocket was altered due to a change in the orientation of Leu_{956} (see Figure 2). Additionally we noticed the primary alcohol formed a hydrogen bond with the glycine rich P-loop (Leu_{828}). It is interesting to note that diasterioisomers **23** and **25** are significantly less potent than **22**. The secondary alcohol of **22** did not appear to make any specific interaction with the protein; however, other crystal structures of JAK3 (e.g., 3LXK) showed the existence of a water network in this region of the protein.

Compound **22** was chosen for additional profiling. **22** demonstrated reduced hERG channel activity compared to **2** (>10 fold improvement) and was deemed to have an acceptable kinome profile. Additionally, **22** was stable in human, mouse, and rat liver microsomes and was permeable in the PAMPA assay. On the basis of this profile, **22** was advanced to an oral mouse PK study. A 10 mg/kg oral dose of **22** (formulated with 5% EtOH, 5% TPGS, 90% PEG 300) provided a plasma C_{max} of >3 μM and a time above the IL2-IFNγ mouseWB IC_{50} of about 3 h. This appeared to be a promising result, although we wanted to explore higher dose PK to allow for greater exposure during the dosing period.

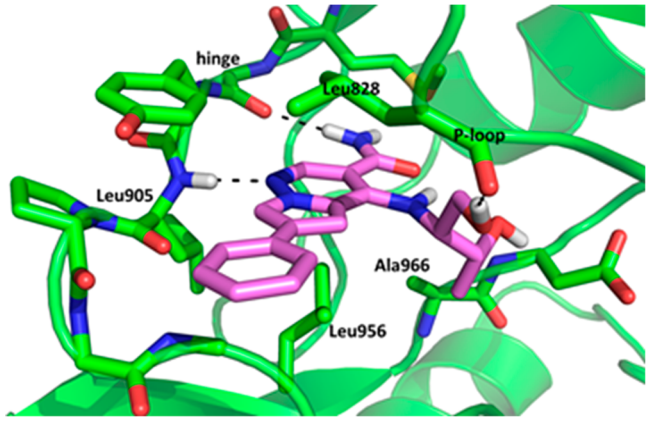


Figure 2. Crystal structure of compound **22** bound to the kinase catalytic domain of JAK3. The carbon atoms are colored green/magenta, oxygens are colored red, and nitrogens are colored blue. Hydrogen bonds are indicated with dashed lines.[24]

After resynthesis of **22**, we planned a PK study at doses of 25 and 50 mg/kg. This new lot failed to dissolve in the original formulation (even at 10 mg/kg dosing concentration). A number of formulation options were explored, but none was

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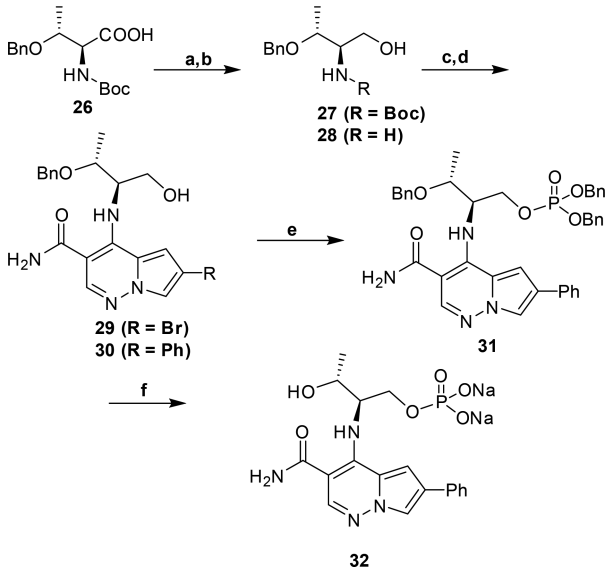
found to be satisfactory. The new lot was found to be crystalline and exhibited poor solubility (<1 μg/mL in 50 mM phosphate buffer). The original amorphous lot was equally insoluble in water. On the basis of these observations, we explored spray dried dispersion but were unable to prevent crystallization at higher drug loads in the matrix. Ultimately we resorted to a microsuspension formulation at a 50 mg/kg oral dose; however, the plasma C_{max} for this study was only 0.1 μM. A single crystal X-ray structure of **22** shows that the compound forms a stable crystal lattice due to an extensive intermolecular hydrogen bonding matrix between neighbor molecules within the unit cell.[25] This observation is also supported by a higher melting point for the dihydroxy analog **22** (280 °C dec) versus the *t*-butylmethyl analog **14** (204 °C dec).

Next we explored the use of a solubilizing prodrug of **22** by preparing and evaluating a phosphate of the primary alcohol (Scheme 2). Benzyl protected Boc-L-threonine (**26**) was

Scheme 2. Synthesis of Phosphate Prodrug (32)



Conditions: (a) LAH, Et_2O, 0 °C (90%); (b) HCl, dioxane, rt (99%); (c) **6**, Hunig's base, DMA, 100 °C (95%); (d) $PhB(OH)_2$, X-Phos, $Pd(OAc)_2$, 2 M K_3PO_4, 100 °C (73%.); (e) (i) dibenzyl diisopropylphosphoramidite, tetrazole, DCM/THF, 0 °C; (ii) 30% H_2O_2, 0 °C (73%); (f) (i) H_2, Pd/C, EtOH (74%); (ii) $NaHCO_3$, MeOH/H_2O (99%).

reduced to the corresponding alcohol, **27**, with LAH in 90% yield. Treatment with anhydrous HCl afforded the amino-alcohol hydrochloride, **28**, which was treated with **6** and Hunig's base at elevated temperature in DMA to afford **29** in a 95% yield over two steps. Suzuki-Miyaura reaction of **29** with phenylboronic acid afforded **30** in 73% yield. Phosphorylation with dibenzyl diisopropylphosphoramidite and hydrogen peroxide, followed by deprotection and salt formation, afforded the disodium salt of the phosphate **32** in good yield over three steps.

The phosphate prodrug (**32**) proved to be freely soluble in water (>1 mg/mL). We progressed the prodrug to PK and found that exposure of the parent was dose proportional (10 mg/kg, AUC ~1 μM h; 40 mg/kg, AUC ~6 μM h). On the basis of this promising result, we designed a mouse collagen induced arthritis study where the low dose was selected to cover the whole blood IC_{50} at trough for 67% of a 24 h period, and the higher dose was selected to cover the whole blood IC_{50} at trough over a 24 h period (mouse IL-15 WB IC_{50}= 810 nM).[26] In addition, **1** was used as a positive control and was dosed to cover the whole blood IC_{50} for 50% of the time. This level of coverage was targeted to approach the exposure observed at the clinical dose of 10 mg BID in humans.[27] The lower dose of **32** demonstrated comparable efficacy to **1**, and we were gratified to observe increased efficacy at the high dose of **32**, which demonstrated that the phosphate prodrug strategy successfully enabled dose escalation (see Figure 3).

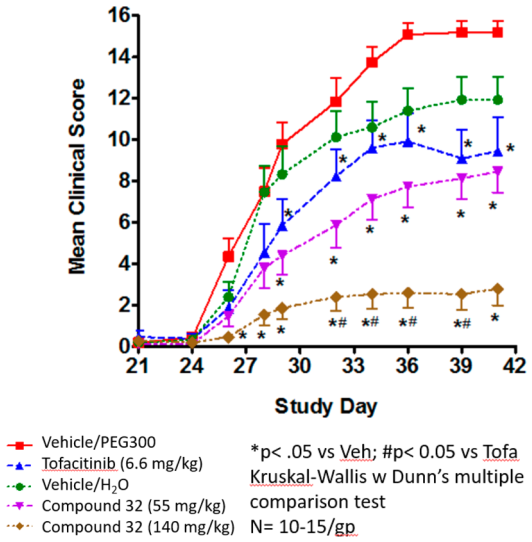


Figure 3. Pseudo-Established Mouse Collagen-Induced Arthritis Study with **32**.* All compounds were dosed orally BID.

Drug levels of the parent **22** after dosing of **32** were close to the initial projections discussed above with the high dose achieving greater than 90% coverage of the whole blood IC_{50} over 24 h. This result clearly demonstrated the utility of a phosphate prodrug strategy to overcome solubility issues associated with the highly crystalline form of the parent.

In summary, we were able to identify novel pyrrolopyridazine diol **22** as a potent inhibitor of JAK1 and JAK3. The compound displayed poor PK properties, which were largely attributed to poor kinetic solubility of a highly crystalline form. However, this limitation was overcome by use of a phosphate prodrug. Prodrug **32** displayed robust dose dependent efficacy in a mouse CIA model, which demonstrated preclinically that a JAK1/3 inhibitor profile may have utility in the treatment of autoimmune disease.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00508.

Synthetic procedures and characterization data for compounds **4**–**32**, *in vitro* profile of **22**, kinome selectivity, biological methods, efficacy study terminal bleed data (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

JAK1, Janus family kinase 1; JAK2, Janus family kinase 2; JAK3, Janus family kinase 3; TYK2, tyrosine kinase 2; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IL-7, interleukin 7; IL-9, interleukin 9; IL-15, interleukin 15; IL-21, interleukin 21; ATP, adenosine triphosphate; PK, pharmacokinetics; hERG, human ether-a-go-go-related gene; PAMPA, parallel artificial membrane permeability assay; SAR, structure activity relationship; GCK, germinal center kinase; GLK, germinal center kinase like kinase; ROCK, rho associated protein kinase

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