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Solution-Phase Parallel Synthesis of Ruxolitinib-Derived Janus Kinase Inhibitors via Copper-Catalyzed Azide−Alkyne Cycloaddition

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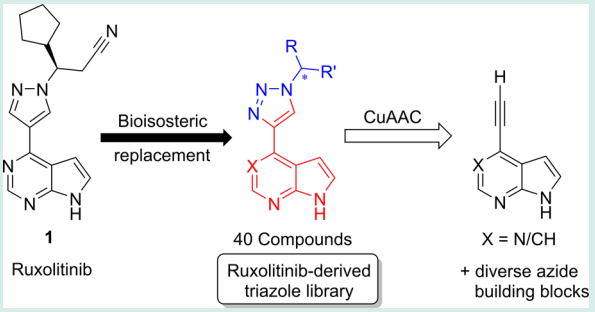
Supporting Information

ABSTRACT: A solution-phase parallel synthesis of triazole-derived ruxolitinib analogues was developed in the current study. The method employs copper-catalyzed azide−alkyne cycloaddition to build up the central triazole template. Product isolation by precipitation and centrifugation is straightforward and yields high purity compounds suited for biological profiling. A simple protocol for accessing the terminal alkyne precursors in high yields was established and a library of ruxolitinib-like triazoles featuring diverse functional groups was prepared. In addition, a model for the binding mode of ruxolitinib to Janus kinase (JAK) 2 is proposed. In contrast to previous models, the pose explains the compound selectivity for JAK1/JAK2 and is in accordance with published structure−activity data. On this basis, a structure-based design hypothesis for inverting the selectivity profile of ruxolitinib is deduced. Application of this strategy identified a moderately potent JAK3 inhibitor (35 nM) with high selectivity against other JAKs, potentially exploiting a covalent binding mode.



KEYWORDS: *Janus kinase inhibitors, heterocycles, triazoles, copper-catalyzed azide−alkyne cycloaddition, click chemistry*

Ruxolitinib (**1**, INCB 018424) is a Janus kinase inhibitor effective in the treatment of several BCR-ABL-negative myeloproliferative neoplasms, such as essential thrombocythemia and polycythemia vera. In 2011, it received marketing authorization in the U.S. and Europe as a first in class drug for treating intermediate to high risk myelofibrosis.[1] Ruxolitinib features a clean selectivity profile within the kinome and was awarded a "high quality chemical probe" for JAK2 inhibition.[2] Despite this status, significant selectivity within the JAK family comprising the four isoforms JAK1−3 and Tyrosine kinase 2 (TYK2) is solely exhibited against JAK3.[3] Since ruxolitinib is the only FDA approved protein kinase inhibitor for which no cocrystal structure in complex with its target has been published, we performed modeling studies to evaluate potential binding modes. Opposing two previous reports,[4,5] our results suggest a binding mode involving a hydrogen bond between the nitrile group of ruxolitinib and the side chain of a serine (Ser936 in JAK2 numbering) in the solvent exposed front region (hydrophobic region II)[6] of the kinase. In contrast to those earlier reports, the presented binding mode is compliant with the structure-activity relationships from several patents, which demonstrated that the nitrile function is crucial for JAK2 activity.[7] Furthermore, the pose explains the approximately 100-fold selectivity against JAK3, which is the only JAK kinase bearing a cysteine (Cys909) residue instead of the mentioned serine (Figure 1).[8]

In a recent study evaluating the structure−activity relationships (SAR) of hinge-binding motifs toward JAK3 inhibition, we found the 7*H*-pyrrolo[2,3-*d*]pyrimidine scaffold to be superior to other mono- and bicyclic *N*-heterocycles possessing the same pharmacophoric interaction pattern.[10] On the basis of these findings, we initiated a discovery program with the aim of identifying ruxolitinib-derived inhibitors with an inverted selectivity profile, which inhibit JAK3 without targeting the other Janus kinases. Considering the binding mode described above, we hypothesized that removing the nitrile function should result in unselective JAK inhibitors, which might subsequently be transformed in JAK3-selective compounds by addressing structural features unique to JAK3. Since JAK3 is this only Janus kinase bearing a cysteine in the solvent exposed front region, attaching an electrophilic covalent tag to replace the nitrile function of ruxolitinib seemed a valid strategy to selectively address this Janus kinase family member. However, for such a structure−activity study, a robust and efficient method for the preparation of a ruxolitinib-derived library lacking the nitrile function was required.

The synthesis reported for **1** is laborious and includes several chromatographic purification steps.[11] The key transformation involves an organocatalyzed enantioselective aza-Michael addition of a free pyrazole to a previously prepared (2*E*)-3-cyclopentylacrylaldehyde, giving high yield and enantiomeric excess (Scheme 1). The obtained aldehyde is subsequently

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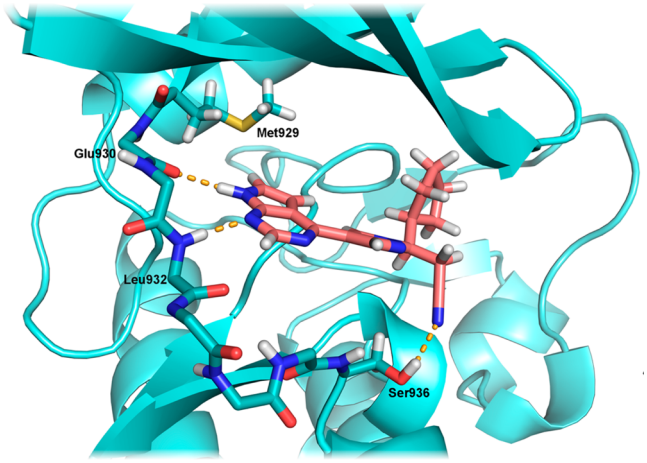


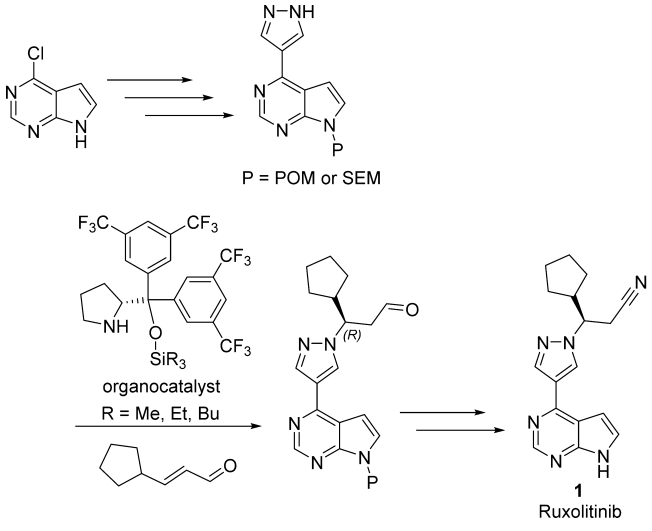
Figure 1. Ruxolitinib (**1**) docked into JAK2 (PDB accession code: 3Q32). The 7*H*-pyrrolo[2,3-*d*]pyrimidine core binds the hinge region of the kinase via a bidentate hydrogen bond. The key feature is a hydrogen bond between the ruxolitinib nitrile function and the Ser936 side chain. Since JAK3 is the only Janus kinase bearing a cysteine instead of this serine, the predicted pose provides a rationale for designing inhibitors with an inverted selectivity profile. The central pyrazole ring linking the ruxolitinib side chain with the hinge-binding adenine mimic acts as a structural template. Since it is not involved in direct interactions with the enzyme, bioisosteric replacement by a triazole ring is a promising strategy to increase synthetic accessibility. Docking was performed using the Gold software[9] and the image generated with PyMol.

transformed to a nitrile function, giving ruxolitinib after an additional deprotection step.

Despite the elegance of this highly optimized approach, its substrate scope is very limited making it unamenable to rapid library synthesis. For preparing a JAK3 screening library of ruxolitinib-derived compounds lacking the nitrile function, we replaced the pyrazole ring found in **1** by a bioisosteric 1,4-substituted triazole accessible by copper-catalyzed azide−alkyne cycloaddition (Figure 2).[12−14] This approach, often referred to as click chemistry, offers substantial advantages, since it is highly flexible, tolerant to multiple functional groups and the products usually do not require purification for initial testing or further synthetic processing.[15] Remarkably, a huge variety of organic azides is commercially available as building blocks from various suppliers. Alternatively, azides may easily be accessed from common precursors, such as halides, amines, alcohols and *α,β*-unsaturated carbonyl compounds using standard literature procedures.[16] Since many natural product derived azides are readily available, chiral ruxolitinib analogues can simply be prepared with high enantiopurity using the presented method without a need for asymmetric synthesis techniques, which often require time-consuming optimization for each individual substrate.

A high yielding synthesis for the terminal alkyne **2** was first developed (Scheme 2). Starting from commercially available 4-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine (**2a**), a halogen exchange

Scheme 1. Literature Synthesis of Ruxolitinib (1) according to Lin et al.[11a]



[a]The key step involves an enantioselective organocatalytic aza-Michael addition with limited substrate scope, succeeded by additional synthetic transformations preventing the combinatorial introduction of diverse building blocks.

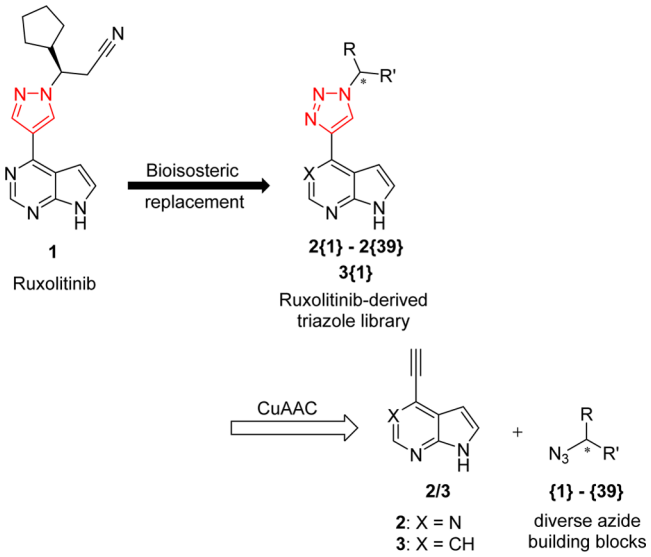
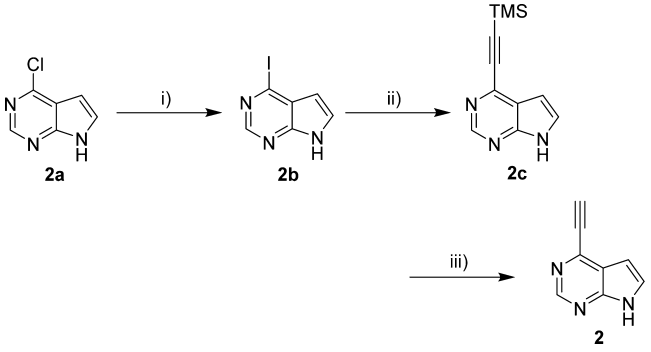


Figure 2. Design of triazole-derived ruxolitinib bioisosteres as potential JAK3 inhibitors accessible via copper-catalyzed azide−alkyne cycloaddition.

Scheme 2. Synthesis of the 4-Ethynyl-Substituted 7*H*-Pyrrolo[2,3-*d*]pyrimidine Scaffold[a]



[a](i) HI_{aq} (58%), rt, 80 h, quantitive yield; (ii) TMS acetylene, cat. CuI, cat. $Pd(PPh)_2Cl_2$, triethylamine, 45 °C, 6 h, 95%; (iii) KF, methanol, rt, 5 h, 74%.

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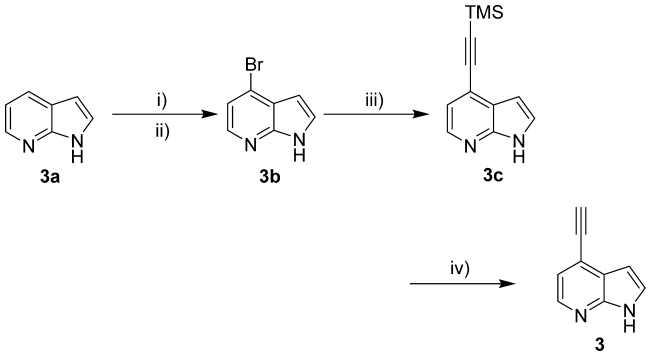
under acidic conditions using aqueous hydrogen iodide was used to obtain 4-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidine (**2b**) in quantitative yields. The heteroaryl iodide was subsequently coupled with trimethylsilylacetylene under standard Sonogashira conditions[17] to give the TMS-protected alkyne **2c**. Conversion proceeded completely and without significant byproduct formation and excellent yields of the pure product (>95% HPLC purity) were obtained. The crude material could be used for the subsequent deprotection after filtering the reaction solution though a short pad of silica gel without the need for previous extraction. Removal of the TMS group was accomplished using potassium fluoride in methanol to obtain 4-ethynyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (**2**) in good overall yields.

The applicability of the method was validated for the 1*H*-pyrrolo[2,3-*b*]pyridine scaffold, which proved to be the second-best bicyclic hinge-binding motif for JAK3 inhibitors in our recent SAR study.[10] According to Scheme 3, 4-bromo-1*H*-pyrrolo[2,3-*b*]pyridine (**3b**) used as the staring materials was prepared from 1*H*-pyrrolo[2,3-*b*]pyridine (**3a**) via *N*-oxidation[18] and subsequent bromination with tetramethylammonium bromide[19] applying literature procedures. Sonogashira coupling with trimethylsilylacetylene and subsequent TMS deprotection were performed in analogy to the synthesis of the parent compound **2** to obtain 4-ethynyl-1*H*-pyrrolo[2,3-*b*]pyridine (**3**).

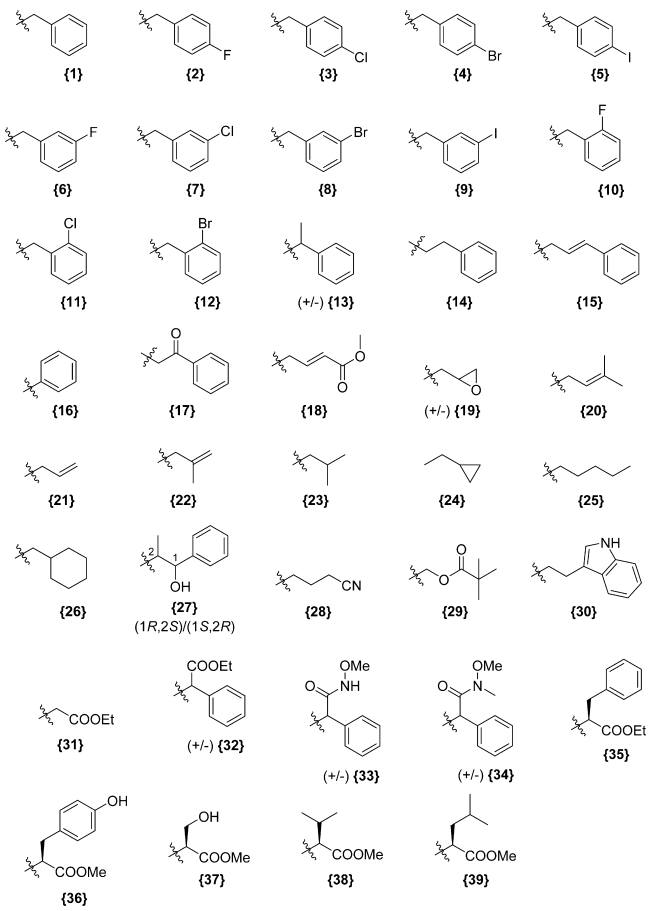
Scheme 3. Synthesis of the 4-Ethynyl-Substituted 7*H*-Pyrrolo[2,3-*b*]pyridine Scaffold[a]



[a](i) mCPBA, DME/*n*-hexane 2:1, rt, 3 h, then $NaHCO_{3(aq)}$, 95%; (ii) tetramethylammonium bromide, methanesulfonic anhydride, DMF, rt, 5 h, 58%; (iii) TMS acetylene, cat. CuI, cat. $Pd(PPh)_2Cl_2$, triethylamine, 45 °C, 12 h, 97%; (iv) KF, methanol, rt, 3 h, 57%.

The azides used throughout this work were either prepared in situ from activated alkyl halides (e.g., benzyl or allyl halides), by literature procedures from simple alkyl halides and sodium azide or from amines using imidazole-1-sulfonyl azide hydrogen sulfate as diazo transfer reagent.[20−22] All azides were used as crude products without chromatographic purification. Copper-catalyzed Huisgen−Meldal−Sharpless cycloaddition was performed using both reactants in equimolar ratio under standard conditions in a 1:1 mixture of *tert*-butanol and water employing 5% mol of the copper(I) catalyst in situ generated from copper(II)sulfate and sodium ascorbate.[14] Conversion was typically clean and complete (HPLC) and workup could easily be performed by complexing the copper catalyst with ammonia and precipitating the product with cold water. Collection of the product was performed by centrifugation or vacuum filtration and followed by washing with water and pentane. The obtained products were sufficiently pure for biological testing or further synthetic transformation. When performed in parallel, the most convenient method was carrying out the reaction in centrifuge tubes, allowing retrieval of the product by centrifugation without the necessity of transferring the suspension after precipitation. Furthermore, the centrifugation approach avoids plugging of filters and is potentially suited for automation. The library prepared using this method is shown in Table 1.

Table 1. Library of Ruxolitinib-Analogous Triazoles Prepared by CuAAC



According to Table 1, the method tolerates a variety of functional groups at the azide building block (i.e., alcohols, epoxides, esters, nitriles, alkoxyamides, α,β-unsaturated esters, and *N*-heterocycles). In contrast to a similar method from Müller et al., employing a Sonogashira coupling−TMS-deprotection−CuAAC sequence to prepare 1,2,3-triazol-5-ylindoles and related *N*-heterocycles,[23] our method does neither require *N*-protection of the heterocycle nor chromatographic purification to obtain compounds sufficiently pure for biological testing (>95% HPLC and NMR purity was achieved in most cases). Furthermore, the presented protocol features a higher functional group tolerance compared to the last mentioned method, which was only applied to simple benzylic azides without any sensitive functionalities incompatible with the Sonogashira coupling or TMS-deprotection step.

The ruxolitinib analogues prepared by applying our protocol were preliminarily tested for their ability to inhibit JAK3 using an ELISA established in our laboratories (Table 2).[24] Many of the compounds inhibited JAK3 with IC_{50} values in the low micromolar range. With the epoxide **2{19}**, which was designed

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Table 2. JAK3 Inhibitory Activity of the Prepared Compounds

cmpd.	IC_{50} ± SEM [μM][a]	cmpd.	IC_{50} ± SEM [μM]
2{1}	9.90 ± 0.20	**2{21}**	10.0 (50%)[b]
2{2}	>10.0 (31%)[b]	**2{22}**	2.66 ± 0.31
2{3}	7.72 ± 0.51	**2{23}**	>10.0 (43%)[b]
2{4}	>10.0 (33%)[b]	**2{24}**	3.22 ± 0.18
2{5}	>10.0 (30%)[b]	**2{25}**	4.98 ± 0.12
2{6}	>10.0 (25%)[b]	**2{26}**	>10.0 (33%)[b]
2{7}	>10.0 (26%)[b]	**2{27}**	4.04 ± 0.48
2{8}	n.i.[d]	**2{28}**	4.09 ± 0.38
2 {9}	>10.0 (24%)[b]	**2{29}**	>10.0 (37%)[b]
2{10}	>10.0 (42%)[b]	**2{30}**	>10.0 (28%)[b]
2{11}	9.96 ± 0.45	**2{31}**	8.75 ± 0.54
2{12}	5.76 ± 0.03	**2{32}**	n.i.[d]
2{13}	>10.0 (38%)[b]	**2{33}**	n.i.[d]
2{14}	n.t.[c]	**2{34}**	9.96 ± 0.35
2{15}	10.0 (50%)[b]	**2{35}**	>10.0 (23%)[b]
2{16}	n.t.[c]	**2{36}**	2.25 ± 0.16
2{17}	>10.0 (48%)[b]	**2{37}**	>10.0 (43%)[b]
2{18}	3.71 ± 0.17	**2{38}**	9.52 ± 0.97
2{19}	0.73 ± 0.06	**2{39}**	>10.0 (25%)[b]
2{20}	9.37 ± 0.38	**3{1}**	n.i.[d]

[a]All IC_{50} values are an average of at least three independent dose–response curves. SEM: Standard error of the mean. [b]Values are reported as % inhibition at an inhibitor concentration of 10 μM. [c]n.t.: Not tested. [d]n.i.: No inhibition (meaning less than 20% inhibition at an inhibitor concentration of 10 μM).

to covalently address Cys909 in JAK3, the library also featured one submicromolar inhibitor in the preliminary testing. Three selected compounds (**2{19}**, **2{22}**, and **2{36}**) were further tested for their selectivity within the JAK family employing a well-proven commercial assay. According to the initial hypothesis, all compounds lost the JAK1/2-selectivity observed for ruxolitinib in favor of JAK3 inhibition (Table 3). The

Table 3. Biological Activities of Selected Compounds against All Janus Kinase Family Members

	IC_{50} [μM][a]		
	2{19}	**2{22}**	**2{36}**
JAK1	2.728	1.479	2.311
JAK2	2.451	0.866	1.429
JAK3	0.035	0.971	0.421
TYK2	5.624	3.433	8.201

[a]IC_{50} values obtained from a commercial radiometric assay (K_m ATP, 5-dose IC_{50} with 5-fold serial dilution starting at 10 μM). Data was obtained from a single determination according to the suppliers standards. Assay details and the respective dose–response curves, are provided in the Supporting Information.

increased inhibitory activity observed in the commercial assay system has previously been discussed.[18] While compound **2{22}** bearing a small lipophilic methallyl residue did not exhibit pronounced selectivity against any of the JAKs, compound **2{36}** derived from *L*-tyrosine methylester is a moderately potent JAK3 inhibitor (IC_{50} = 421 nM) with approximately 6-, 4-, and 20-fold selectivity against JAK1, JAK2, and TYK2, respectively. Racemic compound **2{19}** was highly selective (70–160 fold) against all other Janus kinases. With an IC_{50} value of 35 nM, it is a promising starting point for further optimization to obtain highly potent and selective JAK3 inhibitors. As covalent docking suggests, this compound bearing an electrophilic epoxide tag forms a covalent bond with Cys909 in JAK3 (Figure 3). Further studies have been

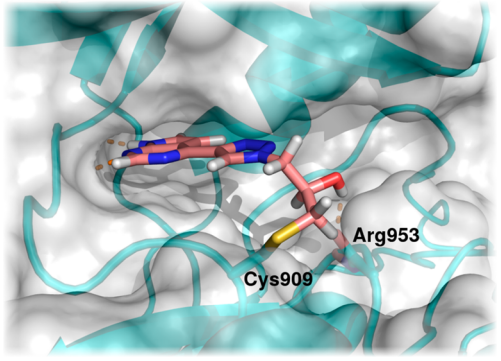

Figure 3. Key compound **2{19}** (*R*-enantiomer) covalently docked into JAK3 (PDB accession code: 3LXK). The 7*H*-pyrrolo[2,3-*d*]pyrimidine core binds the hinge region with the expected bidentate donor–acceptor hydrogen bond. A covalent link is formed between the Cys909 side chain and the terminal carbon atom of the epoxide. The hydoxyl group formed upon epoxide opening is predicted to form a hydrogen bond to the backbone carbonyl function of Arg953. The binding mode is in accordance with the experimental structure–activity relationships observed. Docking was performed using the CovalentDock algorithm of the Schroedinger Small Molecule Drug Discovery Suite (2014–3)[25] and the image generated with PyMol.

initiated to unambiguously assess whether binding of **2{19}** involves the predicted formation of a covalent bond or if selectivity is mediated by noncovalent interactions.

In summary, we have established a convenient method to prepare ruxolitinib analogues in parallel via copper-catalyzed azide-Alkyne cycloaddition. A high yielding synthesis of the alkyne precursors **2** and **3** was developed and their applicability in this click chemistry approach has been demonstrated. The protocol offers a broad scope due to the high functional group tolerance as shown by the prepared library. The commercial availability of diverse azide building blocks makes this method suited for rapid preparation of large and diverse compound collections. In contrast to the published synthesis of ruxolitinib, our approach allows the simple introduction of chiral centers in enantiopure fashion since many natural products (e.g., amino acids) with defined stereochemistry can be used as the azides superseding the use of asymmetric synthesis. Since the reaction is performed in an environmentally friendly solvent system, and it only requires stoichiometric amounts of reactants as well as a cheap and nontoxic catalyst, it is also suited for scale up. Furthermore, workup is simple and the products can be retrieved by centrifugation, in most cases without the requirement for additional purification steps.

With the compound library prepared by this method, we provide evidence supporting our proposed binding mode between ruxolitinib and JAK2. Utilizing the predicted pose in a structure-based design approach, the ruxolitinib nitrile group was replaced by diverse residues. In accordance with the design hypothesis, this modification led to a loss of selectivity for JAK1 and JAK2. By introducing an electrophilic epoxide tag, we identified a potent JAK3 inhibitor with high selectivity against JAK1, JAK2 and TYK2. We suppose that this molecule is capable of covalently targeting Cys909, which is present in JAK3 but not in JAK1/JAK2 and TYK2. As a future research direction, this approach might be used to prepare optimized

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covalent JAK3 inhibitors with increased potency. Furthermore, the application of the presented method to generate non-covalent inhibitors for any JAK isoform exploiting in situ click chemistry[26−28] is envisaged.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis and analytical data of all compounds including precursors **2b**/**2c** and **3b**/**3c**, as well as assay details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BCR-ABL, break point cluster region−Abelson murine leukemia viral oncogene homologue 1 fusion gene/protein; CuAAC, copper-catalyzed azide−alkyne cycloaddition; DME, dimethoxyethane; ELISA, enzyme-linked immunosorbent assay; FDA, U.S. Food and Drug Administration; JAK, Janus kinase; mCPBA, meta-chloroperoxybenzoic acid; PDB, Protein Data Bank; SAR, structure−activity relationship; TMS, trimethylsilyl; TYK2, tyrosin kinase 2

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