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2-Aminopyrazolo[1,5-*a*]pyrimidines as potent and selective inhibitors of JAK2

Mark W. Ledeboer *, Albert C. Pierce *, John P. Duffy, Huai Gao, David Messersmith, Francesco G. Salituro †, Suganthini Nanthakumar, Jon Come, Harmon J. Zuccola, Lora Swenson, Dina Shlyakter, Sudipta Mahajan, Thomas Hoock, Bin Fan ‡, Wan-Jung Tsai, Elaine Kolaczkowski, Scott Carrier, James K. Hogan, Richard Zessis, S. Pazhanisamy, Youssef L. Bennani

Vertex Pharmaceuticals Inc., Cambridge, MA 02139-4242, United States

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ABSTRACT

Constitutive activation of the EPO/JAK2 signaling cascade has recently been implicated in a variety of myeloproliferative disorders including polycythemia vera, essential thrombocythemia and myelofibrosis. In an effort to uncover therapeutic potential of blocking the EPO/JAK2 signaling cascade, we sought to discover selective inhibitors that block the kinase activity of JAK2. Herein, we describe the discovery and structure based optimization of a novel series of 2-amino-pyrazolo[1,5-*a*]pyrimidines that exhibit potent inhibition of JAK2.

Myeloproliferative disorders (MPDs) are a collection of hematological neoplasms including chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis, all of which are characterized by the overproduction of one or more cell types belonging to the myeloid lineage.[1] With the exception of CML, which was shown to be a direct result of activation of the tyrosine kinase 'abl' via a reciprocal translocation between chromosomes 9 and 22 producing the oncogene bcr-abl, the cause of the other disorders was poorly understood until recently. JAK2 is a member of the Janus kinase (JAK) family of non-receptor protein tyrosine kinases, known to play a crucial role in cytokine mediated signal transduction pathways that also include JAK1, JAK3 and TYK2.[2] In early 2005, several groups independently discovered a single point mutation in JAK2 (V617F) common in patients diagnosed with MPDs other than CML. While the mechanism by which the V617F mutation leads to myeloproliferative disorders is not fully elucidated, it is apparent that the mutation results in a gain of function in the JAK2 kinase resulting in pro-proliferative effects via the JAK2 signaling cascades. Currently, this remains an area of intense research to understand the exact mechanism by which the JAK2 kinase is activated via the valine to phenylalanine substitution at position 617.[3,4] In a program directed at discovering the therapeutic potential of attenuating the EPO/JAK2 signaling pathway we sought to identify compounds that would block the kinase activity of JAK2 by targeting the ATP binding site.[5]

Initial screening of our corporate compound collection identified a small set of submicromolar inhibitors of JAK2 containing an aminopyrazolopyrimidine core (APP) (**9–18**, Table 1). Interestingly, compound **12** demonstrated a high affinity for JAK2 and exhibited 40-fold isotype selectivity versus JAK3. To better understand the binding of these compounds, we initiated protein–ligand X-ray crystallographic studies of **12** bound to the kinase domain of JAK2. The co-complex revealed several key binding interactions responsible for binding this ATP active site.[6]

Compound **12** forms two hinge hydrogen bonds with its aminopyrazolopyrimidine (APP) core (Fig. 1). The hydrogen bonds are to the backbone NH and carbonyl of Leu-932. An intramolecular hydrogen bond fixes the pyrimidine conformation, holding it coplanar with the core ring system while directing its amine substituent deep into the active site.

The piperidine ring forms hydrophobic contacts with the glycine-rich loop above it and places the amide substituent against Gly 993 and Asp 994 at the beginning of the activation loop of JAK2. It is these latter interactions that confer selectivity. The

* Corresponding authors. Tel.: +1 617 444 6100.
E-mail addresses: mark_ledeboer@vrtx.com (M.W. Ledeboer), al_pierce@vrtx.com (A.C. Pierce).
† Present address: Agios Pharmaceuticals, 38 Sidney Street, Cambridge, MA 02139, United States.
‡ Present address: Nektar Therapeutics, 201 Industrial Road, San Carlos, CA 94070, United States.

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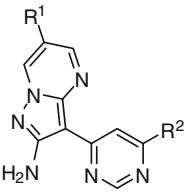
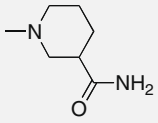
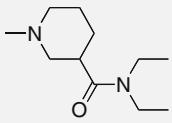
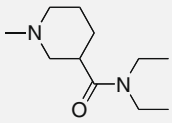
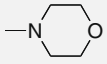
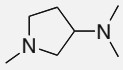
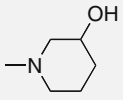
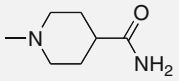
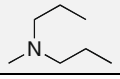
Table 1
Activity of APP analogs



Compd	R^1	R^2	JAK2 K_i (μM)	JAK3 K_i (μM)
9	H		0.020	0.21
10	H		0.044	0.46
11	H		0.084	0.50
12	H		0.017	0.67
13	Me		0.26	2.1
14	H		0.21	1.60
15	H		0.88	4.0
16	H		0.070	0.42
17	H		0.19	1.80
18	H		0.025	0.21

flexibility of the glycine residue of JAK2 allows it to flip into a 'carbonyl-up' conformation, bringing it and the adjacent Asp 994 into closer contact with the amide substituent of 12. The 'carbonyl-down' conformation required by the corresponding Ala-Asp (the green residues in Fig. 1) in JAK3 does not allow the same favorable contacts.[7] Finally, the complex implied a preference for the *S*-enantiomer of the piperidine carboxamide.

In this Letter, we report the elaboration of the APP series to optimize the potency against JAK2, isotype selectivity versus JAK3, and to improve pharmacokinetic properties.

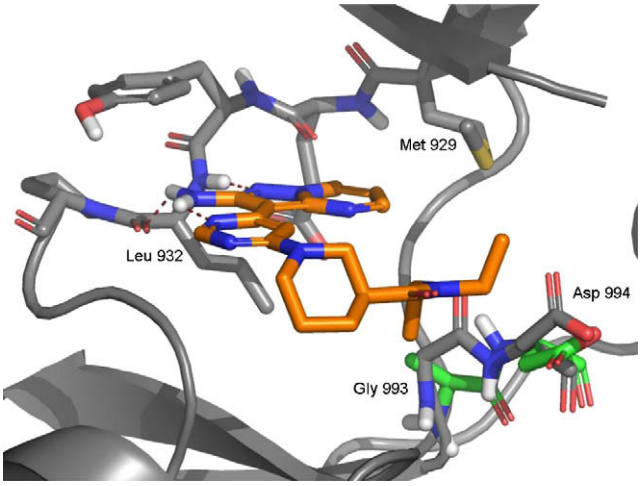


Figure 1. Crystal structure of Compound **12** bound to JAK2. Dashed lines represent hydrogen bonds, both intramolecular and to Leu 932 of JAK2. The Ala and Asp residues of JAK2 that correspond to Gly 993 and Asp 994 are shown in green.

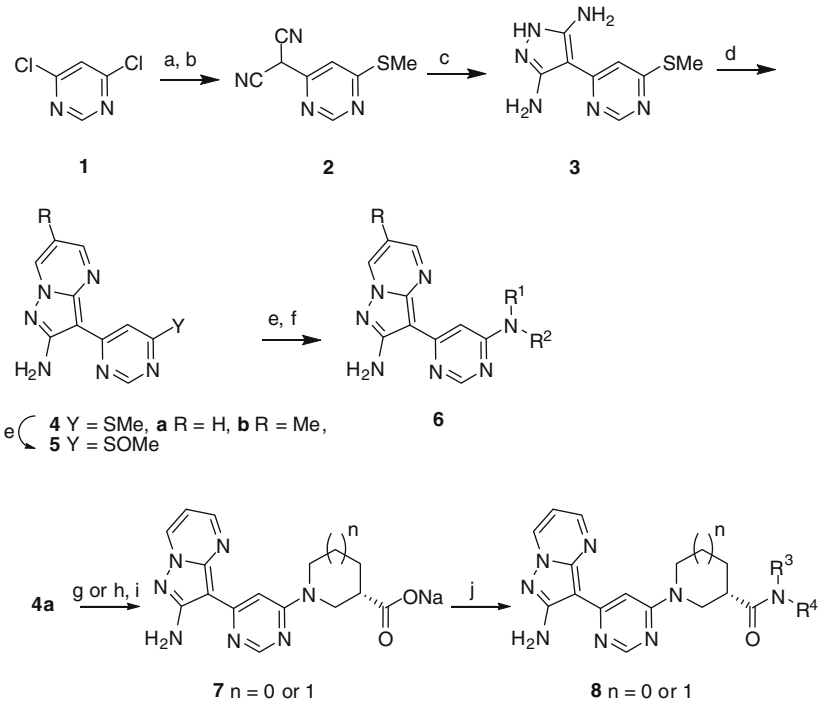
The synthetic approach for this series is outlined in Scheme 1. Treatment of 4,6-dichloropyrimidine with NaSMe generated the corresponding 4-chloro-6-thiomethylpyrimidine,[8] which was added to the sodium salt of malononitrile in a Pd-mediated coupling to provide **2**.[9] Addition of hydrazine hydrate generated diaminopyrazole **3** which could be readily cyclized with either 3-(dimethylamin)acrolein or 3-(dimethylamino)-2-methyl-2-propenal under refluxing conditions to provide the APP core **4a–b**.[10]

Subsequent oxidation of sulfide **4** to the corresponding sulfoxide **5** could be achieved with mCPBA at 0 °C to give **6** ready for further elaboration. The sulfoxide group could be displaced with both primary and secondary amines under thermal conditions or more conveniently on microwave irradiation (NMP, 140–160 °C, typically 10–20 min).[11–13] Compounds of type **7** and **8** were synthesized by sulfoxide displacement of **5a** with the corresponding cyclic piperidine or pyrrolidine carboxylate esters. Hydrolysis of the esters with NaOH provided the corresponding carboxylic acids, which were most conveniently isolated as their corresponding sodium salts **7**. Amide coupling under standard conditions with PyBrop provided final amides **8**.[14]

From the SAR data in Table 1, it is clear that a small substitution at R^1 led to dramatic decreases in potency. Comparing **12** with **13**, addition of a methyl group reduced potency by 15-fold.[15]

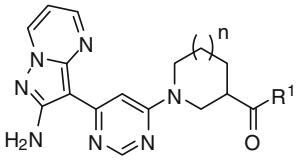
This result is consistent with the observed binding mode for **12**. Extending further into the active site leads to a steric clash with the side-chain of Met-929, resulting in decreased binding affinity (Fig. 1). We identified a variety of potent compounds by varying the amine component (R^2) to include cyclic, acyclic, primary and secondary aliphatic amines and by examining a variety of functional groups including amides, alcohols, ethers and basic amines (see Table 1). Of particular interest was compound **12**, since it exhibited a good potency and isotype selectivity against JAK3. Selectivity of other amines for JAK2 over JAK3 typically ranged from 5 to 10-fold. Therefore, we focused our optimization effort on the SAR of the piperidine carboxamide moiety.

First, we synthesized *S*-enantiomer **22** in order to confirm the stereochemical preference suggested by the X-ray co-complex (table 2). It was gratifying to see that the binding potency of the *S*-enantiomer (**22**) is slightly better than the corresponding racemate (**12**) and maintained the isotype selectivity versus JAK3. Hence, all piperidine-carboxamides were prepared with the *S*-configuration (Table 2). The pyrrolidine containing analogs were prepared as the racemates (**19**, **21**).



Scheme 1. Use Reagents and conditions: (a) NaSMe, THF, 60 °C; (b) malonitrile, NaH, $Pd(PPh_3)_4$, DMSO, 100 °C ; (c) hydrazine hydrate, *i*PrOH, reflux; (d) 3-(dimethylamino)acrolein or 3-(dimethylamino)-2-methyl-2-propenal, HOAc, *i*PrOH, reflux ; (e) mCPBA, DMF, 0 °C; (f) primary or secondary amines, NMP, 140–160 °C, MW, 10–20 min; (g) (*S*)-methyl pyrrolidine-3-carboxylate, 100 °C; (h) (*S*)-methyl piperidine-3-carboxylate, 100 °C; (i) NaOH (aq) THF–H_2O; (j) 3-dimethylamino-2-methyl-2-propenal.

Table 2
Activity of piperidine amide analogs



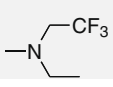
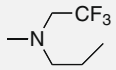
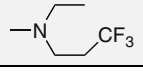
Compd	R^1	*n*	JAK2 K_I (μM)	JAK3 K_I (μM)	TF1-GMCSF IC_{50} (μM)
19	–OMe	0	0.087	0.60	—
20	–OEt	1	0.13	0.57	—
21		0	0.16	2.8	—
22		1	0.007	0.40	>20
23		1	0.20	2.1	—
24		1	0.0036	0.19	6.5
25		1	0.0047	0.22	1.9
26		1	0.004	0.17	1.3

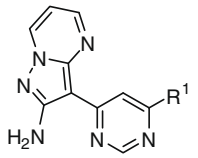
Table 2 (*continued*)

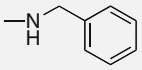
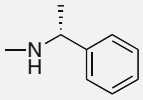
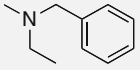
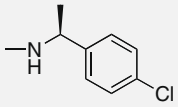
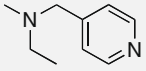
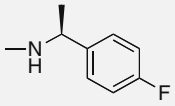
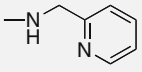
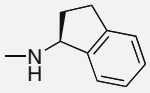
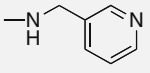
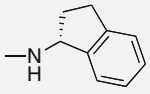
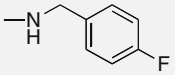
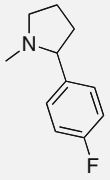
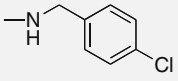
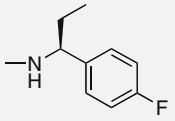
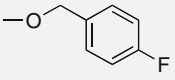
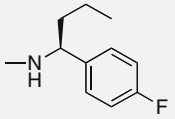
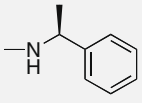
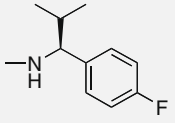
Compd	R^1	*n*	JAK2 K_I (μM)	JAK3 K_I (μM)	TF1-GMCSF IC_{50} (μM)
27		1	0.0031	0.067	1.1
28		1	0.0046	0.20	0.91

Next, we compared ring size of the cyclic amine. The larger piperidine carboxamides are typically 10–40-fold more potent than the corresponding pyrrolidines (**21** vs **24**). The potency and selectivity of the intermediate esters (**19, 20**) was less pronounced, suggesting that the more rigid and planar amide may confer a conformational preference.

A survey of amide substitution suggested that small aliphatic tertiary amines provided the best selectivity. Reducing the size of the *N*-alkyl groups had a detrimental effect on potency. For example, dimethyl amide **23** was 29-fold less potent than diethylamide **22**. The loss in potency was accompanied by a loss in selectivity as well. However, extending one or both of the alkyl groups provided additional high affinity compounds (**24, 25**), which maintained 30-fold selectivity over JAK3 and demonstrated improved cell potency. However, attempts at further improving the cell potency met with limited success. Only replacement of one of the alkyl groups with either a trifluoroethyl (**26**, **27**) or trifluoropropyl (**28**) further improved potency, while again maintaining isotype selectivity. All other attempts at improving affinity or cell potency for this piperidine carboxamide series were fruitless as the use of larger alkyl groups, branching of the alkyl groups, monosubstitution, incorporation of unsaturation or heteroatoms in the alkyl chain all proved detrimental to potency (data not shown).

Table 3
Activity of benzylamine analogs



Compd	R^1	JAK2 K_I (μM)	JAK3 K_I (μM)	TF1-GMCSF IC_{50} (μM)	Compd	R^1	JAK2 K_I (μM)	JAK3 K_I (μM)	TF1-GMCSF IC_{50} (μM)	HT2-IL2 IC50 μM
29		0.006	0.054	>20	**38**		0.021	0.29	18	>20
30		0.034	0.23	—	**39**		0.008	0.081	6.9	11
31		0.56	4.4	—	**40**		0.0002	0.007	0.16	3.2
32		0.022	0.16	>20	**41**		0.052	0.24	—	—
33		0.036	0.049	—	**42**		0.011	0.20	19	>20
34		0.0015	0.039	3.0	**43**		0.0034	0.086	>20	3.1
35		0.0014	0.025	1.5	**44**		0.0006	0.012	0.27	1.9
36		0.0078	0.18	>20	**45**		0.0005	0.010	0.075	2.4
37		0.0014	0.017	4.7	**46**		0.0006	0.012	0.47	4.1

In order to overcome the limited cell potency, we sought alternative substituents that might allow us to drive down the affinity further while maintaining suitable isotype selectivity. A survey of available amines suggested that secondary benzylic amines were potent compounds and provided opportunity for further optimization (see compound **29**, Table 3).

In the benzylic amine series, tertiary amines were not as potent. For example, addition of an *N*-ethyl group (**30**) decreased the potency. Replacing the phenyl group with a pyridine group also lead to loss of potency (**31–33**). However, incorporation of a 4-halo substitution such as F or Cl enhanced enzyme potency fourfold and also improved cell potency (**34, 35**). Importantly, the 4-F-substitution on the phenyl ring improved the isotype selectivity against JAK3 (**34**) by threefold. Replacing the attachment N atom with oxygen led to a fivefold decrease in potency and an even greater loss of cell potency (**36** vs **34**). However, substitution at the benzylic position proved fruitful. Addition of a benzylic methyl boosted potency and further suggested that the *S*-stereochemistry is preferred (*S*-**37** vs *R*-**38**). Combining both 4-F and benzylic methyl groups provided our most potent compound (**40**), which was 35-fold selective over JAK3 and also exhibited submicromolar cellular activity. However, incorporation of the benzylic methyl group decreased potency

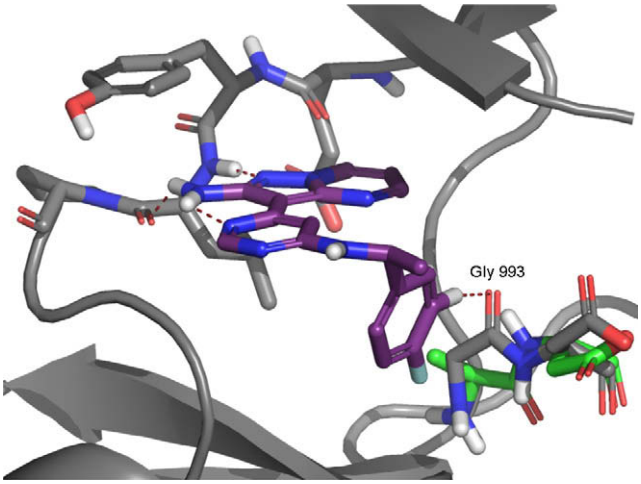


Figure 2. Crystal structure of compound **40**. The CH···O hydrogen bond between the ligand and the carbonyl of Gly 993 is shown in red dashes.

Table 4
Pharmacokinetic parameters determined following single IV administration (1 mg/kg) in male Sprague-Dawley rats

Compds	Cl (mL/min/kg)	$t_{1/2}$ (h)	V_{ss} (L/kg)	AUC_{inf} (g h/mL)
40	104	0.9	5.1	0.16
44	111	0.7	4.5	0.15
45	43.6	3.3	9.5	0.38
46	34.7	2.4	5.1	0.48

Mean values determined from the concentration (as determined using a specific LC/MS/MS method) of each compound in rat ($n = 2$) plasma samples collected prior to dosing up to 8 h post-dose.

when a 4-Cl phenyl group was present (**39**), suggesting size limitations of the pocket. Constraining the compounds to further to enhance potency and to improve the pharmacokinetic profile of the compound did not meet with success and in all cases we saw a drop in enzyme and/or cell potency (**41–43**). Additional exploration of benzylic substitution identified several other potent compounds that retained much of the isotype selectivity against JAK3 (approx. 20-fold) while still exhibiting submicromolar cellular activity (**44–46**).

Next, we assessed the cellular selectivity for JAK2 versus JAK3 mediated STAT-5 phosphorylation. GMCSF stimulation of TF1 cells leads to STAT-5 phosphorylation by JAK2, while IL2 stimulation of HT2 cell lines yields phospho-STAT-5 via the JAK3 pathway.[16] As shown in Table 3, for our most potent compounds a good correlation between the enzymatic and cellular selectivity was observed and cellular selectivities ranged from 8- to 32-fold (**40, 44–46**). Compound **45** was found to be the most selective (32-fold).

Screening of selected compounds against our in-house panel of kinases revealed the series to be quite selective. For example, compound **46** hit only 2 of 24 kinases at K_i <200 nM (FLT3 K_i = 160 nM and ROCK K_i = 190 nM).

To better understand the effect of F-substitution on selectivity, we determined the X-ray crystallographic complex of **40** bound to the kinase domain of JAK2. The selectivity of the 4-F benzylamines arises from a unique CH···O hydrogen bond with Gly 993 in JAK2, as shown in Figure 2.[17] In JAK3 this residue is an Ala, and very few kinases have a Gly in this position as JAK2 does. Because of the flexibility of the glycine side-chain, the protein backbone of JAK2 can flip up to form a hydrogen bond with the aromatic CH of the benzylamine. While the unsubstituted phenyl does make a weak interaction with the carbonyl oxygen, the CH groups adjacent to the fluorine are significantly more polarized, leading to a more favorable interaction with JAK2 that is unavailable in JAK3.

Next, the pharmacokinetic parameters were determined for compounds with good cellular and enzyme potency. It is evident from Table 4 that several compounds exhibit a favorable IV PK profile. While compounds **40** and **44** are rapidly cleared as indicated by high clearances and short T1/2, both compounds **45** and **46** exhibit favorable IV PK profiles with reduced clearance, greater exposure and extended half-lives.

In summary, we have identified potent and selective JAK2 inhibitors with an aminopyrazolopyrimidine core. Specific incorporation of the benzylic moiety dramatically enhanced cell potency while maintaining JAK2 isotype selectivity through a unique CH···O hydrogen bond with Gly 993. In addition, several examples showing promising PK profiles were also identified.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.053.

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