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Discovery of pyrazol-3-ylamino pyrazines as novel JAK2 inhibitors

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ABSTRACT

The design, synthesis and biological evaluation of a series of pyrazol-3-ylamino pyrazines as potent and selective JAK2 kinase inhibitors is reported, along with the pharmacokinetic and pharmacodynamic properties of lead compounds.

The Janus-associated kinase (JAK) family consists of four non-receptor tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) that play pivotal roles in many aspects of cytokine and growth factor mediated signal transduction.[1] While JAK1, JAK2, and TYK2 are ubiquitously expressed, JAK3 is limited to lymphocytes,[2] where it plays an important role in immune system control and maturation. JAK2 is essential for hematopoiesis, platelet formation and other functions that are important in cellular survival, proliferation and differentiation.[1] Cytokine binding to the corresponding cell-surface receptor causes receptor dimerization and facilitates activation of receptor-bound JAK by autophosphorylation. Activated JAK in turn phosphorylates the receptor, stimulating the recruitment of the signal transducers and activators of transcription (STATs). STAT phosphorylation by JAK causes dissociation from the receptor, subsequent dimerization and translocation to the nucleus. A unique, acquired single point mutation (V617F) in the JAK Homology 2-domain of JAK2 has been identified in most Philadelphia chromosome negative myeloproliferative neoplasms (MPNs).[3–5]

These disorders are a group of clonal hematopoietic stem cell disorders that include polycythemia vera, essential thrombocythemia and idiopathic myelofibrosis and in their majority are characterized by the identical JAK2 mutation.[6] The JAK2 V617F protein is capable of modulating downstream signaling in the absence of cytokine stimulation. Although it is uncertain how the same somatic mutation could result in the various phenotypes associated with these disorders, the 'dosage' of JAK2-V617F mutation has been implicated with the corresponding clinical outcome.[7] Currently there are several JAK2 inhibitors being tested in clinical trials. INCB-18424,[8] XL019,[9] TG101348,[10] and SB1518[11], are among the reportedly selective, orally bioavailable JAK2 kinase inhibitors.

Significant reduction in spleen size and improvement in the quality of life of treated patients has been reported during the clinical evaluation of early JAK2 inhibitors.[12] We have recently described the pyrazolo nicotinonitrile, AZ960 **1** (Fig. 1) as a novel small molecule JAK2 inhibitor,[13] inhibiting JAK2 enzymatic activity with an IC_{50} <0.003 μM. Although AZ960 showed excellent JAK2 inhibitory activity we sought to expand our efforts by transferring the pyrazol-3-ylamine hinge binding motif onto a pyrazine scaffold. In this Letter, we describe the discovery of pyrazol-3-ylamino

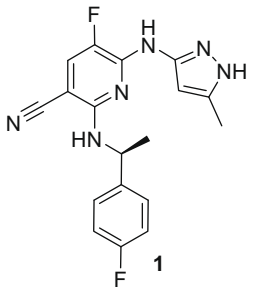

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Figure 1. Structure of AZ960.

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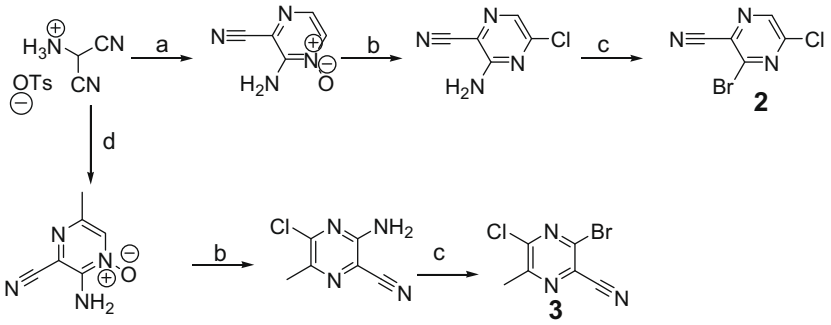


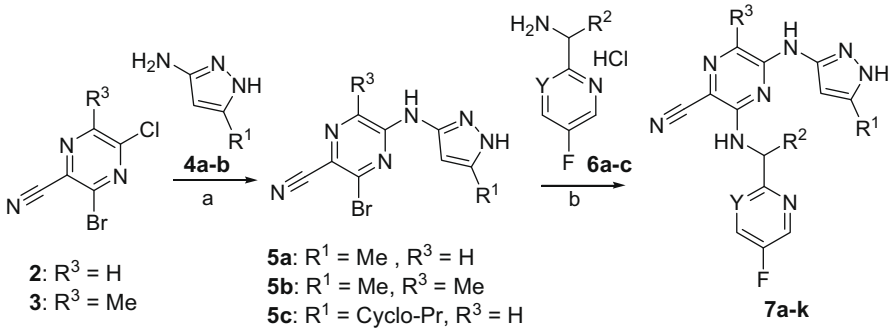
Scheme 1. Preparation of pyrazine scaffolds. Reagents and conditions: (a) glyoxal (30% w/w in H_2O), acetone oxime; (b) $POCl_3$, 80 °C; (c) *t*-BuONO, $CuBr_2$, 65 °C; (d) isonitrosoacetone, *i*-PrOH, rt.

pyrazines as potent and selective JAK2 inhibitors. The synthetic route to the pyrazine scaffolds is illustrated in Scheme 1. Reaction of aminomalonitrile *p*-toluenesulfonate with glyoxal in the presence of acetone oxime gave the corresponding pyrazine *N*-oxide. Concomitant deoxygenation and chlorination of the *N*-oxide with $POCl_3$, followed by Sandemeyer reaction afforded compound **2** in good yield. In a similar manner, pyrazine **3** was prepared from the coupling reaction aminomalonitrile *p*-toluenesulfonate with isonitroso acetone followed by subsequent chemical manipulation described for **2**.[14–16]

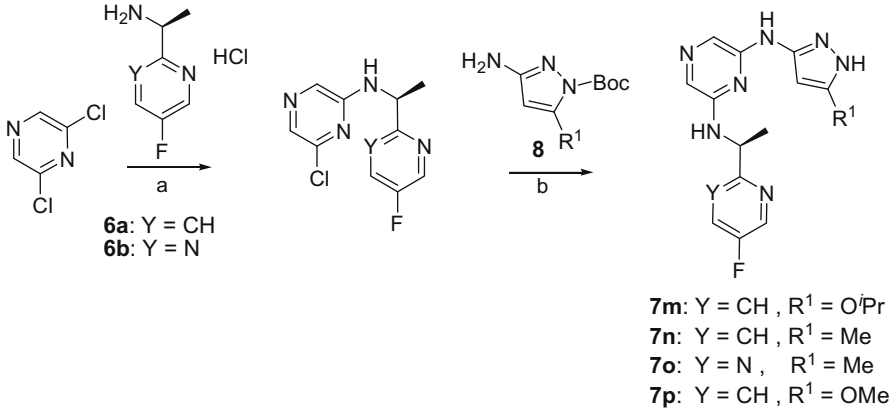
Pyrazines **2** and **3** were reacted with aminopyrazoles **4** (R^1 = cyclo-Pr, Me) in the presence of Hünig's base to give intermediates **5a–c** in good yield. Nucleophilic displacement of the bromide, under microwave irradiation, with α-methyl heteroarylamines **6a–c** (**6a**: Y = CH, R^2 = (*S*)-Me; **6b**: Y = N, R^2 = (*S*)-Me; **6c**: Y = CH, R^2 = (*rac*)-Me) gave the desired final products **7a–k** (Scheme 2 and see Table 4 for R^1, R^2, R^3, and R^4 definitions). Ref. 18 provides detailed experimental procedures for all analogs described in Table 4.

In the absence of the cyano group on the pyrazine scaffold, the attachment of the aminopyrazole proved to be sluggish under several examined conditions. In order to circumvent this issue, the heteroarylamines (*S*)-5-fluoropyridin-2-yl-ethanamine **6a** and (*S*)-5-fluoropyrimidin-2-yl-ethanamine **6b** were first installed by microwave heating and subsequent palladium-catalyzed amination with Boc-protected aminopyrazoles **8** (Scheme 3) afforded the corresponding compounds (**7m**, **7n**, **7o**, and **7p**). It is noteworthy that the Boc protecting group was cleaved under the microwave conditions employed. The Boc- protected aminopyrazoles **8** prepared were either commercially available (R^1 = Me) or synthesized according to previously reported procedures (R^1 = OMe, O^{i}Pr).[17]

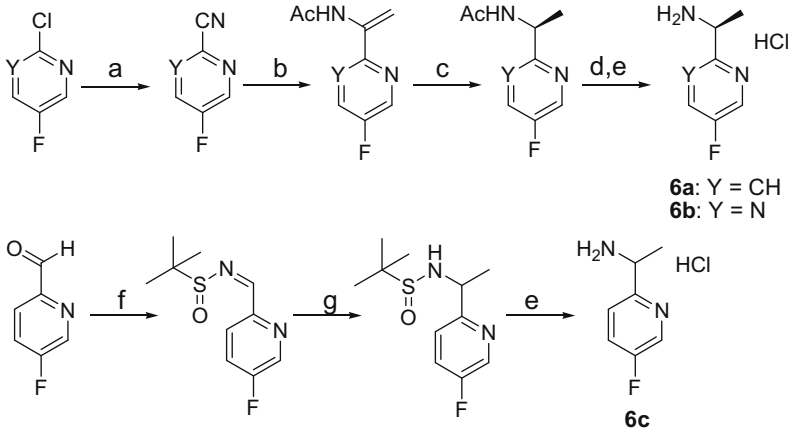
The synthesis of (*S*)-5-fluoropyridin-2-yl- and (*S*)-5-fluoropyrimidin-2-yl-ethanamines (**6a** and **6b**, respectively) has been previ-



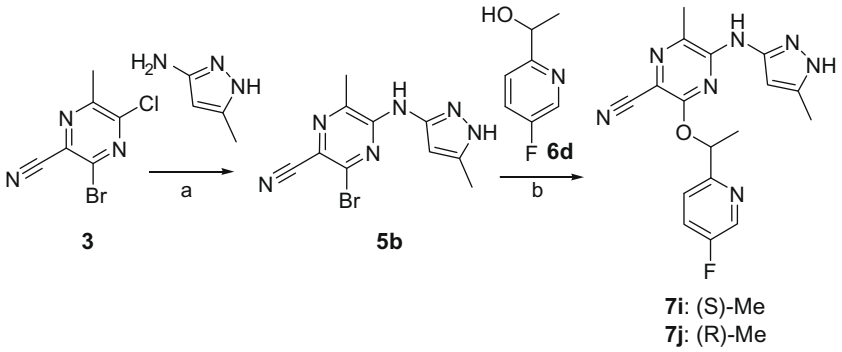
Scheme 2. Reagents and conditions: (a) Hünig's base, 65 °C; (b) Hünig's base, 160 °C, microwave.



Scheme 3. Reagents and conditions: (a) NMP, Hünig's base, 180 °C, microwave; (b) Cs_2CO_3, $Pd_2(dba)_3$, Xantphos®,dioxane, 100 °C, microwave.

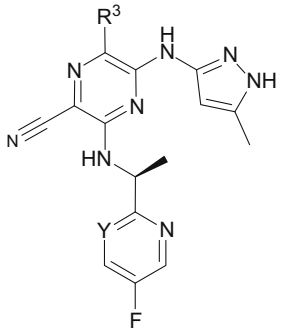


Scheme 4. Reagents and conditions: (a) $Pd_2(dba)_3$, $Zn(CN)_2$/Zn, dppf; (b) MeMgBr, Ac_2O (c) H_2, *S,S*-Et-Duphos-Rh-OTf, 150 psi (d) (i) Boc_2O, 180 °C; (ii) LiOH, THF/H_2O; (e) 4 N HCl in dioxane/MeOH; (f) *t*-BuS(O)NH_2, $CuSO_4$, DCM; (g) MeMgBr, THF.



Scheme 5. Reagents and conditions: (a) Hünig's base, 65 °C; (b) NaO-*t*-Bu, *t*-BuOH, 90 °C.

Table 1
Cell and selectivity data of lead compounds



Compound	R^3/Y	TEL-JAK2 GI_{50} (μM)	TEL-JAK3 GI_{50} (μM)	EPOR-STAT5 EC_{50} (μM)	CDK2/AurB IC_{50}(μM)	TRKA IC_{50} (μM)
7a	H/N	0.16	1.3	0.04	0.05/1.84	1.44
7c	Me/N	0.22	7.4	0.04	0.41/0.85	1.48
7e	Me/CH	0.11	2.1	0.02	0.46/0.27	0.58
7h	H/CH	0.06	1.1	NT	0.03/0.68	0.67

Table 2
Pharmacokinetic properties of lead compounds

Compound	Rat[a]			Dog[b]		
	CL ml/min/Kg	V_{dss} l/Kg	$T_{½}$ h	CL ml/min/Kg	V_{dss} l/Kg	$T_{½}$ h
7c	13	1.6	1.6	<2	1.1	7.0
7h	25	2.2	1.2	NT[c]	NT[c]	NT[c]

[a] Han Wistar rat male; 10 mg/kg po (0.1% HPMC); 3 mg/kg iv (DMA/PEG/saline = 40/40/20).
[b] Male Beagle; 2.5 mg/kg iv, 70%TEG:30% Captisol (1:1).
[c] Denotes not tested.

Table 3
Pharmacodynamic data on lead compounds

Compound	P-STAT5 inhibition (%) at 2 h post dose		
	5 mpk	10 mpk	25 mpk
7c	>80%	>90%	>95%
7h	>80%	>90%	>90%[a]

[a] At 4 h post dose.

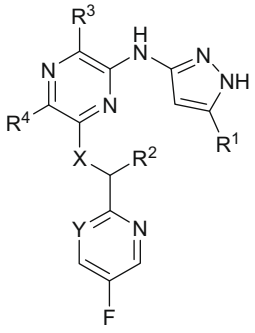
ously described.[18] Cyanation of the α-halo aza substrate using $Zn(CN)_2$ (Scheme 4) in the presence of palladium catalyst followed by acetyl trapping of the Grignard intermediate gave the corresponding vinyl acetamides. Asymmetric hydrogenation in the presence of (*S*,*S*-Et-Duphos-Rh-OTf),[19] followed by protecting group manipulation, generated the desired amines in excellent overall yield (>70%). The chiral integrity of these intermediates has been unambiguously determined.[18] In order to confirm that no stereochemical erosion occurred upon the installment of the chiral amine, the racemic material **7g** (Table 4) was prepared. In this case, the racemic heteroarylamine **6c** was readily accessible via Ellman's sulfinamide chemistry.[20] Sulfinamide formation from the commercially available 5-fluoro-2-formylpyridine under standard conditions, Grignard addition and subsequent removal of the protecting group under acidic conditions furnished the desired amine as the corresponding hydrochloride salt (Scheme 4).

To our surprise, determination of the enantiomeric purity of **7e** revealed partial racemization of the chiral center. Similarly, attachment of (*S*)-5-fluoropyrimidin-2-yl-ethanamine **6b** to intermediate **5b** gave a mixture of enantiomers that could be readily isolated by chiral chromatography (compounds **7c** and **7d**, see Table 4). In contrast, during formation of compound **7h**, no appreciable scrambling of the chiral center was observed using similar reaction conditions. We speculated that the introduction of the chiral amine in the desmethyl pyrazine intermediates was faster than in the methyl analogs, presumably permitting enough time to allow scrambling of the chiral center. However, the exact mechanistic pathway that leads to the partial racemization is unclear.

To further expand our SAR, benzyl alcohol **6d** was treated with sodium *t*-butoxide in *t*-BuOH to generate the corresponding alkoxide, and subsequent reaction with **5b** afforded the products **7i** and **7j** (Scheme 5). Finally, in an attempt to convert the cyano group to the corresponding primary amide, compound **7l** (Table 4) was formed upon exposure of **7e** to aqueous base, presumably via hydrolysis to the acid and consequent decarboxylation. For this series of compounds, it was anticipated that the pyrazole moiety would interact with the ATP-binding site (hinge) whereas the C-5 substituent would be directed towards the selectivity pocket (gatekeeper). The rather large gatekeeper residue (methionine) in JAK2 would disfavor the introduction of large substituents at the pyrazole C-5 position. Accordingly, analog **7m** had reduced JAK2 enzymatic activity when compared to the methyl analog **7h** (Table 4). In several cases, when testing at K_m concentrations of ATP, IC_{50} values for JAK2 inhibition were at the detection limit of the assay[13] making it difficult to prioritize compounds for cascade progression. Thus, assays using high concentrations of ATP (5 mM) were introduced in our enzyme-screening cascade (JAK2 and JAK3),[21] in order to allow better differentiation of the various analogs. It should be noted that most of the compounds portrayed excellent selectivity against JAK3 (>200-fold, Table 4).

Interestingly, the replacement of NH (compound **7e**) with oxygen (**7i)** seems to be costly in terms of activity, while the absence of CN at R^4 does not alter the potency significantly (**7l**–**p** see Table 4).

Table 4
Pyrazol-3-ylamino pyrazines SAR



Compound	R^1	R^2	X	Y	R^3	R^4	JAK2 (Km ATP) IC_{50} (μM)	JAK2 (5 mM ATP) IC_{50} (μM)	JAK3 (5 mM ATP) IC_{50} (μM)
7a	Me	(*S*)-Me	NH	N	H	CN	<0.003	0.006	5.03
7b	Me	*rac*-Me	NH	CH	H	CN	<0.003	0.005	12.77
7c	Me	(*S*)-Me	NH	N	Me	CN	<0.003	0.087	26.48
7d	Me	(*R*)-Me	NH	N	Me	CN	0.050	10.42	>30
7e	Me	(*S*)-Me	NH	CH	Me	CN	<0.003	0.011	15.67
7f	Me	(*R*)-Me	NH	CH	Me	CN	0.075	0.907	>30
7g	Me	rac-Me	NH	CH	Me	CN	<0.003	0.047	25.05
7h	Me	(*S*)-Me	NH	CH	H	CN	<0.003	0.003	5.17
7i	Me	(*S*)-Me	O	CH	Me	CN	0.004	0.142	>30
7j	Me	(*R*)-Me	O	CH	Me	CN	0.108	4.666	>30
7k	Cyclopropyl	(*S*)-Me	NH	CH	H	CN	<0.003	0.025	26.88
7l	Me	(*S*)-Me	NH	CH	Me	H	<0.003	0.018	3.66
7m	O′Pr	(*S*)-Me	NH	CH	H	H	0.010	0.421	15.22
7n	Me	(*S*)-Me	NH	CH	H	H	<0.003	0.003	11.71
7o	Me	(*S*)-Me	NH	N	H	H	<0.003	0.003	13.73
7p	OMe	(*S*)-Me	NH	CH	H	H	0.003	0.008	NT[a]

[a] NT: not tested.

Compounds **7a**, **7c**, **7e**, and **7h** were tested in a TEL-JAK2 proliferation assay in Ba/F3 cells[13] or EPOR-STAT5 assay.[22] In addition, their selectivity versus Aurora B, CDK2 and TRKA enzymes was evaluated. As it can be seen in Table 1, the introduction of the Me group on the pyrazine ring results in improved selectivity versus CDK2 while the other kinase activities evaluated were much weaker than the corresponding JAK2 activity. Compounds **7c** and **7h** were used as examples in order to assess the in vivo pharmacokinetic properties in pre-clinical species (Table 2). Both compounds had low intrinsic clearance in rat microsomes and hepatocytes (**7c**: 18 ml/min/mg and 3 ml/min/10^{-6} cells and **7h**:12 ml/min/mg and 3 ml/min/10^{-6} cells, respectively) that translated into low in vivo clearance. Compound **7c** was therefore chosen to progress to dog pharmacokinetic studies. The compound showed excellent plasma stability when it was dosed at 2.5 mg/kg (iv) with reasonable exposure and long half-life.

Based upon the favorable pharmacokinetic properties compounds **7c** and **7h** were profiled in our pharmacodynamic model. In nude mice implanted with TEL-JAK2 transfected Ba/F3 cells the compounds demonstrated a dose-dependent effect, as indicated by the reduced levels of STAT5 phosphorylation in splenic infiltrates of leukemia cells (Table 3).[23]

In conclusion, we have demonstrated that a series of 6-pyrazol-3-ylamino pyrazines are potent and selective JAK2 inhibitors. These compounds possess good pharmacokinetic properties and several lead compounds were identified as suitable candidates to further explore the hypothesis that modulation of JAK2 activity could be beneficial in the treatment of MPNs and other cancers. Further evaluation of such compounds will be reported in due course.

Acknowledgments

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21. To measure JAK2 kinase activity at 5 mM ATP, 150 pM JAK2 enzyme (Upstate Biotechnology MA) was incubated with 80 nM biotinylated TYK2 peptide substrate (Cell Signaling Technology, MA) and adenosine triphosphate (ATP 5 mM) for 60 min at room temperature in reaction buffer containing 10 mM $MgCl_2$, 1 mM DTT, 50 mM HEPES, and 0.025% Tween20. Similarly, JAK3 kinase activity was assayed using 600 pM JAK3 enzyme (Upstate Biotechnology MA), 5 nM biotinylated poly-EY peptide substrate (HTRF CisBio) and 5 mM ATP in reaction buffer for 60 min at room temperature. Reactions were stopped by addition of Detection mix consisting of 20 mM HEPES, 102 mM ethylenediamine tetraacetic acid, 1.65 mg/ml BSA, 136 mM NaCl, 40 μg/ml Streptavidin donor beads (Perkin Elmer, MA), and 40 μg/ml phosphotyrosine-specific antibody coated acceptor beads (Perkin Elmer, MA). Phosphorylated substrate was detected after overnight incubation in an EnVision plate reader (Perkin Elmer, MA) 680 nm excitation, 520–620 nm emission. Data was graphed and IC_{50}s calculated using Xlfit4 4.2.2 (Microsoft).
22. EPOR-STAT5 assay was used as a mode of action assay, which measured the relocalization of STAT5 upon the stimulation of Erythropoietin (EPO). U2OS/EPOR-STAT5-zsGreen stable cell line was generated in house. The cells were seeded at 17,500 cells/well in 96 well plates and incubated at 37 °C/5% CO_2 for 24 h. Cells were dosed with compounds for 30 min and then EPO (0.5 U/ml final concentration, R&D System) for 1.5 h. Cells were then fixed with paraformaldehyde (4% final concentration, Sigma) and the nuclei were stained with Hoechst 33,342 (Invitrogen). The relocalization of STAT5/zsGreen were measured by ImagXpress 5000.
23. Female NCr mice (Taconic Farms, Germantown, NY), aged 5–6 weeks were implanted intravenously with Ba/F3 TEL-JAK2 cells. Ten days post-implantation animals received a single oral dose of vehicle, reference or test compound. Spleens were harvested and snap frozen in liquid nitrogen at specified time points post-compound administration for analysis of pharmacodynamic markers. In addition, blood samples were collected via cardiac puncture from the same animals for pharmacokinetic analysis. Lysates of each spleen sample were prepared by homogenizing tissue samples in lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40 substitution, and 1 mM Sodium Vanadate (phosphates inhibitor set II, CALBIOCHEM #524625)) using a Qiagen Tissue Lyser. Protein values were quantitated using the BCA Protein Assay Kit (Pierce, cat #23227). Lysates were prepared at equivalent protein concentrations in Laemmli Sample Buffer and loaded onto Novex 4–12% Tris–Glycine gels (Invitrogen) for SDS PAGE. Gels were transferred to Nitrocellulose (Invitrogen) and blocked in Blocking Buffer (Licor). Western blots were processed with primary antibodies that recognize phospho-STAT5 (BD Transduction Labs) or total-STAT5 (Epitomics) and then treated with appropriate secondary antibodies (Licor). Phospho-signal was quantitated using Odyssey laser imaging (Licor). % Phospho-STAT5 inhibition was calculated using vehicle and control inhibitors for max and min value using Microsoft Excel. Mice used in the studies were maintained under specific pathogen-free conditions and were used in compliance with protocols approved by the Institutional Animal Care and Use Committees of AstraZeneca, which conform to institutional and national regulatory standards on experimental animal usage.