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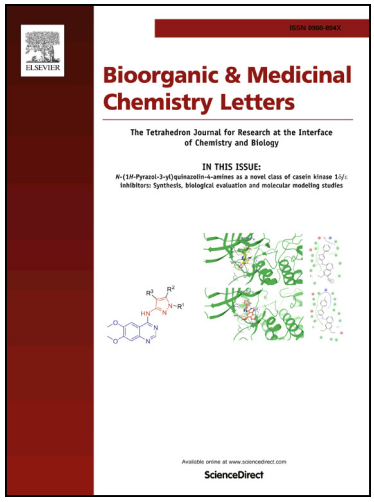

Discovery of highly potent, selective, covalent inhibitors of JAK3

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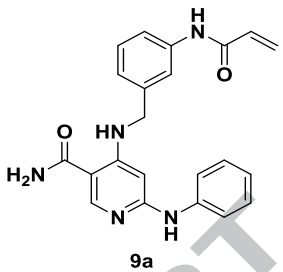

9a

James Kempson,* Damaso Ovalle, Junqing Guo, Stephen T. Wrobleski, Shuqun Lin, Steven H. Spergel, James J-W. Duan, Bin Jiang, Zhonghui Lu, Jagabandhu Das, Bingwei V. Yang, John Hynes Jr., Hong Wu, John Tokarski, John S. Sack, Javed Khan, Gary Schieven, Luisa M. Salter-Cid, Aberra Fura, Yuval Blatt, Charu Chaudhry, Joel C. Barrish, Percy H. Carter and William J. Pitts.

Bristol-Myers Squibb Research and Development, Princeton, NJ 0854-4000, USA

A useful and novel set of tool molecules have been identified which bind irreversibly to the JAK3 active site cysteine residue. The design was based on crystal structure information and a comparative study of several electrophilic warheads.

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Abstract— *A useful and novel set of tool molecules have been identified which bind irreversibly to the JAK3 active site cysteine residue. The design was based on crystal structure information and a comparative study of several electrophilic warheads.*

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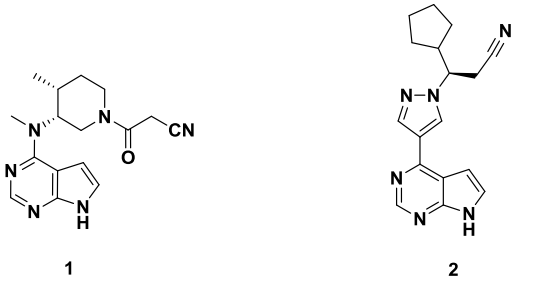
The Janus kinases (JAKs) play a central role in regulating the immune system.[1] The JAK family of kinases is comprised of four family members, JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2), each of which can bind to distinct cytokines and/or growth factor receptors. More specifically, JAK3 is required for signaling by cytokines which include interleukin 2 (IL-2), IL-4, IL-7, IL-9, IL-15, and IL-21, which act via receptors that contain the common gamma chain (γ_c) cytokine receptor subunit. JAK3 associates with the γ_c receptor in the cytoplasm and works in concert with JAK1 to activate/phosphorylate the Signal Transducers and Activators of Transcription (STAT) proteins. Once phosphorylated, these STAT proteins then dimerize and translocate to the nucleus to initiate gene transcription which specifically activates T, B and NK cell immune responses.



1 2

Figure 1: FDA approved JAK inhibitors.

Significant drug discovery efforts have resulted in the development of several JAK inhibitors including tofacitinib[2] (**1**, pan-JAK inhibitor) which has been approved for the treatment of rheumatoid arthritis and ruxolitinib[3] (**2,** JAK1/2 inhibitor) which has been approved for the treatment of myelofibrosis (Figure 1). Several other JAK family inhibitors are currently in clinical trials.[4] Due to the highly conserved nature of the enzyme active sites across all JAK family members, the traditional ATP-competitive inhibitor approach has found difficulty in obtaining a high degree of selectivity within the family. However, the active site of JAK3 is unique as it contains a cysteine (Cys_{909}) which if properly engaged could provide access to JAK3 selective inhibitors. Recently there have been several reports of selective covalent JAK3 inhibitors.[5] Indeed, studies with one of these inhibitors[5c-d] suggests a therapeutic potential for JAK3 selective inhibition as demonstrated by both potent inhibition of *in-vitro* cellular signalling through the γ_c cytokines in addition to a high degree of efficacy in a variety of *in-vivo* inflammatory disease models. Our efforts to covalently target JAK3 are described below.

As part of our efforts to identify JAK1/3 inhibitors, we identified two lead compounds (**3**, **4,** Figure 2) which were potent reversible inhibitors of JAK3 and showed a degree of selectivity over other JAK family members. We reasoned these would be good starting points to design selective JAK3 covalent inhibitors based on their similar predicted binding orientations in JAK3 from early modeling efforts.

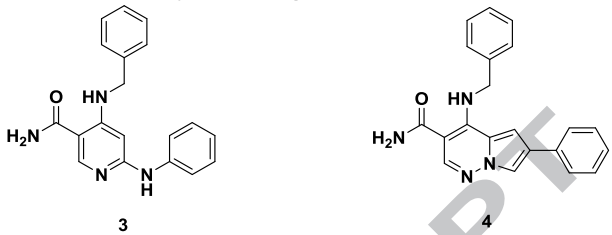


Figure 2: Reversible inhibitors of JAK3

Docking[7] of **3** to a published JAK3 X-ray structure[8] led to a model that suggested placement of an electrophile at the *meta*-position of the benzylamine would provide a suitable vector to target Cys909 (Figure 3). Based on these considerations, we designed and prepared compounds **9a,** containing an acrylamide group as the electrophile, and the propanamide **9b** as an isosteric, but unreactive comparator.

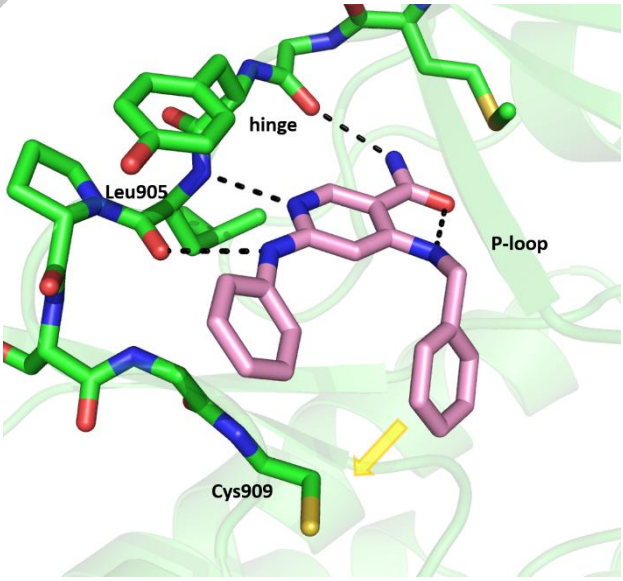
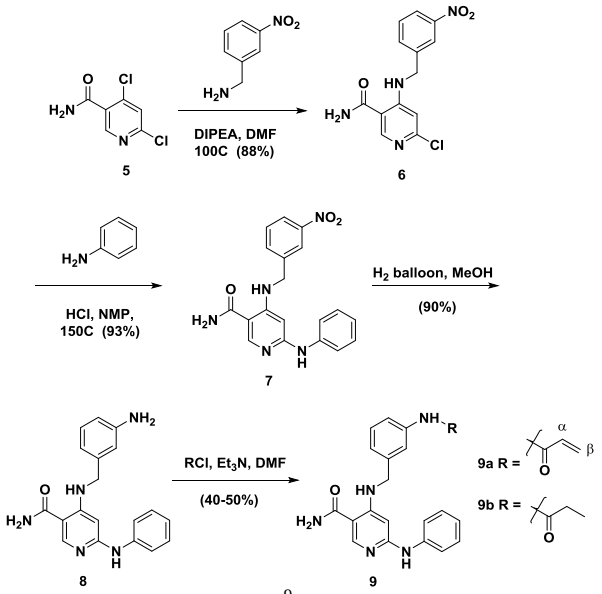


Figure 3 : Model of compound **3** docked in JAK3 kinase domain showing a close-up of the binding site. Hydrogen bond interactions are shown with dotted lines. JAK3 ribbon and carbons in green. Carbons of **3** in magenta. Yellow arrow indicates vector towards Cys909.



Scheme 1: Synthetic route to **9a**[9] and **9b**.

The synthesis of **9** (Scheme 1) was accomplished in 4 steps starting from intermediate **5** and the addition of *meta*-nitro benzylamine to first give **6** in 88% yield. Subsequent addition of aniline under acidic conditions then produced **7** which was then subjected to hydrogenolysis using Pd/C as catalyst. The resulting aniline was then acylated with acroyl chloride to give **9a**. Acylation of **8** with propionyl chloride provided the comparator compound **9b**.

Testing for JAK3 inhibition in a fixed time point enzymatic assay,[10] **9a** was found to be a highly potent (JAK3 IC_{50} < 1 nM, *n*=3) inhibitor and exhibited time-dependent inhibition (100% inhibition at 15 minutes). In contrast **9b**, was moderately potent (JAK3 IC_{50} = 100 nM, *n*=1) with no change in IC_{50} after pre-incubation. The JAK family selectivity of **9a** was significantly improved compared to the reversible leads **3**, **4** (**9a** enzyme data: JAK2 IC_{50} = 1,300 nM, *n*=3; JAK1 IC_{50} = 1,100 nM, *n*=3; TYK2 IC_{50} > 5000 nM, *n*=3) and exhibited good cellular potency (IL2 driven T-Cell proliferation IC_{50} = 22 nM, IL2 driven pSTAT3 IC_{50} = 51 nM) and human whole blood potency (IL-2 driven IFNγ production hWB IC_{50} = 490 nM). **9a** also possesed a high degree of selectivity over a JAK2 cellular endpoint (EPO driven pSTAT5 IC_{50} = 11 μM) and a JAK1/TYK2 cellular endpoint (IFNα driven pSTAT3 IC_{50} = 4.9 μM) To assess broad kinome selectivity, we evaluated **9a** in an external panel screen against >350 kinases which showed excellent overall kinome selectivity (Kinases < 10% of control at 1 μM = JAK3, FMS, BMPR2).[11] This selectivity result is somewhat surprising, as there are ten other protein kinases with cysteines at this location in the active site including the TEC family (e.g. BTK) and several other kinases such as EGFR.[12]

This finding was confirmed using an in-house kinase caliper screening panel which showed that **9a** only inhibited FMS (IC_{50} =180 nM, *n*=2), while inhibition of BTK (IC_{50} > 2.5 μM, *n*=3) and EGFR (IC_{50} > 10 μM, *n*=1) was weak at the concentrations tested. As IC_{50} data is less than ideal for determining selectivity of covalent inhibitors, we proceeded to examine **9a** using a time-dependent assay in which BTK inhibition was measured at four time points (Figure 4). EC_{50} values are noted in μM and reveal the time-dependent nature of inhibition against BTK with ~10-fold increase in potency during the course of the experiment as target (Cys) engagement takes place. However, after 120 minutes, the EC_{50} for BTK inhibition is still greater than 1 μM.[9] While irreversible inhibitors are often characterized using IC_{50} values, these measurements are time-dependent. The efficiency of covalent inhibitors can be more accurately expressed as a function of the kinact/Ki ratio.[13] To accurately gauge JAK3 vs BTK selectivity for Cmpd **9a**, we determined corresponding kinact/Ki values using a continuous fluorescence assay. Consistent with predictions from our preincubation experiment results, kinact/Ki for cmpd **9a** at JAK3 (0.3 uM-1 sec-1) was at at least 3 orders of magnitude larger than at BTK (0.00005 uM-1 sec-1), revealing a significant selectivity window for this compound. Additionally **9a** had an IC_{50} greater than 10μM in a a BCR-stimulated CD69 expression assay in human whole blood.[14]

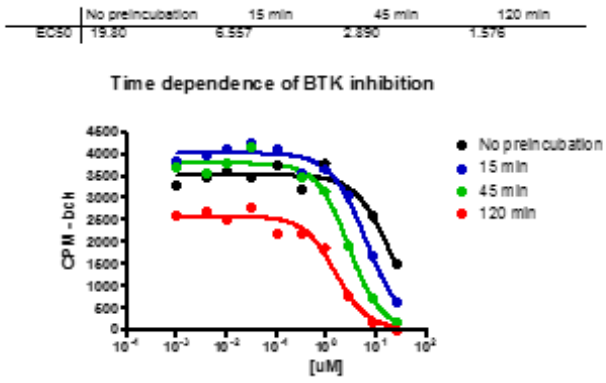


Figure 4: BTK pre-incubation enzyme assay 9a

Pharmacokinetic evaluation revealed that **9a** had low bioavailability (<30%) and high clearance (significantly greater than hepatic blood flow) in the mouse. While high clearance desirable attribute for covalent inhibitors, the plasma levels achieved were only very briefly above the whole blood IC_{50} which precluded advancement to *in vivo* studies. Thus we sought to extend this proof-of-concept work in the nicotinamide series to the closely related pyrazolopyridazine series **4** where we had generated analogs with an improved pharmacokinetic profile and demonstrated efficacy in a mouse model of arthritis.[15]

Utilizing the same acrylamide probe within this series provided **13a** (Figure 5),

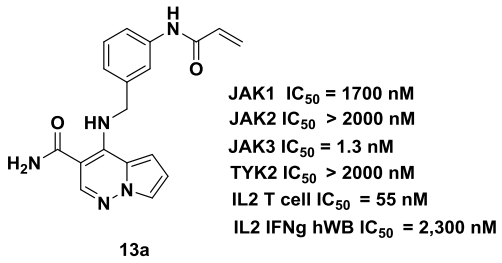


Figure 5: Structure and *in-vitro* profile for compound **13a**

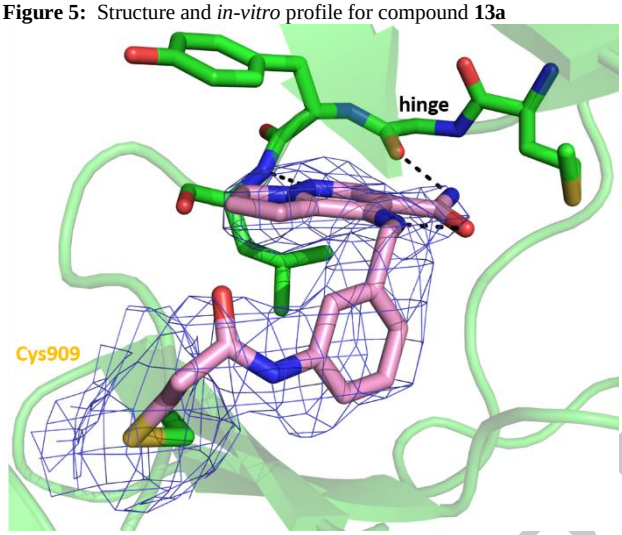
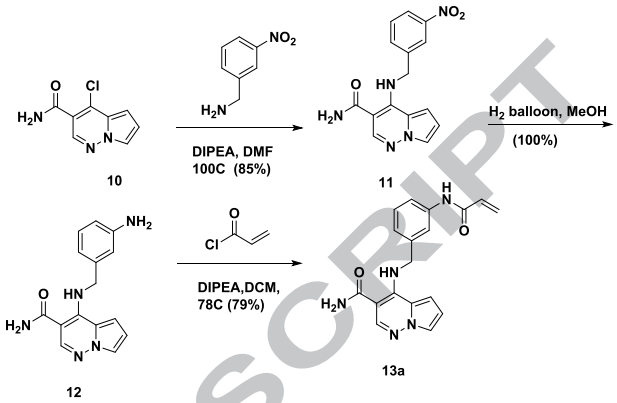


Figure 6: X-ray co-crystal structure of JAK3 kinase domain bound with compound **13a** showing a close-up of the binding site. PDB code: 5WFJ. Hydrogen bond interactions are shown with dotted lines. JAK3 ribbon and carbons in green. Carbons of **13a** in magenta. The electron desnisty map around the inhibitor and Cys909 are shown in blue mesh at a sigma level = 1.0. The density is continuous between the acrylamide warhead and Cys909 indicating a covalent bond.

which was also found to give a selective JAK3 inhibition profile. We were able to confirm the structural basis for achieving selectivity with this inhibitor by solving the co-crystal structure of the JAK3 kinase domain in complex with **13a** at a resolution of 2.9Å (Figure 6). In this structure, the primary amide and pyrazolopyridazine core binds to the hinge region with the benzylamine orientated under the P-loop. Continuous electron density is observed between the acrylamide group and Cys909, suggestive of covalent bond formation.

Table 1... SAR with respect to JAK family inhibition.

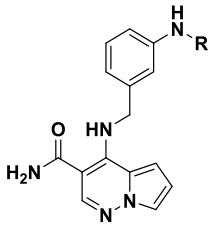
With the activity of **13a** confirmed, our focus shifted to exploration of alternative electrophilic groups[16] (Table 1) utilizing similar chemistry to that depicted in Scheme 2 for the synthesis of **13a**.

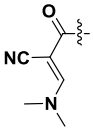
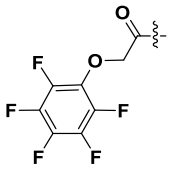
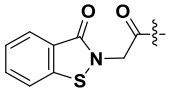


Scheme 2. Synthetic route to **13a**

Compounds were evaluated for their ability to inhibit the JAK family of kinases and also evaluated in an IL-2 driven cell T cell proliferation assay (IL-2 T-Cell). The structure-activity relationships for the inhibition of JAK family and cell based activity are summarized in Table 1.

In comparison to **13a,** addition of a beta-methyl group to the acrylamide group (**13b)** resulted in significant loss of JAK3 potency presumably due to steric hinderance along the trajectory for interaction with Cys909. The vinyl sulfone electrophile **13c** displayed similar enzymatic potency to **13a**, but this was not reflected in the cellular assay (IL-2 T-Cell = 6700 nM). Incorporation of nitrile functionality (**13d**) in an attempt to reversibly engage Cys909 resulted in a modest decrease in JAK3 potency compared to **13a**. Incorporation of larger electrophiles (**13e-13f**) led to a decrease in JAK3 potency but exhibited excellent selectivity against JAK1/2, and across the kinome. We also explored the benzisothiazolone motif[12b] (**13g**) which was more potent against JAK3 compared to (**13e,f**) but given the similarity in size to **13f** we were somewhat surprised to see an erosion of selectivity against JAK1/2 and TYK2.



Compd	R	JAK3 IC_{50} nM	JAK2 IC_{50} nM	JAK1 IC_{50} nM	TYK2 IC_{50} nM	IL-2 T-Cell IC_{50} nM
13b		175	>2000	1422	>2000	26600
13c		3.5	1270	974	>2000	6700
13d		35	658	264	2000	8450
13e		942	>2000	>2000	>2000	14400
13f		743	>2000	>2000	>2000	15400
13g		30	745	196	240	5480

In summary, we have identified acrylamide **9a** as a potent, selective JAK3 covalent inhibitor which may be a useful addition to the *in vivo* tool molecules and clinical compounds reported by others.[5,6] In a closely-related pyrrolopyridazine series, we were able to confirm a covalent interaction with Cys909 in the JAK3 active site, by both kinetic evaluation and x-ray crystallography studies using **13a**. As a result of these studies, future efforts are currently focused on the continued optimization of irreversible JAK inhibitors within this novel series and additional results will be reported in due course.

Acknowledgements

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