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Janus Kinase 2 Inhibitors. Synthesis and Characterization of a Novel Polycyclic Azaindole†

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Abstract: The synthesis and characterization of a novel polycyclic azaindole based derivative is disclosed, and its binding to JAK2 is described. The compound is further evaluated for its ability to block the EPO/JAK2 signaling cascade in vitro and in vivo.

The Janus kinases (JAK[a]) are members of a family of intracellular nonreceptor tyrosine kinases that play a crucial role in the cytokine-mediated JAK-STAT signaling pathway.[1] There are four known mamalian kinases in the JAK family: JAK1, JAK2, JAK3, and TYK2. Of particular interest is the emergence of JAK2 as a potential new therapeutic target for myeloproliferative disorders (MPDs).[2] The MPDs include several hematological neoplasms that include polycythemia vera (PV), essential thombocythemia (ET), idiopathic myelofibrosis (IMF), and chronic myeloid leukemia (CML). Recently a common gain-of-function mutation in JAK2 (V617F) was discovered among patients diagnosed with PV, ET, or IMF but not CML. This has resulted in intense efforts to understand the exact mechanism by which the proproliferative effects, resulting from the activation of the JAK STAT pathway by valine to phenylalanine mutation at position 617, lead to these MPDs.[3,4]

In our program directed at identifying agents that can attenuate the EPO/JAK/STAT pathway we sought to block the kinase activity of JAK2 by targeting its ATP binding site.[5,6]

Screening of our corporate compound collection identified several low molecular weight hits with respectable potency

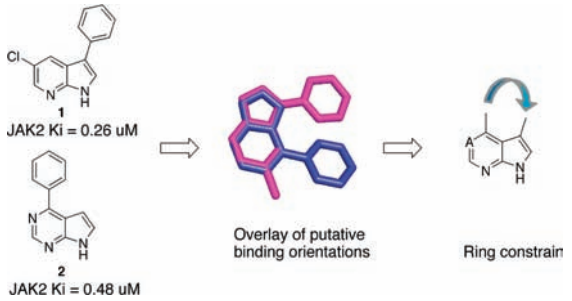
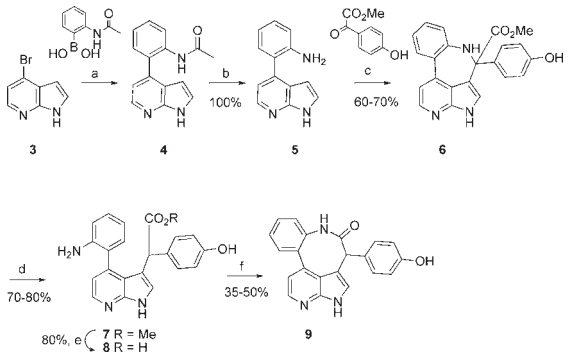


Figure 1. Overlay of putative binding orientations in JAK2.

Scheme 1[a]



[a] (a) $Pd(PPh_3)_4$, K_2CO_3, DME; (b) conc HCl, reflux; (c) HCl−dioxane, MeOH; (d) H_2, Pd−C, HCl, MeOH; (e) 6 M HCl (aq), reflux; (f) HATU, HOAt DIEA, DMF.

containing an azaindole (**1**) or deazapurine (**2**) hinge binding motif similar to CP-690,550.[7] Overlaying the putative ATP binding site orientations of **1** and **2**, we were intrigued by the possibility of creating novel chemotypes with improved potency for JAK2 by cyclizing the 3- and 4-positions (Figure 1).

Here, we detail the synthesis, characterization, and activity profile of **9**, which contains an eight-membered central lactam ring and exhibits in vitro potency against JAK2 mediated end points and in vivo efficacy in a JAK2 dependent mouse model of disease (vide infra).

Previously, SAR studies on related compounds established that compounds containing a 3,4-fused seven-membered central ring were indeed potent inhibitors of JAK2 and that the related phenolic OH plays a dual H-bond acceptor and H-bond donor role which is required to achieve maximum potency. Compounds without the phenolic OH or with alternative H donor or acceptor groups at the 4-position, such as halogen, methoxy, carboxylic acid, aniline groups, were all less potent on the enzyme (data not shown).[8]

Compound **9** with an eight-membered central ring was prepared following the methods described in Scheme 1. Thus, Suzuki coupling of 4-bromoazaindole **3** with the requisite boronic acid gave 4-aryl azaindole **4**. Deprotection of the acetamide was achieved by heating **4** in 6 N aqueous HCl to give the cyclization precursor **5**. Pictet−Spengeler like condensation of **5** with the appropriate ketoester provided cyclized product **6**. Reductive ring-opening under hydrogenation conditions gave ester **7**, which was hydrolyzed under acidic conditions (6 N HCl) to provide the requisite precursor

†PDB ID: The atomic coordinates and structure factors have been deposited in the Protein Data Bank for compound **9** and the kinase domain of JAK2 (PDB code 3JY9).

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[a] Abbreviations: JAK, Janus kinase; TYK, tyrosine kinase 2; STAT, signal transducer and activator of transcription; MPD, myeloproliferative disorder; PV, polycythemia vera; ET, essential thombocythemia; IMF, idiopathic myelofibrosis; CML, chronic myeloid leukemia; EPO, erythropoeitin; EEC, EPO-independent endogenous erythroid colonies; CFU-E, erythroid colony forming unit; GM-CSF, granulocyte-macrophage colony stimulating factor.

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Table 1. JAK2 and JAK3 Mediated Enzyme and Cell Activities for Atropisomers **9A** and **9B**

	K_I, μM		IC_{50}, μM	
compd	JAK2	JAK3	TF1-GMCSF	HT2- IL2
9A	0.0010 ± 0.00016	0.0055 ± 0.0014	0.27 ± 0.16	1.53 ± 1.5
9B	0.001	0.006		

Figure 2. Conformational isomers of **9**. The conformations with the cis-amide are shown in green and orange. The conformation with the trans-amide is shown in pink.

8. Lactam formation was most conveniently carried out with HATU/HOAt to provide **9A** in moderate yield. However, a second cyclization product **9B** was also isolated with identical mass and whose NMR was also consistent with the cyclized product. When the cyclization was carried out at higher temperature (60 °C), more of **9B** formed. Conversely, at ambient temperature the reaction yielded a higher proportion of **9A** albeit at a slower rate. While **9A** and **9B** were separable, there was always a small amount of cross-contamination present.[9]

We speculated that these products might be atropisomers. To confirm this, we monitored for interconversion of the atropisomers, and we found that each isomer, when kept in DMSO solution at room temperature, independently equilibrated to a 2:1 mixture of isomers. The major isomer **9A** equilibrated over a period of 1 week, while the minor isomer **9B** equilibrated more rapidly over the course of approximately 1−2 days. However, when stored in solid form, little or no equilibration of atropisomers was seen over a period of several months. For subsequent studies we used samples freshly prepared from solid **9A**.

Testing of both products against the target enzyme demonstrated that samples of **9A** and **9B** were equipotent inhibitors of JAK2 with similar preference over JAK3 (Table 1).[10,11] In addition, **9A** showed similar fold selectivity for JAK2 vs JAK3 mediated STAT 5 phosphorylation in TF1 and HT2 cells, respectively.[12]

Computational analysis suggests that **9** has two conformations with similar energies but a high barrier to interconversion. Both conformations contain cis-amides, as the trans-amide conformations require a relatively flat, strained conformation of the eight-membered ring. The two cis-conformations (green and orange) and one trans-conformation (pink) can be seen in Figure 2. Given the roughly 2:1 ratio of atropisomers at equilibrium, the energy difference between the two should be approximately 0.4 kcal/mol. This is in reasonably good agreement with the MMFF94s calculated energy difference of 1.6 kcal/mol, with the discrepancy likely due to solvent effects.[13] There are no established methods for calculating the transition state energy for a ring-flip such as the one that interconverts the two atropisomers of **9**. However, the flat, trans-amide conformation (depicted in pink) serves as a reasonable approximation of the flat conformation that would be a transition between the amide-up (green) and amide-down (orange) conformations of **9**. This trans-amide conformation is approximately 25 kcal/mol above the energy of the amide-up conformation, so 25 kcal/mol makes a good lower-bound estimate of the transition energy. Although this is only a rough estimate, a barrier of this magnitude would certainly lead to slow interconversion rates such as those described above.

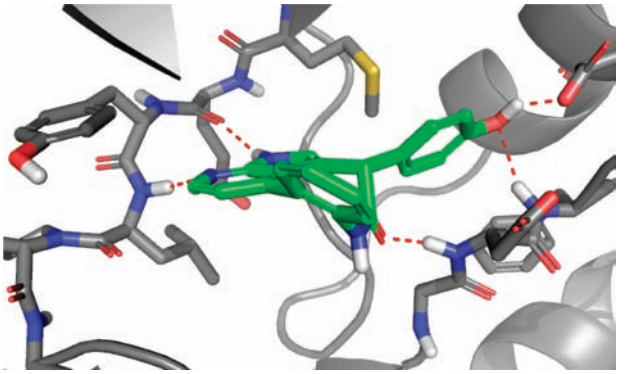

Figure 3. X-ray cocrystal structure depiction of the cocomplex of **9** and the kinase domain of JAK2. Hydrogen bonds (red) are shown between the ligand and (from left to right) Leu-932, Glu-930, Asp-994, Phe-995, and Glu-898.

In order to determine preferred stereochemistry and amide conformation of **9** for binding to JAK2, we determined the crystal structure cocomplex between JAK2 and **9**.[5,7] The structure revealed several key hydrogen bonds to backbone residues (Figure 3).

Compound **9** adopts an open conformation similar to that depicted in Figure 2 (shown in green) and forms two hinge hydrogen bonds with its azaindole moiety to the protein (Figure 1). The hydrogen bonds are to the backbone NH of Leu-932 and the carbonyl of Glu-930. The phenyl ring forms some hydrophobic contacts with the glycine-rich loop above it. The eight-membered ring is puckered such that the lactam carbonyl is in proximity and forms a H-bond with the backbone NH of Asp-994 at the beginning of the activation loop of JAK2. The importance of the phenolic OH is revealed, as it acts both as an H-donor, by forming an H-bond with carboxylate of Glu-898, and as an H-acceptor, by forming a H-bond with Phe-995.

Finally, the complex implies a preference for the *S*-stereochemistry at the benzylic carbon. Modeling studies suggest that the *R*-isomer is likely to be far less active. All of our attempts at docking the *R*-enantiomer in the active site were unsuccessful, suggesting that all the observed activity is ascribed to the *S*-enantiomer and that the *R*-enantiomer is likely to be inactive. As such, we did not attempt to separate the enantiomers of **9**.

Screening of **9** against a panel of kinases revealed the selective nature of the compound, hitting only 3 out of 36 kinases at $K_i < 500$ nM (ALK $K_i = 0.017\,\mu$M and cKit $K_i = 0.19\,\mu$M, GCK $IC_{50} = 0.34\,\mu$M).

Because of the favorable potency and selectivity for JAK2 of **9**, we set out to characterize its in vivo pharmacokinetic parameters. The data are summarized below (see Table 2). Compound **9** exhibited moderate clearance, had long

Table 2. Pharmacokinetic Parameters of **9** Determined in Male Charles River Sprague-Dawley Rats and Fox Chase SCID (CB-17) Mice[a]

	rat		mouse	
	iv dose	po dose	iv dose	po dose
dose (mg/kg)	1.8	10	9.0	10
C_{max} (μg/mL)	0.70	1.08	7.3	1.0
T_{max} (h)		3		2
AUC_{0-inf} (μg·h/mL)	1.26	7.7	12.5	8.3
$T_{1/2}$ (h)	3.4	3.6	2.7	3.2
CL ((mL/min)/kg)	23.8		12.1	
V_{ss} (L/kg)	5.7		2.3	
F (%)		100		66.1

[a] Mean values determined from the concentration (as determined using a specific LC/MS/MS method).

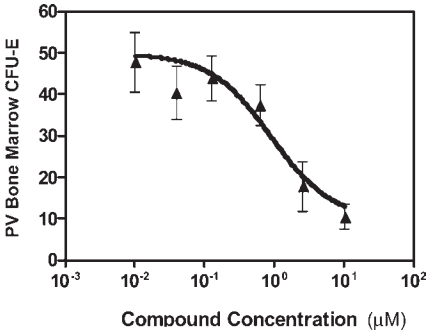

Figure 4. Effect of **9** on EPO-independent erythroid colony formation and growth of a PV patient bone marrow aspirate cultured in vitro.

half-lives, and was 100% orally bioavailable when dosed in Charles River Sprague−Dawley rats. Compound **9** showed 66% oral bioavailability in Fox Chase SCID (CB-17) mice and a similar half-life.

Next, we evaluated the ability of **9** to inhibit the JAK2/STAT pathway. Formation of EPO-independent endogenous erythroid colonies (EEC) is a proposed diagnostic criterion for patient with polycythemia vera.[14] Thus, we established an in vitro assay using PV patient bone marrow aspirate to evaluate the effects of **9** on EPO-independent erhythroid colony formation and proliferation as a surrogate for JAK2/STAT pathway inhibition. As shown in Figure 4, **9** inhibited the spontaneous growth of erythroid colonies (colony forming unit or CFU-E) from a PV patient bone marrow specimen with confirmed JAK2 (V617F) mutation in a dose-dependent manner. The average IC_{50} from two independent assays using different PV patient samples with confirmed JAK2 (V617F) mutation was 0.87 ± 0.48 μM. These results corroborate potency against the intended target and indicate effective inhibition of the JAK2/STAT pathway in a disease-relevant model system.

To measure the in vivo efficacy of **9**, a survival study was performed using the mutant (V617F) JAK2-dependent Ba/F3 murine leukemia cell line (mJAK2-Ba/F3).[15] To induce leukemia, one million mJAK2-Ba/F3 cells were intravenously injected into two groups of BALB/c mice ($n = 12$/group). The first group was dosed orally twice per day (15 mg/kg, b.i.d.) with **9** suspended in a 5% methylcellulose solution (vehicle); the second group received vehicle alone. The results of this study are summarized in a Kaplan−Meier survival plot (see Figure 5). Animals that received vehicle became moribund approximately 23 days after receiving the leukemic implant. Treatment with **9** provided a significant ($P < 0.05$) survival

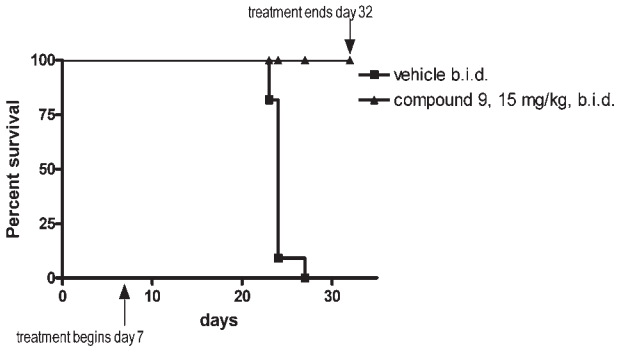

Figure 5. Treatment began 1 week after implantation of 1 million mJAK2-Ba/F3 leukemia cells ($n = 12$/group). Animals were treated by oral gavage with vehicle (5% methylcellulose solution, MC) or **9** (15 mg/kg, suspension in 5% MC) twice per day until day 32 or achieving moribundity. All **9** treated animals survived until day 32. Two animals experienced cage deaths that were not related to treatment or disease; thus, data from these animals were censored.

advantage, prolonging survival to 32 days (study termination). Following sacrifice on day 32, spleens were harvested and weighed to assess the degree of leukemic burden in treated animals versus controls. Animals treated with **9** experienced significantly reduced splenomegaly when compared to vehicle controls ($P < 0.05$): 216 ± 15 mg vs 466 ± 26 mg, respectively.

In summary, we have characterized the binding of **9**, a selective and potent new polycyclic azaindole based inhibitor, of JAK2, which revealed key interactions responsible for its potency against the target protein. We have demonstrated the ability of **9** to block the JAK2/STAT pathway in vitro in an EPO-independent erhythroid colony formation assay. With its ability to elicit a pharmacological benefit in a mutant (V617F) JAK2-dependent Ba/F3 murine leukemia model, we have shown the utility of **9** as a tool compound for the study of relevant models of disease.

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Supporting Information Available: Synthetic procedures; ^{1}H NMR and LC/MS data for all compounds and HRMS data for both atropisomers of **9**; assay protocols for K_i and IC_{50} determinations and colony forming assays; crystallographic information; protocols for cloning, expression, and purification of JAK2. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) The purity of the combined atropisomers **9A** and **9B** as an equilibrium mixture in DMSO was >95% as judged by orthogonal HPLC methods. See Supporting Information for full characterization data.

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(11) The JAK2 data in Table 1 was obtained using a truncated kinase domain form of the enzyme. Identical results were obtained for **9A** using alternative forms of JAK2 comprising the combined kinase and pseudokinase domains having valine or phenylalanine at amino acid 617 (unpublished results).

(12) GM-CSF stimulation of TF-1 cells leads to STAT-5 phosphorylation by JAK2, while IL2 stimulation of HT-2 cell lines yields phospho-STAT-5 via the JAK3 pathway. For full details regarding IC_{50} determinations using TF1 and HT2 cells refer to Supporting Information.

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