Virtual Screening to Successfully Identify Novel Janus Kinase 3 Inhibitors: A Sequential Focused Screening Approach

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In an effort to identify novel Janus kinase 3 inhibitors, a sequential focused screening approach was adopted to search our in-house chemical database. By biologically testing only 79 selected compounds, we successfully identified 19 compounds showing $IC_{50} < 20\ \mu M$, with four of them in the nanomolar range. Particularly, a 3,5-disubstituted pyrazolo[4,3-*d*]pyrimidine scaffold emerged as a promising candidate for further lead optimization. With the advantages of efficiency and flexibility, this approach may be utilized to identify leads for other therapeutic targets.

Introduction

Janus kinase 3 (JAK3[a]) is a member of the Janus family of protein tyrosine kinases, which also includes JAK1, JAK2, and TYK2.[1,2]It is composed of multiple topological domains, with the C-terminal kinase domain being a functional tyrosine kinase. Although the whole Janus family is involved in the cytokine receptor-mediated intracellular signal transduction, JAK3 is unique in its expression and association. JAK3 is highly expressed in hematopoietic cells, while the other Janus family members are expressed almost ubiquitously. JAK3 binds specifically to the common gamma (γc) chain of the interleukin receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, which may cause the proliferation and differentiation of lymphocytes.[3] For these reasons, JAK3 has been intensively studied as a potential therapeutic target for the treatment of severe combined immunodeficiency (SCID),[4] asthma,[5] and some other immunological disorders. Furthermore, JAK3 specific inhibitors have also been developed as immune suppressants in human organ transplantation.[6] Multiple chemical series of JAK3 inhibitors have been reported,[7] including benzylidenemalonitriles,[8] quinazolines,[9] naphthyl ketones,[10] and tetracyclic pyridones.[11]

Biological screening of chemical databases, which may contain hundreds of thousands to millions of compounds, has become a routine effort for lead identification in today's pharmaceutical industry.[12] In general, there are two different strategies that can be applied for this purpose: broad screening and focused screening. Broad screening, represented by high throughput screening (HTS) technology,[13] attempts to sample the whole chemical space as broadly as possible and typically involves testing hundreds of thousands of diverse compounds in a biological assay. In contrast, focused screening identifies the region of chemical space that is relevant to the biological target and selects a much smaller set of compounds from that region for biologically testing, usually through a virtual screening (VS) process.[14] Although both strategies were applied successfully in many lead identification projects, they have also demonstrated their own limitations. Broad screening is often associated with a very low hit rate and may end up with few meaningful hits. Focused screening is obviously biased to the existing structure−activity information and thus may not be successful in identifying highly novel lead scaffolds.

However, a sequential screening approach may provide us a simple but effective way to alleviate or even overcome these limitations. Sequential screening is unique in that it conducts screening in an iterative fashion. Instead of attempting to find all the desirable hits in a single round of screening, it runs screening in multiple rounds and selects the screening candidates of each round based on the new structure−activity data coming out of the previous round of screening. This approach has demonstrated its efficiency and flexibility in several recent reports. For example, Jones-Hertzog and coauthors described a sequential screening process using recursive partitioning as the data mining technique on a panel of 14 GPCR assays.[15] Engels and Venkatarangan reported a retrospective study of a cluster-based sequential screening approach on 18 different cancer cell lines.[16] Young and co-workers studied the influence of initial compound selection on the outcome of a sequential screening exercise.[17] Recently, Blower and coauthors did a more extensive study on several other factors that might affect the performance of a sequential screening approach.[18]

Interestingly, all these reported efforts were conducted in the context of HTS. In other words, the sequential screening strategy was introduced mainly to increase the efficiency or hit rate of a broad screening project. However, the same strategy can also be applied into a focused screening project to increase the structural novelty of screening hits, as pointed out by Bajorath.[19] Herein, we report the application of a sequential focused screening approach, which performs focused screening in a sequential fashion, to successfully identify novel JAK3 inhibitors out of the Johnson & Johnson (J&J) corporate database of about one million searchable chemical structures.

Results

Our JAK3 screening effort started from following the lead compound reported by Merck (**1**, Figure 1).[11] Compound **1** is a reversible ATP-competitive kinase inhibitor based on steady-state kinetic studies, showing significant specificity for the JAK family over other kinases and limited selectivity within the JAK

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[a] Abbreviations: CDK, cyclin-dependent kinase; EGFR, epidermal growth factor receptor; HTS, high throughput screening; JAK, Janus kinase; JNK, c-Jun *N*-terminal kinase; IL, interleukin; VS, virtual screening; GPCR, G-protein coupled receptor.

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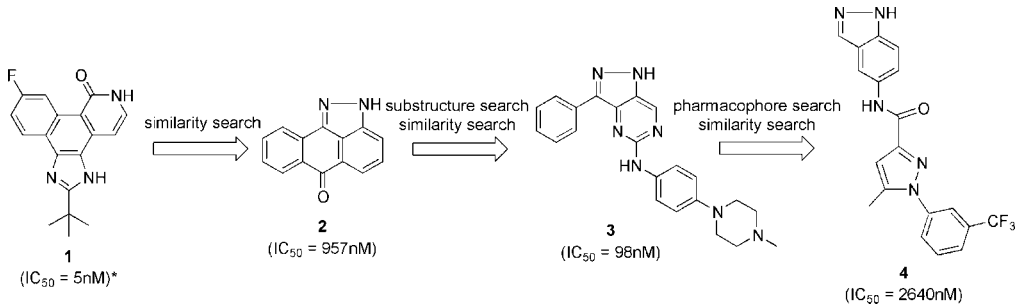

Figure 1. Illustration of the sequential focused screening approach as applied in the JAK3 lead identification effort reported here, with the representative hits (**2**−**4**) identified from each round of screening.

Table 1. Summary of Active Compounds Identified in Each Round of Sequential Screening

	number of tested compounds	number of JAK3 active compounds		
		$IC_{50} \leq 1\ \mu m$	$IC_{50} \leq 10\ \mu m$	$IC_{50} \leq 20\ \mu m$
1st round	40	1	4	6
2nd round	10	3	3	4
3rd round	29	0	4	9
total	79	4	11	19

family itself (IC_{50} = 15 nM for JAK1, 1 nM for JAK2, 5 nM for JAK3, and 1 nM for TYK2). Because no more structure−activity data had been published for this series of tetracyclic pyridones, a similarity search was adopted for the first round of virtual screening and conducted by our in-house program PRISM,[20] which is designed to identify chemical compounds with similar topological structures. As a result, 40 J&J compounds were selected and tested in the JAK3 biological assay (Table 1). Four of them showed IC_{50} values less than 10 μM, with the most potent one (**2**, Figure 1) having an IC_{50} value close to 1 μM (IC_{50} = 957 nM). A literature search revealed that this particular anthra[1,9-*cd*]pyrazol-6(2*H*)-one structure is actually SP600125, a compound originally developed as a reversible ATP-competitive inhibitor of JNK,[21] a protein serine/threonine kinase. Since then, compound **2** has been profiled against various kinases and found to be a rather nonspecific inhibitor with comparable potencies against multiple kinases.[22,23] Although no reports on the properties of compound **2** as a JAK3 inhibitor had been found, its poor selectivity profile demanded that we continue looking for more scaffolds for JAK3 inhibition, hopefully with improved novelty and selectivity.

Homology modeling and molecular docking were conducted to study the possible binding modes of compound **2** in the ATP-binding site of JAK3 kinase domain. As shown in Figure 2, docking results indicated that compound **2** may use its two pyrazolyl nitrogens to form two hydrogen bonds with the JAK3 backbone at the kinase hinge region. Interestingly, although the two possible tautomers of compound **2** may form hydrogen-bond interactions with different residues, they orient themselves in similar ways and put the same phenyl ring inside the ATP-binding pocket. On the basis of our knowledge of other kinase inhibitors, we believed that an aromatic ring located in that region is usually important for kinase binding. Therefore, we designed a pair of substructure search queries as shown in Figure 3 that contain a key pyrazolyl ring and an adjacent 6-membered aromatic ring. A simple substructure search conducted by the program ISIS,[24] followed by a PRISM similarity search, led to 10 J&J compounds being selected and tested in the JAK3 biological assay (Table 1). Three of them showed IC_{50} values in the nanomolar range. The most potent hit (**3**, Figure 1) had an IC_{50} value of 98 nM. This represents about 1 order of magnitude improvement of potency compared to the initial hit **2**. Furthermore, the 3,5-disubstituted pyrazolo[4,3-*d*]pyrimidine scaffold represented by this structure is sufficiently novel in terms of kinase inhibition. To the best of our knowledge, the only two other pyrazolopyrimidine series that have been reported for kinase inhibition are 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines as CDK1 inhibitors,[25] and 4-(phenylamino)pyrazolo[3,4-*d*]pyrimidines as EGFR inhibitors.[26] These scaffolds possess either a different heteroatom pattern on the core structure or a different substitution pattern on the side chains and should adopt completely different binding modes in kinases as observed in other series of kinase inhibitors.[27]

By following the same strategy, a molecular docking study was conducted on the newly identified hit **3**. As shown in Figure 4, the docking results suggested that the piperazinyl nitrogen of **3** may pick up an additional hydrogen-bond interaction with the side chain of Arg953. Consequently, a pair of four-point pharmacophore search queries was formulated, as shown in Figure 5. They were composed of a pair of hydrogen bond donors and acceptors located on the pyrazolyl ring, an aromatic center located on the pyrimidinyl ring, and a hydrogen bond acceptor located on the piperazinyl nitrogen. A pharmacophore search was done by the program Catalyst,[28] followed by a PRISM similarity search. As a result, 29 J&J compounds were submitted to the JAK3 biological assay (Table 1). Four of them showed IC_{50} values less than 10 μM, with the most potent hit (**4**, Figure 1) having an IC_{50} value around 2.5 μM. Because compound **4** displayed a much weaker potency and also a highly similar scaffold compared to compound **3**, our sequential screening effort starting from the Merck JAK3 inhibitor **1** was discontinued.

In summary, by biologically testing only 79 J&J compounds selected by the virtual screening of J&J corporate database of about one million searchable chemical structures, we successfully identified 19 compounds showing JAK3 inhibition with IC_{50} values less than 20 μM (Table 1). This represents an impressive 24% hit rate. Four of these 19 hits are in the nanomolar range and belong to two new chemical series for JAK3 inhibition, pyrazolanthrones (**2**) and pyrazolopyrimidines (**3**). Between them, the 3,5-disubstituted pyrazolo[4,3-*d*]pyrimidine scaffold looks particularly promising to serve as a starting point for further lead optimization.

In a parallel in-house HTS effort of JAK3 screening, approximately 200000 J&J compounds that were preselected from the whole J&J corporate database by a clustering-based diversity analysis were biologically tested, followed by the strategy of retesting all the compounds in the same clusters as the initial active hits.[16] The overall hit rate turned out to be less than 0.1%, a number very similar to what have been reported by other HTS efforts.[29,30] Furthermore, compound **2**

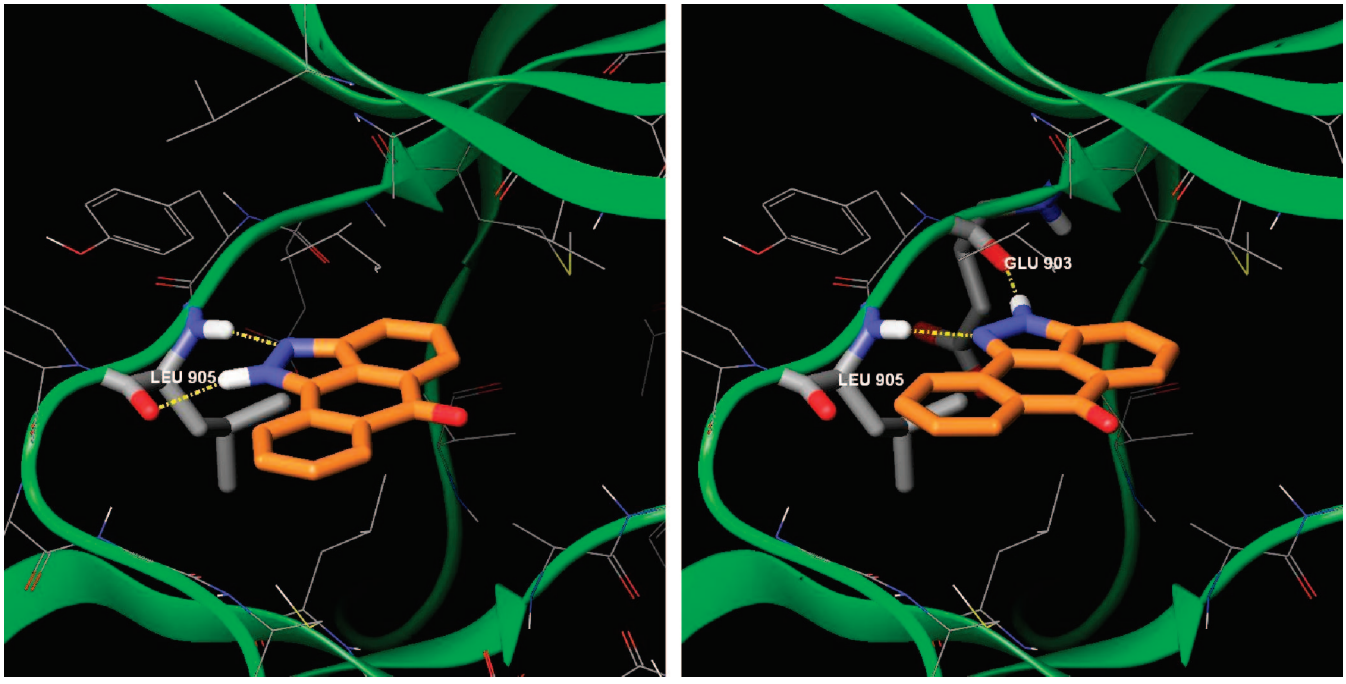

Figure 2. Illustration of the binding modes of compound **2** in the ATP-binding site of JAK3 proposed by the molecular docking study. The ligands and the key residues that act as their hydrogen-bond partners are represented in the tube model, while the proteins are in the cartoon model. The hydrogen bonds are indicated by the yellow dotted lines. (Atom color scheme: hydrogen in white, oxygen in red, nitrogen in blue, carbon on ligands in orange, and carbon on proteins in gray).

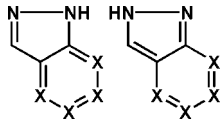

Figure 3. Illustration of the search queries used for the ISIS substructure search (X represents any heavy atom).

was identified as the most potent JAK3 inhibitor by the HTS screening, but compound **3** was completely missed. Remarkably, at least in this case, the VS strategy outperformed the HTS strategy in terms of the both hit rate and hit quality.

Discussions

The work reported here provides an example of how a sequential screening approach can be adopted in a focused screening project to successfully address the challenge of structural novelty. By adjusting the search focus of each round of screening, a focused screening process can be guided to explore a much broader region in chemical space while still maintaining the advantage of screening efficiency. This approach is essentially a kind of decrease-and-conquer strategy,[31] which can be described as reducing the instance of a problem into a smaller instance, solving the smaller instance, and then trying to convert the solution into a solution of the original instance of the problem. In this case, instead of trying to discover highly novel hits from a single round of screening, we managed to increase the scaffold novelty of screening hits gradually through multiple rounds of screening with the help of iteratively applying computational analysis and search techniques.

This approach has the obvious advantages of efficiency and flexibility. By focusing the biological screening only to the region of chemical space that is identified as relevant to the screening target, a much higher screening efficiency can usually be achieved in comparison to a typical broad screening effort. A higher screening efficiency will lead to not only a higher hit rate in general but also a higher chance of finding novel hits in the practical sense. It may arguably be the most important factor that need be considered in a screening project. Moreover, because the whole screening process is divided into multiple rounds, different computational analysis and search techniques can be utilized at different stages. This will introduce significant flexibility, allowing us to choose the most appropriate computational/biological screening technique(s) based on the new structure−activity data that become available at that time. This characteristic is actually critical for any screening approach to be useful in current drug discovery practice.

However, as is the case with other compound screening strategies, the actual performance of this approach highly depends on the content of the chemical database that it is to screen. It cannot be understated that any given database needs to possess desirable hits before any screening effort is able to identify them. Furthermore, this particular approach requires the pre-existence of some "intermediate" hits that bridge the initial lead structure and the final screening hit in the database, otherwise, it will not be able to achieve a "scaffold hopping"[32] by itself. Another potential disadvantage of this sequential approach is the prolonged time frame because the screening candidates of each round can be selected only after the previous round of biological screening is conducted and its results are analyzed. In practice, this kind of intermittent delay time can become significant if project management is not properly conducted and streamlined.

Conclusions

By applying a sequential focused screening approach, we not only successfully identified a couple of novel scaffolds for JAK3 inhibition but also achieved these findings with a quite high level of screening efficiency. As a general strategy for chemical compound screening, this approach can be applied to any chemical databases, including virtual and nonproprietary databases, greatly reducing potential acquisition and synthesis costs. It can be used in association with various computational and biological screening techniques with great flexibility, making it a practical tool for

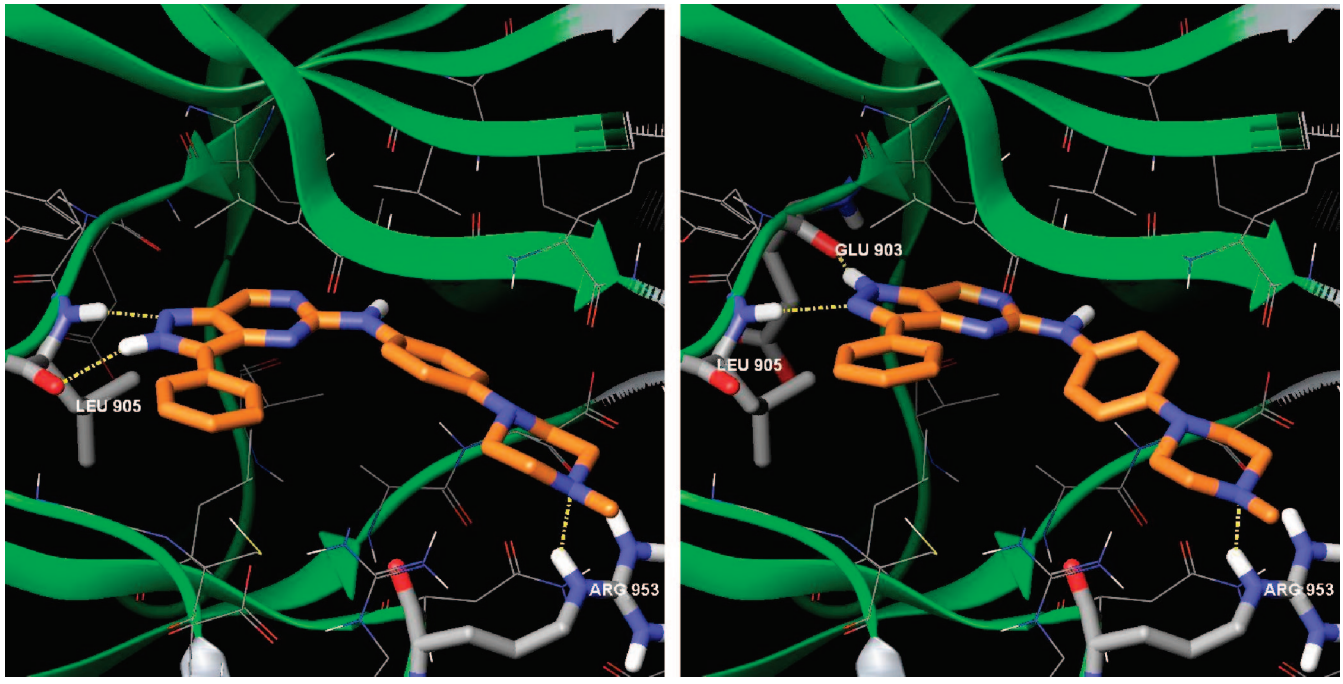

Figure 4. Illustration of the binding modes of compound **3** in the ATP-binding site of JAK3 proposed by the molecular docking study. The ligands and the key residues that act as their hydrogen-bond partners are represented in the tube model, while the proteins are in the cartoon model. The hydrogen bonds are indicated by the yellow dotted lines. (Atom color scheme: hydrogen in white, oxygen in red, nitrogen in blue, carbon on ligands in orange, and carbon on proteins in gray).

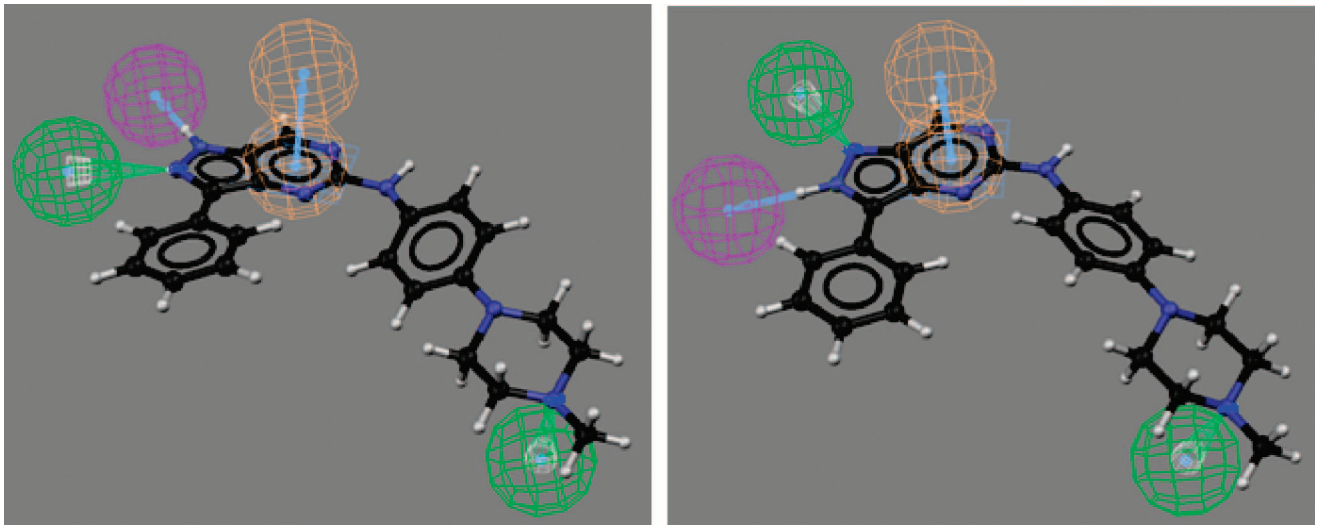

Figure 5. Illustration of the search queries used for the Catalyst pharmacophore search, aligned with compound **3**. Key pharmacophore points are position-constrained within a sphere of 1 Å radius, respectively. (Atom color scheme: hydrogen bond donor in purple, hydrogen bond acceptor in green, aromatic center in orange).

current lead identification processes. We hope to see more applications and studies of this promising approach in the future.

Experimental Section

Similarity searches were conducted by our in-house program PRISM,[20] which represents a chemical structure as a collection of topological linear fragments and calculates the similarity between two chemical structures based on the numbers of their common and unique fragments. More specifically, chemical structures are represented by topological atom sequences and similarity values are calculated by using the Dice coefficient (see the Supporting Information for more details on the PRISM program). Substructure searches and Pharmacophore searches were done, respectively, by the programs ISIS[24] and Catalyst.[28] The Catalyst pharmacophore search queries were manually defined based on the JAK3 binding conformations of compound **3** suggested by docking studies. A spherical position constraint of 1 Å radius was added to each key pharmacophore point. During the Catalyst search, a database compound must match all the features of a pharmacophore search query to be treated as a hit. The output of a substructure or pharmacophore search was further prioritized by PRISM similarity search so that the compounds that also showed a higher overall resemblance to the lead compound would receive a higher priority to be selected for biological testing. This selection process was done by visually inspecting each chemical structure coming out of a virtual screening, based on the considerations such as lead-likeness, stability, synthesizability, and novelty.

The JAK3 homology model was built by using the program Prime.[33] The EGFR X-ray structure (PDB ID: 1M17) was selected as the template[34] because it has the closest amino acid sequence, with about 33% identity and 51% similarity. Molecular docking

was conducted by using the program Glide.[35] The same computational procedure as described in ref 27 was used in this work. Each compound for the Glide docking study was preprocessed by the program Ligprep,[36] which generated all the possible structures of different tautomers, stereoisomers, and ionization states at neutral pH. The complex structure that gave the best Glide score was selected as the possible binding mode.

Enzyme activity of JAK3 was determined in terms of enzyme phosphorylation as described in ref 37.

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Supporting Information Available: More information on the PRISM algorithm, purity data for the screening hits **3** and **4** as reported in this paper. This material is available free of charge via the Internet at http://pubs.acs.org.

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