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JAK2 JH2 Fluorescence Polarization Assay and Crystal Structures for Complexes with Three Small Molecules

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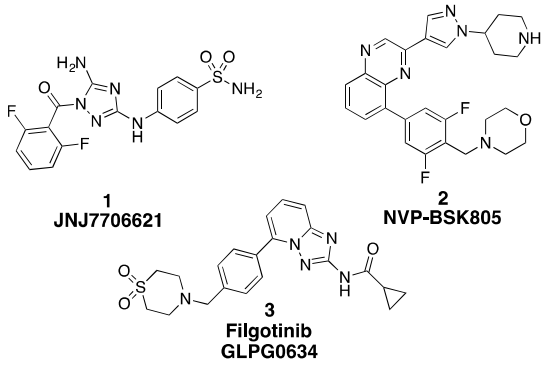
KEYWORDS: *Janus Kinase 2*, JAK2, *JAK2 JH2*, *Pseudokinase* Domain, Fluorescence Polarization, FP, JNJ7706621, NVP-BSK805, filgotinib (GLPG0634), protein crystallography.

ABSTRACT: A competitive fluorescence polarization (FP) assay is reported for determining binding affinities of probe molecules with the pseudokinase JAK2 JH2 allosteric site. The syntheses of the fluorescent **5** and **6** used in the assay are reported as well as K_d results for 10 compounds including JNJ7706621, NVP-BSK805 and filgotinib (GLPG0634). X-ray crystal structures of JAK2 JH2 in complexes with NVP-BSK805, filgotinib, and a diaminopyrimidine **8** elucidate the binding poses.

Janus kinases (JAKs) are non-receptor tyrosine kinases involved in the regulation of hematopoiesis, the immune system, and cellular metabolism. In mammals, the family has four members: JAK1-3 and tyrosine kinase 2 (TYK2). Each protein contains an N-terminal FERM domain, an SH2-like domain, a pseudo-kinase domain (JAK homology 2 or JH2) and the C-terminal kinase domain (JH1).[1]

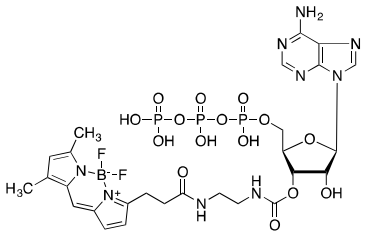
Though JH2 domains are similar in structure to kinase domains and include an ATP binding site, they lack or possess negligible catalytic activity and serve a primarily regulatory role on JH1 kinase activity.[2-5] Notably, multiple diseases are associated with mutations in JAK2 JH2 domain; in particular, V617F has been linked to polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (PMF).[6-10] Moreover, though disruption of ATP binding in JH2 showed only minor effects on JAK2 wild-type (WT) activity, it did inhibit the hyperactivity of the pathogenic V617F mutant.[11] This suggests that small molecules that bind at the JAK2 JH2 ATP site have potential for therapeutic drug development.

Although potent inhibitors of Janus kinases have been reported in the literature,[12-13] drug discovery efforts have not been able to address diseases caused by mutated JAK2.[11] The need for binders that selectively target JAK2 JH2 domain is therefore pressing. Since the JH2 domain is not catalytic, an accurate and rapid direct binding assay is needed for gauging potency. To this end, we report here such an assay using fluorescence polarization (FP). This binding assay contrasts conventional JAK assays (e.g., autophosphorylation[14-16] and proliferation[17]) by providing quantitative measurements of binding constants, K_d.[18] The use of standard microplate readers, the ability to reanalyze assay plates, and the fact that substrates or radiolabeled reagents are not needed makes FP attractive for drug discovery. Ten compounds, including the well-known multikinase inhibitors JNJ7706621 **1**,[19] NVP-BSK805 **2**,[20] and filgotinib **3**,[21] were tested, and three X-ray crystal structures were obtained to elucidate the binding poses of JH2 complexes.

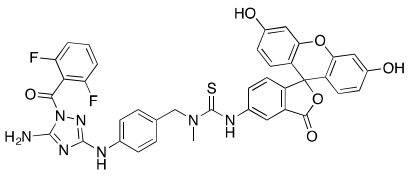


A prerequisite for FP is a fluorescent probe with high affinity for the binding site. For the JAK2 JH2 domain, the initial choice was BODIPY-ATP **4** (Invitrogen, ThermoFisher Scientific supplier), a fluorophore attached to a ribose ring.[22] However, the affinity of tracer **4** towards JAK2 JH2 was weak (K_d = 7 μM) and therefore could not be used to determine accurately affinities in the nanomolar range. In addition, the concentration of protein (7 μM, Table 1) needed for the assay is not desirable for high-throughput screening.

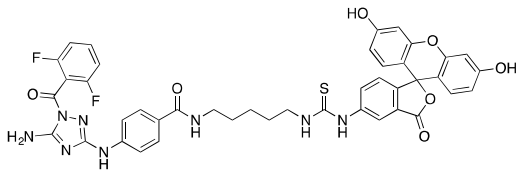
To overcome these problems, we designed and synthesized tracers **5** and **6** (Schemes 1 and 2) by attaching a fluorescein isothiocyanate (FITC) to two amino-analogs of **1**, namely **18** and **24**. A detailed description can be found in the Supporting Information. Compound **1** was found through an *in vitro* screen at the Yale Small Molecule Discovery Center and determined to have a K_d of 106 nM by isothermal titration calorimetry (ITC) with JAK2 JH2.[22] The minimum tracer concentration that retained a satisfactory signal-to-noise ratio with **5** was found to be 1.5 pM.



4
BODIPY-ATP



5



6

Table 1. Concentration of tracer and JAK2 JH2 protein needed when using tracers **4** and **5** in FP assays.

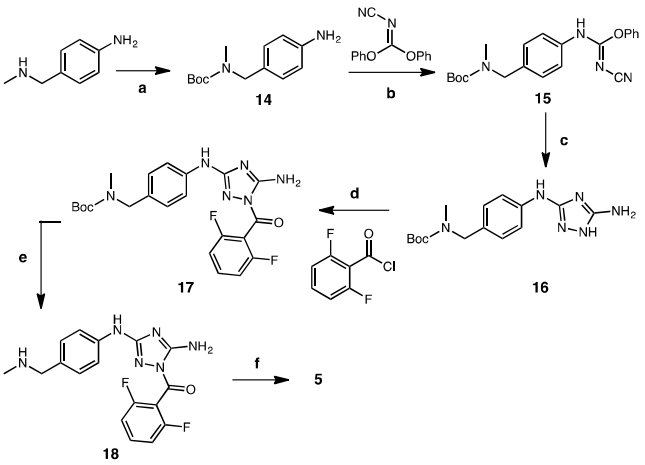
	Tracer 4	Tracer 5
[tracer]	5 nM	1.5 pM
[protein]	7 μM	200 nM

Saturation experiments, which involved adding incremental amounts of JAK2 JH2 (0 to 4.8 μM) to the tracer solution, were then carried out with measurements over 90 minutes and showed stable K_d values over time. The dissociation constants of tracers **5** and **6** were determined to be roughly equivalent near 0.2 μM (Figure 1B), a significant improvement over tracer **4**. The lower K_d values translate to correspondently lower protein concentrations needed in the competitive assay (Table 1). Interestingly, tracer **5** showed a greater ΔFP over the range of protein concentrations than tracer **6** (2.5-fold vs 1.5-fold, Figure 1A). This suggests that the fluorescent properties of tracer **6** are affected by its binding to the protein, so **5** was selected for the subsequent competitive assays.

In view of the structure of **1**, exploratory studies led to preparation of potential JH2 binders containing diamino-substituted heterocycles with terminal 4-cyanophenyl substituents such as **7-13** (synthetic schemes are in the Supporting Information). These compounds along with **1** – **3** were studied using the optimized FP assay (Table 2). The most active compound, **1**, has a K_d of 0.8 μM in the FP assay. This value is 8-fold weaker than obtained from ITC, reflecting differences in conditions including use of 50 mM Hepes vs 20 mM Tris-Cl buffer, and less glycerol (10% vs. 20%) and more protein (ca. 5 μM vs. 6 nM) for ITC.[22] Filgotinib (**3**), **7**, **9**, **10**, and **13** showed less than ca. 10% binding in an initial screen at a concentration of 50 μM, so precise K_d values were not determined. Though the simple pyrimidine **7** was inactive, expansion at C6 did provide active compounds with **8** showing the lowest K_d.

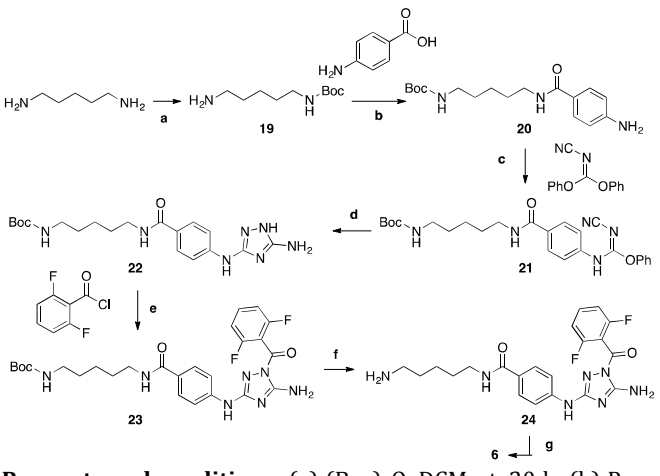
To complement these studies and provide a solid basis for structure-based design, X-ray crystal structures were pursued for complexes of the small molecules with JAK2 JH2. Success was obtained for several compounds including **2**, **3**, and **8** at 2.0, 1.9, and 1.6 Å resolution, respectively. Full details are provided in the Supporting Information. The binding poses are shown in Figure 2 and confirm the expected positioning in the hinge region of the JH2 ATP site. **2** only has one hydrogen bond (yellow dashed line) with the backbone NH of Val629, while **3** adds one with the carbonyl group. However, the complex with **8** appears well-packed and has three additional hydrogen bonds with the side-chain carbonyl group of Gln626, the backbone carbonyl group of Glu627, and one between its terminal hydroxyl group and the sidechain of Asn678. The lower K_d for **2** than **8** suggests that replacement of the hydroxymethylpyrazole with larger, more hydrophobic substituents could lead to enhanced binding.

Scheme 1. Synthesis of tracer **5**



Reagents and conditions: (a) $(Boc)_2O$, THF, 23 °C, 20 h; (b) THF, 65 °C, 20 h; (c) Hydrazine, THF, 70 °C, 2h; (d) Py, 18 h; (e) THF, TFA, 50 °C, 3h; (f) Fluorescein-NCS, DIPEA, DMF, 23 °C, 1 h.

Scheme 2. Synthesis of tracer **6.**



Reagents and conditions: (a) $(Boc)_2O$, DCM, rt, 20 h; (b) PyBOP, HOBt, Et_3N, THF, rt, 20 h; (c) THF, 65 °C, 20 h; (d) hydrazine, THF, 70 °C, 2 h; (e) Py, rt, 18 h; (f) THF, TFA, 50 °C, 3h; (g) Fluorescein-NCS, DIPEA, DMF, rt, 1 h.

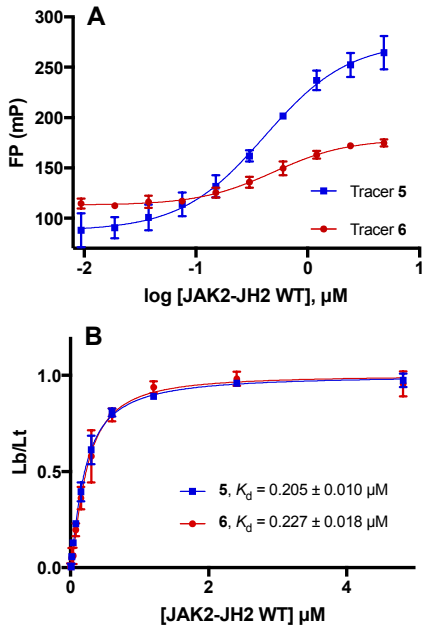


Figure 1. Determination of binding affinities for tracers **5** and **6** (1.5 pM) through saturation experiments. (A) Variation of FP values as a function of JAK2 JH2 WT concentration. (B) K_d determination for tracers **5** and **6**. Lb/Lt = ratio of ligand bound to the total. Data from quadruplicate experiments in three independent assays. Mean ± SEM plotted for all data.

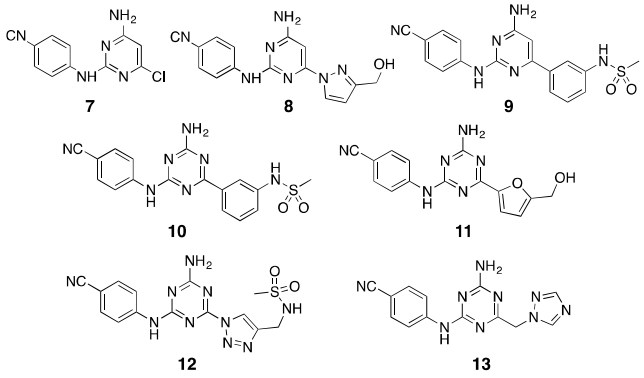


Table 2. Binding affinity values (K_d, μM) from the FP Assay.

Compd	K_d (μM)[a]
1 JNJ7706621	0.80 ± 0.05
2 NVP-BSK805	42.0 ± 3.5
3 filgotinib (GLPG0634)	9% (50 μM)
7	0% (50 μM)
8	57.3 ± 2.8
9	3% (50 μM)
10	11% (50 μM)
11	122.3 ± 18.5
12	106.0 ±18.8
13	3% (50 μM)

[a] K_d or % bound at indicated concentration in parentheses. Data shown from quadruplicate experiments in three independent assays. Mean ± SEM.

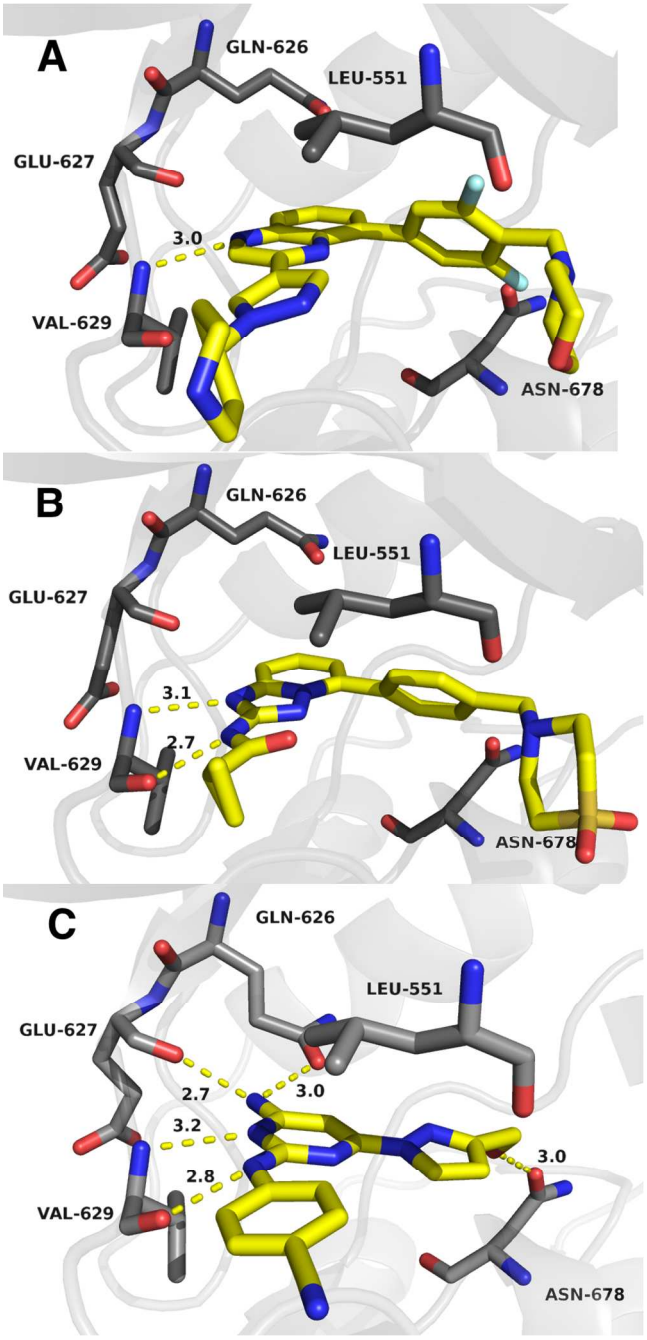


Figure 2. Renderings from the crystal structures of NVP-BSK805 **2** (A), filgotinib **3** (B), and **8** with wild type JAK2 JH2. Carbon atoms of **2**, **3**, and **8** are colored yellow. The PDB codes are 5UT4, 5UT5, and 5UT6.

In summary, the desire to discover small molecules that selectively target the JH2 domain of JAK2 led us to pursue a fluorescence polarization assay. This competitive binding assay allows fast and accurate measurements of K_d values, suitable for high-throughput screening. To this end, two fluorescein-labeled ligands (**5**, **6**) were synthesized. The high affinity of tracer **5** (K_d = 0.2 μM) allowed for low con-

centrations of both tracer and protein in the FP assay. The FP assay was then applied to three well-known JAK inhibitors (**1**-**3**) and seven synthesized compounds (**7**-**13**). In conjunction with the reported crystal structures for complexes with JAK2 JH2, application of structure-based and computer-aided design to the discovery of potent JH2 binding molecules has a firm foundation.

ASSOCIATED CONTENT

Supporting Information. Full synthetic procedures and spectral characterization data for all intermediates and final compounds **5-13**; crystallographic data for complexes **2**, **3**, and **8** with JAK2 JH2 have been deposited in the RCSB Protein Data Bank with the PDB codes 5UT4, 5UT5, and 5UT6; experimental details of FP assays. This information is available free of charge via the Internet at http://pubs.acs.org .

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS

JAK, Janus Kinases; JH2, pseudo-kinase domain; FP, Fluorescence Polarization; Boc, *tert*-Butyloxycarbonyl protecting group; DCM, dichloromethane; THF, tetrahydrofuran; DMF, dimethylformamide; Py, pyridine; TFA, trifluoroacetic acid; DIPEA, N,N-diisopropylethylamine, PyBop, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; HOBt, hydroxybenzotriazole.

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