Leukemia (2017), 1–16

www.nature.com/leu

REVIEW

Rethinking JAK2 inhibition: towards novel strategies of more specific and versatile janus kinase inhibition

E Leroy and SN Constantinescu

Janus kinases (JAKs) are required for cytokine receptor signaling. Since the discovery of the highly prevalent JAK2 V617F mutation in myeloproliferative neoplasms (MPNs), JAK2 became a prime target for inhibition. Only one approved JAK2 inhibitor exists, with positive, but not curative effects in MPNs, and promising effects in autoimmune diseases and cancer. On the basis of recent advances in the structural features regulating both normal and mutant JAKs, as well as in small-molecule targeting, we review the current state of JAK2 inhibitor development and present novel avenues of selecting JAK2 inhibitors, with broad and narrow specificities and extend these approaches to other JAKs.

Leukemia advance online publication, 17 February 2017; doi:10.1038/leu.2017.43

JAK2, THE ACHILLES' HEEL OF MPNS

Myeloproliferative neoplasms (MPNs) are characterized by an excessive proliferation of megakaryocytic, erythroid and granulocytic progenitors due to a dysregulation of the JAK-STAT signaling pathway.[1] JAKs (JAK1-3 and TYK2) are characterized by tandem kinase domains: a typical tyrosine kinase domain (JH1) and a pseudokinase domain (JH2). The former is responsible for the catalytic activity and mediates signaling by several cytokine receptors.[2] JAK2 associates with homodimeric type-I cytokine receptors such as the thrombopoietin[3] and the erythropoietin receptors[4] to regulate proliferation, differentiation, survival and apoptosis of myeloid cells during hematopoiesis, and also trafficking of these receptors to cell surface.[5] JAK2 became an intensive focus in the field of MPN after the discovery of an activating mutation in its pseudokinase domain, namely JAK2 V617F that encompasses 70% of MPNs.[6–9] The three major BCR-ABL-negative MPNs are polycythemia vera, essential thrombocythemia and myelofibrosis (MF). Patients with MPNs develop splenomegaly, thrombosis, bleeding and can transform to acute myeloid leukemia. Other driver mutations in MPNs are activating mutations in the gene *c-MPL* encoding the thrombopoietin receptor (TpoR) such as TpoR W515K/L/S/A or TpoR S505N found in 4–5% of essential thrombocythemia or patients,[10] and mutations in calreticulin gene (*CALR*) found in 20–30% of essential thrombocythemia and MF patients.[11,12] Mutations in MPL/TpoR lead to ligand-independent receptor dimerization and constitutive activation of wild-type JAK2. CALR mutants induce constitutive activation of the JAK-STAT signaling pathway through intracellular and cell surface activation of the TpoR.[13] All things considered, JAK2 remains at the center of these disorders,[14] and MPN patients can respond to JAK inhibitors regardless of their mutational status.[15,16]

Development of potent JAK2 inhibitors for MPN treatment needs to satisfy several criteria. First, they must be specific to JAKs over other kinases to avoid any off-target toxicity. Drug exploitation of different features of particular kinase ATP-binding pockets such as the gatekeeper residue side chains, non-covalent interactions, salt bridges and the DFG-loop position allows specificity to be achieved.[17] Second, the inhibitors should display specificity to JAK2 compared to other JAKs (JAK1-3 and TYK2). Third, and case specific, in order to treat patients harboring the most predominant mutation in MPN, JAK2 V617F, the compounds should be specific to the mutant form of the protein and spare JAK2 WT.

TYPE-I INHIBITORS

Type-I inhibitors target the ATP-binding site of kinases in their active conformation. Naturally, targeting the catalytic activity of JAK2 has become a conceivable approach. The dual JAK1/JAK2 inhibitor, Ruxolitinib, was the first molecule developed and the first targeted therapy applied in MPN, as it was approved for treatment of MF after the COMFORT-I/II trials[18,19] and more recently for treatment of polycythemia vera after the RESPONSE trial.[20] Although Ruxolitinib reduces MF symptoms such as splenomegaly, it does not significantly decrease or eliminate the MPN clones. Ruxolitinib does not penetrate the blood–brain barrier. Furthermore, Ruxolitinib can induce thrombocytopenia and anemia, and to a lesser extent immunosuppression.[21] As genetic studies showed that JAK2 can exert a negative regulatory role in thrombopoiesis at the end of megakaryocyte differentiation, thrombocytopenia might also be linked to other actions of Ruxolitinib that are not understood.[22] This strongly encouraged the development of novel compounds showing more selectivity for JAK2 over the other JAK family members and thereby different safety profiles. Type-I JAK2 inhibitors have been tested in preclinical and clinical studies. Fedratinib (SAR302503), Lestaurtinib (CEP-701), Momelotinib (CYT387), Pacritinib (SB1518), Gandotinib (LY2784544), BMS-911543, XL019 have been developed for MPN treatment. These inhibitors appear to target myeloid progenitors, both mutated and non-mutated, but there is no evidence that they can eliminate mutated HSCs, which are the

Christian de Duve Institute, Ludwig Institute for Cancer Research and Université catholique de Louvain, de Duve Institute, Brussels, Belgium. Correspondence: Professor SN Constantinescu, Christian de Duve Institute, Ludwig Institute for Cancer Research and Université catholique de Louvain, de Duve Institute, Avenue Hippocrate 74, Brussels B1200, Belgium.
E-mail: stefan.constantinescu@bru.licr.org
Received 20 October 2016; revised 16 December 2016; accepted 10 January 2017; accepted article preview online 25 January 2017

reservoir of the disease. Yet, persistent activation of STAT5 has been established as a driver of enhanced HSC renewal[23,24] and could contribute to clonal dominance. Novel JAK2 inhibitors in the future might achieve targeting of mutated HSCs if their JAK2 inhibition is strong, specific and achieve complete inhibition of STAT5 in mutated HSCs, sparing normal HSCs. Clinical development of Fedratinib was terminated after reports of Wernicke's encephalopathy in MF patients, likely due to inhibition of the B1 vitamin transporter by the inhibitor.[25] Interestingly, this toxicity represented by insufficient central nervous system B1 was probably potentiated by the severe gastrointestinal problems induced by all JAK2 inhibitors that also inhibit FLT3, such as Fedratinib, Pacritinib and others. The reasons behind this gastrointestinal toxicity are not clear. Such inhibitors might inhibit off-target tyrosine kinases in intestinal epithelial cells, or might target receptors on gamma/delta T cells that are present in the intestinal mucosa. More studies are required to decipher the mechanisms linked to these toxicities, but new inhibitors should not inhibit FLT3 or B1 transporter.

In the COMFORT-I and II trials with Ruxolitinib,[18,19] it was reported that 96% of patients exhibit some degree of splenic response, indicating resistance is seen in 4% of patients. If the stringency of spleen response is increased to at least 10% volumetric reduction, then resistance or insufficient response is seen in 15% of patients. It is too early to have publication data on the Phase III trials with Momelotinib and Pacritinib, but resistance percentage appears to be the same, making it unlikely that changing the type-I JAK2 inhibitor with another one would restore sensitivity.

Importantly, Ruxolitinib inhibits JAK2 in receptor complexes other than those relevant for MPNs. For example, inhibition of interferon (IFN) gamma receptor 2 signaling is likely involved in the reactivation of herpes-zoster infection in MPN patients treated with Ruxolitinib; having molecules that do not inhibit JAK2 in the IFN gamma receptor complex would prevent such immunosuppression.

Tofacitinib (CP-690,550) and Baricitinib (LY3009104) were developed for rheumatological disease, and block JAK3/JAK1 and JAK2/JAK1, respectively. Finally, CEP-33779 was investigated in solid cancers (Table 1 and Supplementary Table S1). All these compounds are ATP mimetics recognizing the ATP-binding pocket in the so-called active conformation of the kinase. In this active conformation, the DFG phenylalanine of the activation loop makes hydrophobic contacts with the N-lobe αC helix and the catalytic loop, thereby adopting a 'DFG-in' conformation (Figure 1). Some of these compounds show higher specificity to JAK2, such as BMS-911543 and Gandotinib (Table 1 and Supplementary Table S1).

Among inhibitors targeting active JH1, compounds NS-018 and Gandotinib appear to be more effective to inhibit proliferation of JAK2 V617F cells versus cytokine-stimulated JAK2 WT cells.[26,27] An interaction between both these molecules and G993 (preceding the DFG motif) was visualized by crystal structures.[26,27] However, G993 seems to be close in structure to several of the other non-V617F-specific JAK inhibitors. Whether these molecules show preference to JAK2 V617F is due to targeting G993 in JH1, or addiction of transformed cells to JAK2 signaling remains to be determined. An alternative explanation could reside in higher rates of proliferation of JAK2 V617F-expressing cells.

By occupying the ATP-binding pocket, type-I inhibitors abolish phosphoryl transfer from ATP to the substrate. Andraos *et al.* showed that this binding mode induces a paradoxical increased phosphorylation of the activation loop Y1007 of JAK2 despite inhibition of downstream signaling likely due to trans-phosphorylation by other JAK family members or other kinases.[28] This has been a complicating issue in many studies, as phosphorylation of Y1007 is a read-out of activation.[29] Paradoxical phosphorylation upon inhibitor addition was already reported for other kinases such as BRAF.[30,31] For JAK2, such an open active conformation might explain why under inhibitor pressure, tumor overexpressed JAK2 can heterodimerize with JAK1 or TYK2 leading to a functional resistance to type-I inhibitors denoted as 'persistance'.[32,33] Finally, germ-line JAK2-activating mutants were identified, which exhibit longer half-life and resistance to type-I inhibitors.[34] For all these resistance situations, type-II inhibitors that bind to the inactive kinase conformation might be more effective.

TYPE-II INHIBITORS

Type-II inhibitors recognize the inactive conformation of kinases and bind to the ATP-binding pocket and to the extra 'DFG-out' pocket only accessible in the conformation of inactive kinases (Figure 1). The so-called 'DFG-out' pocket is created by the DFG phenylalanine adopting an 'out' conformation, which means that its side chain is out of a hydrophobic spine found in the active conformation (Figure 1). Using crystallographic data and a better understanding of kinase regulation, the growing field of structure-based drug design could identify this second class of kinase inhibitors preferentially binding to the unphosphorylated inactive conformation of the kinase. By exploiting supplementary binding sites contiguous to the ATP-binding pocket (the DFG- pocket), type-II inhibitors can gain in specificity and hence in selectivity.[35] Importantly, the extra pocket targeted by type-II inhibitors is less conserved within the kinome than the canonical ATP-binding pocket, thus promising better selectivity of the compound and minimizing adverse effects due to inhibition of unintended kinases. This second generation of inhibitors prompted considerable interest for the treatment of Brc-Abl-positive CML after the introduction of the earliest type-II inhibitor, Imatinib (Gleevec, STI-571). The solved structure of the Bcr-Abl co-crystallized with Imatinib (PDB: 1IEP)[36] actually served as a model for further type-II drug design.

To date, two compounds have been described to bind JAK2 in a type-II binding mode, namely NVP-BBT594 and subsequently NVP-CHZ868 (Novartis; Figure 1). Strikingly, both drugs were described to retain the capacity to inhibit persistence cells.[32,33,37] Andraos and colleagues described that, in contrast to type-I inhibitors, the type-II inhibitor NVP-BBT594 stabilizes the inactive conformation of JAK2, which results in an unphosphorylated activation loop likely achieving a stronger inhibition.[28] Moreover, this work reported the first structure of the JAK2 kinase domain in its inactive state by co-crystallization of JH1 with NVP-BBT594, where the DFG phenylalanine is moved by ~ 10 Å from its position in the active state (PDB: 3UGC; Figure 1). NVP-BBT594 recognizes structurally distinctive regions within the kinase cleft compared to type-I inhibitors such as Ruxolitinib (Figure 1). This atomic structure will likely help the design of future type-II inhibitors. The dihydroindole NVP-BBT594 was originally developed as an inhibitor of the Imatinib-resistant Bcr-Abl T315I allele,[38] and was later found to inhibit JAK2. However, it retains some limitations in selectivity and its pharmacokinetic properties that render it unsuitable for *in vivo* use.[37] For this reason, another type-II inhibitor was developed with improved physicochemical and pharmacokinetic properties, NVP-CHZ868, which shows strong efficacy against activated forms of JAK2 responsible for MPN development. Two accompanying publications reported the effect of NVP-CHZ868 in MPN[33] and in B-ALL[37] mouse models, showing that NVP-CHZ868 suppresses signaling of transformed cells resistant to type-I inhibitors and even displayed a significant reduction of the allele burden in polycythemia vera and MF murine models. NVP-CHZ868 exhibited selectivity for pathogenic JAK2 activation against JAK2 WT activation and remarkably, it did not only affect V617F-mediated but also the pathological-persistent MPL W515L-mediated activation of JAK2 WT.[33] NVP-CHZ868 is not a clinical candidate, but represents a first prototype type-II JAK2 inhibitor, that can be used in preclinical models to

Table 1. JAK2 inhibitors historically studied

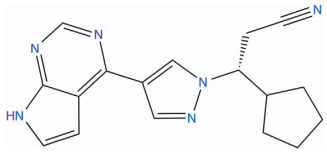
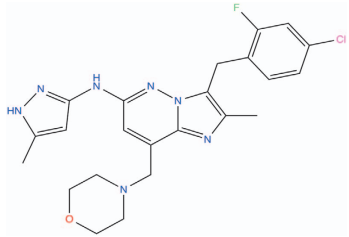
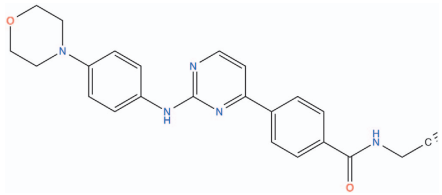
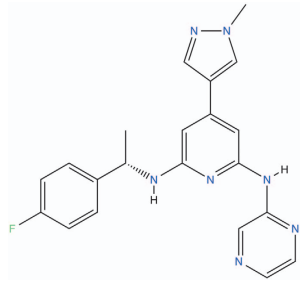
Inhibitor	*Other names*	*Company*	*Compound Structure*	*Scaffolds*	*Type*	*Disease*	*JAK2 IC50 (nM)*[a]	*Other Targets*	*Status*	*Side effects*	*Ref.*
INCB018424	Ruxolitinib Jakavi	*Incyte/Novartis*		Pyrrolo-pyrimidine	Type-I	MF, PV	2.8	JAK1	FDA approved MF (2011) PV (2015)	Thrombocytopenia, Anemia, Neutropenia, Myelo-suppression	18–20
LY2784544	Gandotinib	*Eli Lilly*		Substituted imidazo-pyridazine	Type-I	ET, PV, MF Solid tumor	3	JAK1, JAK3	Phase I/II, ongoing Preclinic	Renal impairment, tumor lysis syndrome, gastrointestinal toxicity, anemia	27
CYT387	Momelotinib GS-0387	*Cytopia, acquired Gilead*		Amino-pyrimidine	Type-I	PMF, post PV MF, post ET MF	11	JAK1, TYK2 TBK1/IKK-ε, ALK-2	Phase III, ongoing	Withdrawal symptoms, neurological side effects (peripheral neuropathy)	96
NS-018		*Nippon Shinyaku Co*		Pyridine based	Type-I	MF, post PV MF, post ET MF	< 1	Src	Phase I/II, ongoing		26

Table 1. (Continued)

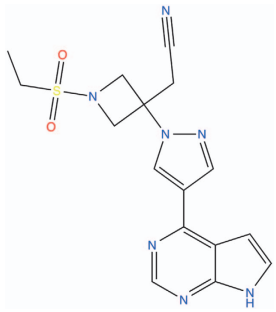
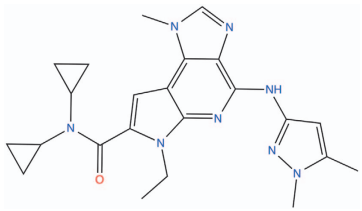
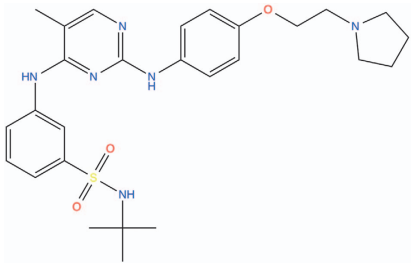
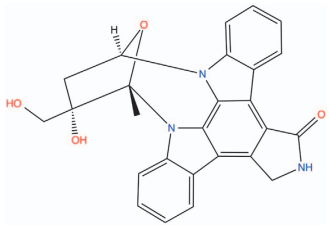
Inhibitor	*Other names*	*Company*	*Compound Structure*	*Scaffolds*	*Type*	*Disease*	*JAK2 IC50 (nM)*[a]	*Other Targets*	*Status*	*Side effects*	*Ref.*
INCB028050	Baricitinib LY3009104	*Incyte/Eli Lilly*		Pyrrolo-pyrimidine	Type-I	RA, Psoriasis	5.7	JAK1	Phase III (RA), Phase II (psoriasis)		[89]
BMS-911543		*Bristol Myers Squibb*		Imidazo-pyrrolo-pyridine	Type-I	MF	1	JAK1 JAK3 TYK2	Phase I/II, completed	Leukopenia, thrombocytopenia, neutropenia	[97]
TG101348	Fedratinib SAR302503	*TargeGene Sanofi Aventi*		Amino-pyrimidine (thiamine analog)	Type-I	MF	3	FLT3 RET	Phase III, development terminated in, Nov 2013	Wernicke's encephalopathy (due to ressemblance of fedratinib with thiamine, VitB1, whose absorption was reduced by hTHTR2)	[25]
CEP-701	Lestaurtinib	*Cephalon*		Indolo-carbazole (staurosporine analog)	Type-I	MF,ET, PV	0.9	FLT3	Phase I/II, development terminated	Gastrointestinal toxicity, high pharmacokinetic variability	[98]

Table 1. (Continued)

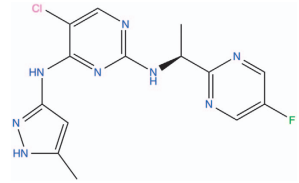
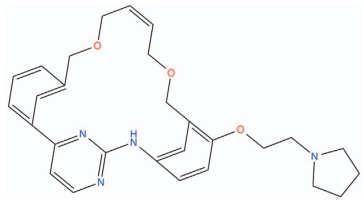
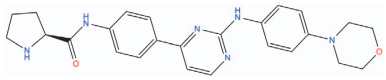
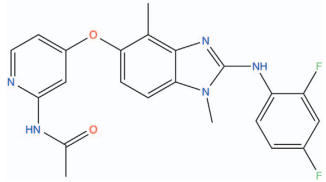
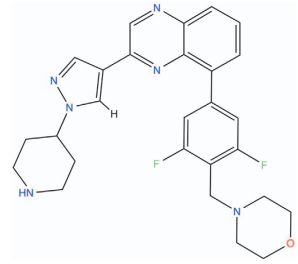
Inhibitor	*Other names*	*Company*	*Compound Structure*	*Scaffolds*	*Type*	*Disease*	*JAK2 IC50 (nM)*[a]	*Other Targets*	*Status*	*Side effects*	*Ref.*
AZD-1480		*AstraZeneca*		Pyrazol-pyrimidine	Type-I	Primary MF, post PV MF, post ET MF,	3	JAK1 Aurora-A FGFR1 FLT4	Phase I, development terminated	Neurological toxicities	99
SB1518	Pacritinib	*S*BIO, CTI biopharma*		Macrocyclic pyrimidine-based	Type-I	MPN, AML, MDS	23	FLT3, IRAK1	Phase III, development terminated in Feb 2016	Intra-cerebral hemorrhage, cardiac failure and arrest, gastrointestinal toxicity	100
XL019		*Exelixis*		Pyrimidine based	Type-I	PV, MF	2	JAK1 JAK3 TYK2	Phase I, development terminated	Neurotoxicty (peripheral neurophathy)	101
NVP-CHZ868		*Novartis*		Benzimidazole	Type-II	MPN	110	KIT, as well as PDGFR and VEGFR family kinases	Preclinic		33,37
NVP-BSK805		*Novartis*		Subsituted quinoxaline	Type-I	MPN	0.48	JAK1 JAK3 TYK2	Preclinic		92,102

Table 1. (Continued)

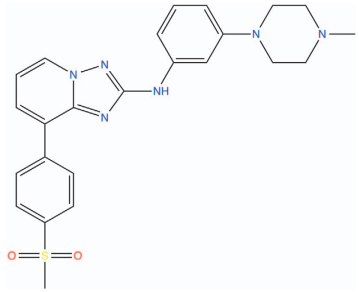
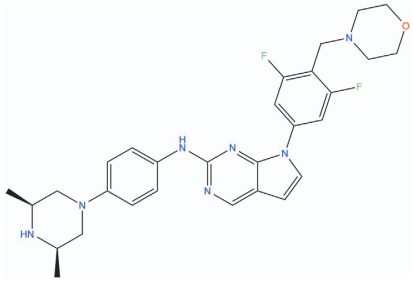
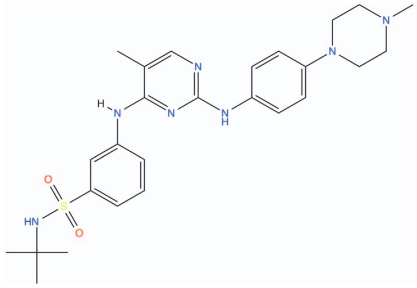
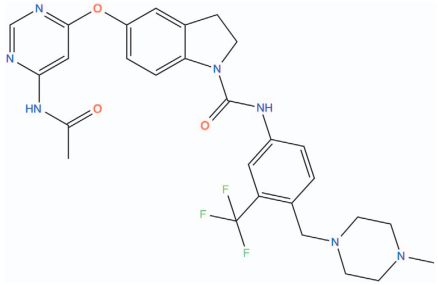
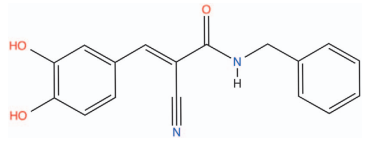
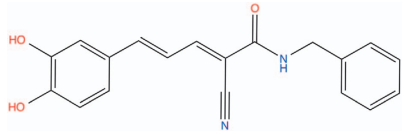
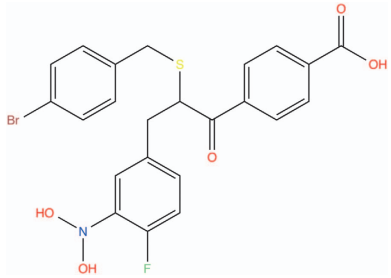
Inhibitor	*Other names*	*Company*	*Compound Structure*	*Scaffolds*	*Type*	*Disease*	*JAK2 IC50 (nM)*[a]	*Other Targets*	*Status*	*Side effects*	*Ref.*
CEP-33779		*Cephalon*		Pyridine derivative	Type-I	Colorectal Tumor	1,3	NA	Preclinic		[91]
NVP-BVB808		*Novartis*		Pyrrolo-pyrimidine	Type-I		0.35	FLT3	Preclinic		[102]
TG101209		*TargeGen*		Pyrimidine-based	Type-I		6	JAK3, FLT3, RET	Preclinic		[103]

Table 1. (Continued)

Inhibitor	*Other names*	*Company*	*Compound Structure*	*Scaffolds*	*Type*	*Disease*	*JAK2 IC50 (nM)*[a]	*Other Targets*	*Status*	*Side effects*	*Ref.*
NVP-BBT594		*Novartis*		Dihydroindole	Type-II		990	Brc-Abl KDR FLT3 RET	Research only	Limits in specificity for JAK2, pharamaco-kinetics unproper for *in vivo* use	28,33
AG490		*Tocris Bioscience*		Tyrphostin (first generation)	Type-I		100	EGFR	Research only (*First JAK2 inhibitor*)	Pharamacokinetics unproper for *in vivo* use	104
LS104				Dienamide	Allosteric			Bcr-Abl RET	Research only		46
ON044580		*Onconova Therapeutics*		α-benzoyl styryl benzyl sulfide	Allosteric			Bcr-Abl	Research only		45,47

**NA: not available. (See Supplementary Table S1 for complete list of references). The bold values indicate the names of the inhibitor compounds. [a]IC50 values from cell-free assays.

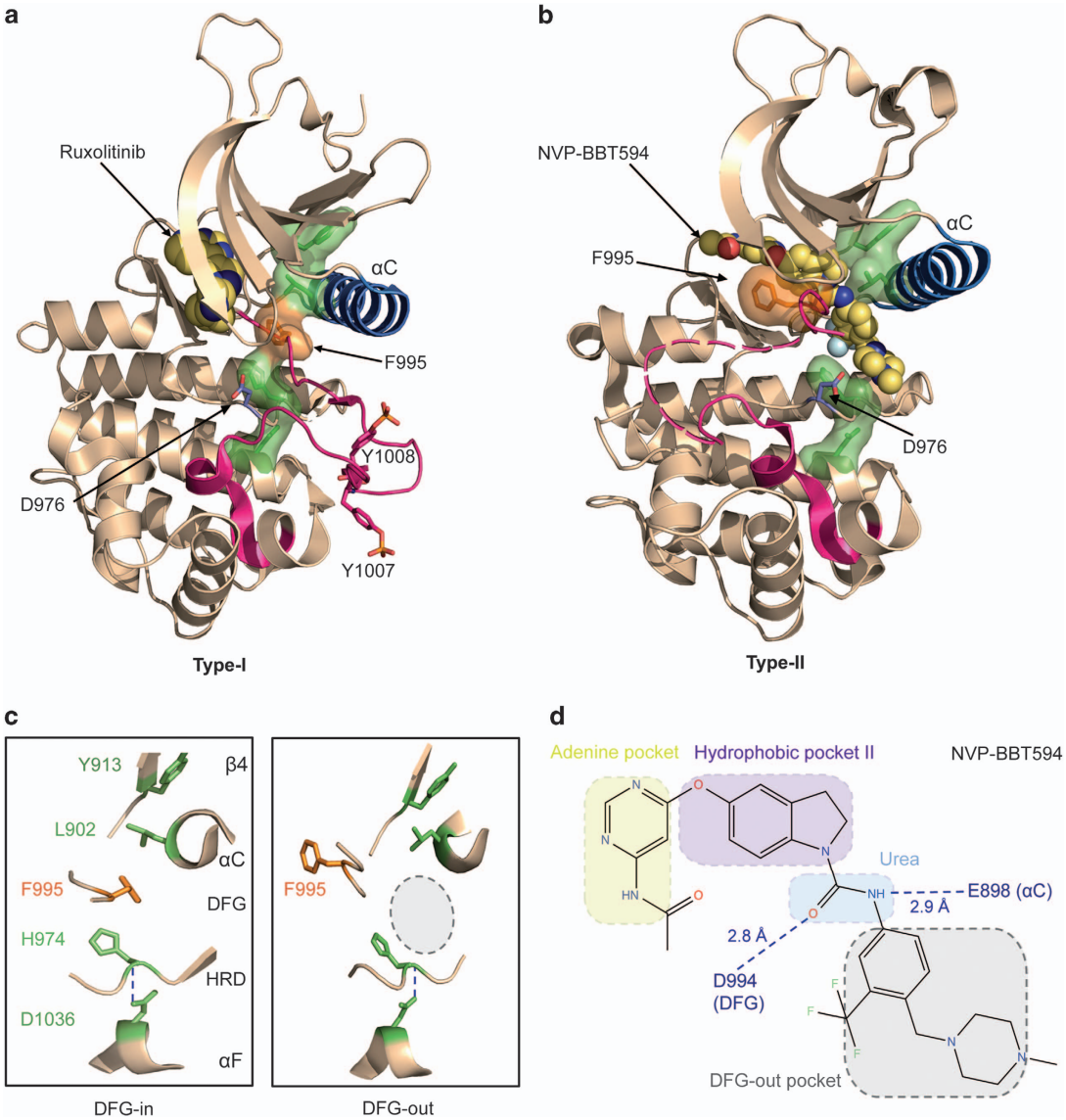


Figure 1. (**a**) Type-I binding mode. Ruxolitinib (shown is spheres) binding to the active conformation of JAK2 JH1 (PDB code: 3KRR[92]) has been modeled based on the co-crystal structure of Ruxolitinib bound Src (PDB: 4U5J[93]). The αC is colored in blue and the activation loop, colored in pink, adopts an open (active) conformation. D976 from the catalytic loop (HRD motif) colored in light blue is turned toward the active site. F995 from the DFG motif, located at the outset of the activation loop, is colored in orange and adopts an 'in' position in the hydrophobic regulatory spine depicted in green. (**b**). Type-II binding mode. Ribbon diagram of the co-crystal structure of NVP-BBT594 bound JAK2 JH1 (PDB: 3UGC[28]). The inhibitor is shown in spheres and binds JH1 that exhibits an activation loop in a closed (inactive) conformation (pink dashed lines). D976 is turned outward of the active site, while F995 is translocated away from it original pocket and now resides 'out' of the regulatory spine. (**c**). A regulatory hydrophobic spine is composed of a series of 4 hydrophobic residues from β4, αC, DFG and HRD. These residues are only correctly aligned in the active conformation of kinases and that is stabilized by phosphorylation of the activation loop (left panel). This so-called regulatory spine is bound to the C-lobe α helix F (αF) via a conserved hydrogen bond between H974 and D1036 (dashed line). F995 from the DFG occupies its pocket and is aligned in the spine in the active stat (DFG-in). In the inactive conformation (right panel), the regulatory spine is 'broken' since F995 is moved away from its original position in the spine leaving the pocket (gray oval) empty. This DFG-out pocket can now be occupied by type-II inhibitors. (**d**). Schematic drawing of some important contacts specific of type-II inhibitor binding to kinase. Type-II inhibitors, such as NVP-BBT594, usually present an amide or an urea group in the molecule that makes hydrogen bonding with a conserved αC glutamate side chain and with the DFG aspartate backbone (blue dashed lines). D994 from the DFG motif makes a hydrogen bond to the oxygen atom of the urea group of NVP-BBT594 via its backbone nitrogen atom. The pyrimidine group of NVP-BBT594 occupies the adenine site of the ATP-binding site (yellow) while the dihydroindole moiety occupies the adjacent hydrophobic pocket II (purple) in opposition to the hydrophobic pocket I occupied by type-I inhibitors (not shown). The Tri-fluoro-methyl-phenyl and the N-methylpiperazine occupy the DFG-out pocket (gray).

interrogate effects on disease of such effective inhibition. NVP-CHZ868 still exhibits off-target activity, for example, on VEGF receptor tyrosine kinase and further medicinal chemistry efforts will be necessary to bring this molecule to clinical development. Recently, virtual screens and optimization efforts have been reported with respect to obtaining new type-II JAK2 inhibitors.[39]

One JAK2 kinase domain mutation conferring resistance to type-II inhibitors, L884P, was isolated by a mutagenesis approach conducted with the B-ALL-associated JAK2 R683G mutant, and was found to prevent inhibition by NVP-CHZ868.[37] This mutation is thought to disorganize the hydrophobic network regulating the shape of the ATP-binding cleft and to prevent binding of the

type-II inhibitor by changing the position of the αC helix. Interestingly, this mutation was also described as an activating mutation in JAK2 and JAK3 (L857P).[37,40] This mutation appears to intrinsically activate JH1, as it does not require receptor activation.[40] Thus, the mutation obstructs the type-II inhibitor-binding pocket, and stabilizes the active state of the kinase.

Even though type-II inhibitors can provide better inhibition efficiency, they cannot discriminate between wild-type and the mutant forms.

ALLOSTERIC INHIBITORS

Allosteric inhibitors can present significant advantages compared to ATP-site binders. An allosteric site is defined as a region distinct from the kinase active site and where ligand binding can either positively or negatively regulate the enzyme activity.[41] This holds the potential of higher selectivity because every kinase possesses specific regulatory mechanisms. Moreover, targeting regions outside of the ATP-binding site will allow the development of compounds with different and improved physicochemical properties.[42] Another asset of allosteric inhibitors would be the possibility to overcome drug resistance by targeting two different sites in combination, as drug resistance usually emerges when mutations arise to prevent the compound from binding to one site.[43]

Indirect targeting of the catalytic function of kinases has already been investigated for some kinases. For instance, the compound GNF-2 was shown to inhibit Bcr-Abl by binding to the C-terminal myristoyl pocket of Abl kinase domain.[44]

Allosteric inhibitors that are defined as molecules binding to a region on the kinase that does not overlap with the ATP-binding site are also referred as type-III inhibitors[43] (Table 2, Figure 2 and Supplementary Table S2). To date, no such molecules have been clearly identified for JAK2. Two molecules, LS104 and ON044580 were described as allosteric inhibitors of JAK2[45–47] and demonstrated inhibitory activity against native JAK2 and JAK2 V617F. However, their allosteric mechanism was never confirmed, and their development was not continued. At present, structural and mechanistic studies[48–51] indicate that allosteric inhibition of JAK2 could be achieved by targeting other domains of JAK2, distinct from the kinase domain. This new class of compounds, sometimes referred as type-IV inhibitors[43] (Table 2 and Figure 2), could be possibly developed in order to achieve JAK2 V617F-specific inhibition.

Targeting the pseudokinase ATP-binding site

JAKs are intriguing proteins, as they possess both a pseudokinase and an active kinase domain in their C-terminal part (Figure 3). Together with GCN2 (general control nonderepressible-2), they are the unique proteins to share this feature within the kinome. The pseudokinase domain (JH2) exhibits important regulatory functions in JAKs, playing key roles in both activation and inhibition of the kinase domain.[52–54] An inhibitory function of the JH2 domain in JAKs was indicated by early studies and by recent molecular dynamic simulation (for JAK2), and structural X-ray structure determination (for TYK2).[55,56] The lab of S Hubbard solved for the first time the atomic details of JH2 in both WT- and V617F-mutated forms and found that it adopts a typical protein kinase fold.[57] Interestingly, JH2 was shown to bind ATP[58] in a non-canonical manner;[57] however, the high-affinity binding and weak hydrolysis of ATP implies a structural rather than catalytic role of JH2 nucleotide binding.[50] Indeed, ATP seems to act as a pseudokinase stabilizer through which it mediates auto-inhibition in the absence of stimulation.

Results from structure-guided mutagenesis and biochemical studies indicate that ATP binding to JH2 is critical for hyperactivity of JAK2 mutants, and apparently to a lesser extent for the activity of JAK2 WT.[50] Hence, abrogating ATP binding on JH2 with the use of nucleotide mimicking compounds might be a good approach in order to achieve mutant-specific inhibition. JAK2 V617F appears to be essential in amplifying clonal MPN stem and early progenitor cells.[59] Specifically targeting JAK2 V617F could potentially have a profound effect on the MPN clones and its progeny while sparing the cells with wild-type JAK2. Moreover, the same would be predicted for MPNs with TpoR W515 mutations.[60]

To our knowledge, no ATP competitors were discovered to bind JH2 and modulate the activity of JAK2, but the pseudokinase

Table 2. The different types of kinase inhibitors

Type[a]	*Description*	*Examples*
Type -I	ATP competitive. Reversible. Bind to the ATP-binding pocket. Target the active conformation or 'DFG-in' conformation where the Phe is in the hydrophobic regulatory spine and the HRD Asp is facing into the active site (illustrated in Figure 2a).	Ruxolitinib (JAK2)
Type-II	ATP competitive. Reversible. Bind to the ATP-binding pocket and the DFG pocket. Target the inactive conformation or 'DFG-out' conformation where the Phe is out of the regulatory spine and the HRD Asp is extending outward from the active site (illustrated in Figure 2b).	Imatinib (BCR-ABL), NVP-CHZ868 (JAK2), NVP-BBT594 (JAK2)
Type-III	Reversible. Bind exclusively to an allosteric pocket, located on the kinase domain, close to the ATP-binding pocket but does not overlap it (illustrated in Figure 2c).	PD184352 (MEK)
Type-IV	Reversible. These inhibitors are truly allosteric inhibitors that bind exclusively to an allosteric pocket, distant of the ATP-binding pocket (illustrated in Figure 2d). These molecules can also bind to other domains of the target protein or to other interaction partners of the kinase (illustrated in Figure 2e).	GNF-2 (BCR-ABL)
Type-V	Reversible. These molecules are bivalent inhibitors that bind to two distinct sites on the target kinase protein. Composed of an ATP competitor molecule connected to a ligand that targets a region remote from the active site.	Bivalent inhibitors (c-Src)
Covalent	Bind irreversibly to the kinase active site by reacting with a highly reactive nucleophilic residue, usually a cysteine residue, located in the ATP-binding site.	Neratinib (HER2) Ibrutinib (BTK)

A fifth group includes bivalent inhibitors that use a combination of one or more of the four preceding binding modes. (See Supplementary Table S2 for complete list of references). [a]The common *reversible* inhibitors are classified in four different groups from I to IV (illustrated in Figure 2).

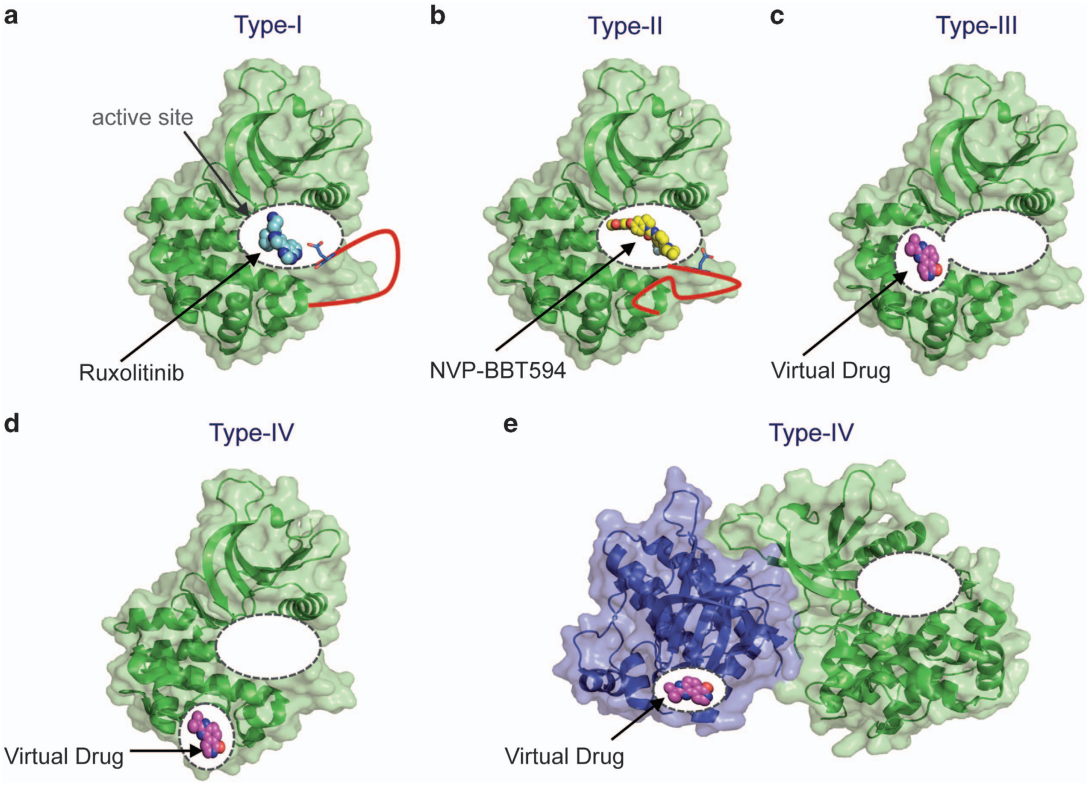


Figure 2. The common reversible inhibitors. The common reversible inhibitors can be classified in four different groups and are represented here. The target kinase is represented with ribbon diagram and transparent surface. The activation loop is represented in red. The numbering from **a**–**e**. corresponds to the description in Table 2.

domains of TYK2 and JAK1 have been described to bind such compounds.[61,62] In addition, a co-crystal structure of TYK2 JH2 with a pyrazine compound confirmed that the non-canonical ATP-binding pocket of the pseudokinase domain is actually accessible to ATP competitors,[63] but whether this would lead to inhibition has to be clarified. Tokarski and colleagues provided the first evidence that signaling inhibition can be reached by pharmacologically targeting of TYK2 JH2.[61] It is not clear whether this approach could be translated to the other JAK family members, especially JAK2.

The latter functions in homodimeric receptor complexes,also contains two unique autoinhibitory phosphorylation sites, namely S523 and Y570.[58] As abrogating auto-phosphorylation sites in JH2 lead to weak basal activation of the JAK2 kinase domain,[58] JH2 targeting could also raise the basal kinase level constitutively while inhibiting mutant JAKs. The development of JH2 binders, along with our increasing comprehension of the mechanistic role of JH2 might bring new inhibition strategies for JAK2.

Targeting specific allosteric sites involved in V617F activation mechanism

The V617F mutation is located in the loop connecting β4–β5 strand in the N-lobe of JH2 (Figure 3). It induces activation of the kinase domain of JAK2, so in one sense itself is an allosteric regulator of the JH1 kinase domain. Guided by the discovery that a large hydrophobic residue was required at position 617 to cause JAK2 activation[64] and inspection of available kinase structures, it was suggested that F617 could interact with the middle αC helix phenylalanines, F545 and F595. It was established that these two residues are necessary for the activation by V617F and suggested that the three aromatic residues are involved in a π–π stacking interaction, which impact the conformation of the αC helix of JH2.[48] This change of conformation was visualized by the X-ray crystal structure of the pseudokinase domain containing V617F.[57] Small molecules able to disrupt the π–π stacking interaction that only exist in the mutant conformation would represent a valuable approach in order to achieve mutant-specific inhibition.

Another key element of V617F-mediated activation is an absolute requirement for the negative charge on the face of helix C opposite to F594/F595, and facing JH1 and the SH2-JH2 linker.[51] Reversing the charge of this face or of one residue, E596, via an E596R/K mutation, restores wild-type phenotype to JAK2, namely absent constitutive activity and normal response to cytokine stimulation. Further studies indicated that V617F activates JAK2 through a community of residues across JAK2 (JH2 helix C F595-E596; SH2-JH2 linker F537, JH2 N-lobe E543-D569-Y570 arriving at JH1 K883 and K857). Mutation of any residue in the circuit activates JAK2, and this can be prevented by an E596R mutation, like for V617F. Identical circuits exist for active JAK1 and TYK2 homologous mutants. Small molecules interfering with the aromatic π–π stacking or neutralizing the charge at 596, or the face containing it should be explored.

V617F was shown to induce a rearrangement of the SH2-JH2 linker (Figure 3), repositioning the highly conserved linker residue F537.[49] F537 was found to be necessary for the hyperactivity of V617F,[49] but not for physiological JAK2 function.[51] Yet, targeting this linker appears difficult due to its flexibility and potential to induce activation of JAK2.[49,51,55,65]

Potential new allosteric sites in the mutant JAK2 V617F are summarized in Figure 3. Whether these regions correspond to druggable pockets on the protein surface still has to be investigated. Future efforts will focus these allosteric sites and the development of compounds able to shift the regulatory interactions toward auto-inhibition in the case of mutant JAK2.

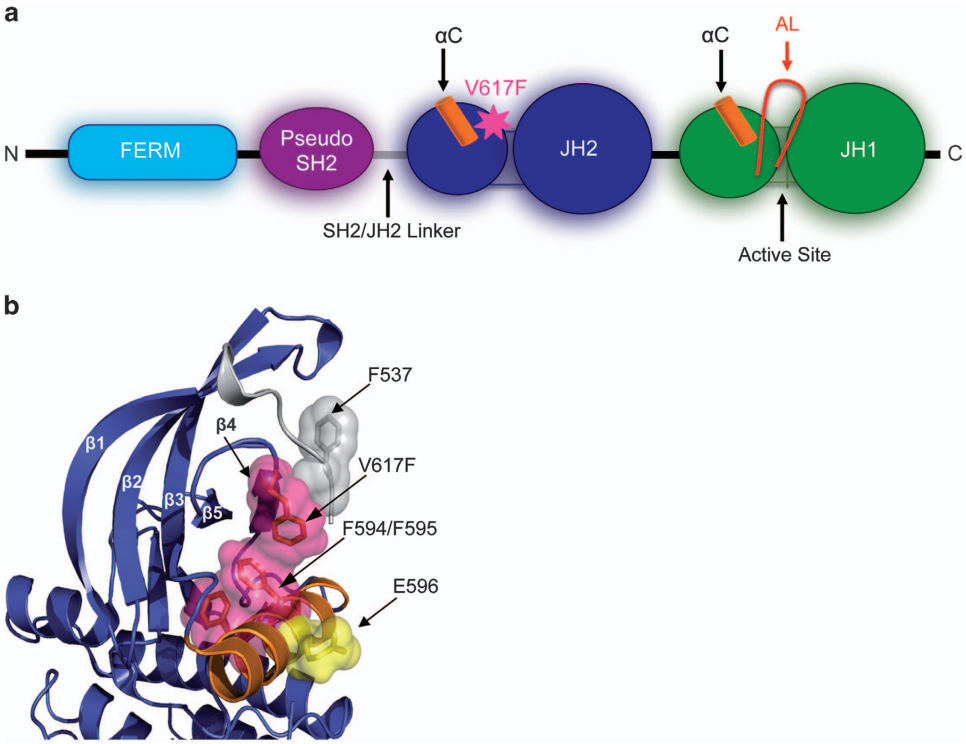


Figure 3. JAK2 structures and the possible allosteric sites. (**a**). Representation of the primary domain structure of JAK2 that is composed, from N-term to C-term, of a FERM domain, a SH2-like domain, a pseudokinase domain (JH2) and a kinase domain (JH1) from N-term to C-term. JH1 and JH2 are composed of a N-terminal smaller lobe mainly composed of β sheets and C-terminal larger lobe mainly helical. The activating mutation V617F is located in the smaller N-lobe of JH2. (**b**). The N-term lobe of JH2 is shown in blue ribbon diagram (PDB code: 4FVR[57]). The αC is shown in orange. The three possible allosteric sites of JH2 are represented by sticks and surface in three different colors. The first allosteric site, shown in gray, is the region around F537 located on the SH2/JH2 linker. The second one, shown in pink, is the phenylalanine triad maintained by an aromatic stacking. F594 and F595 from the αC interact with V617F located in the β4-β5 turn. A third possible site, shown in yellow, is the glutamate 596 located on the opposite face of αC than F594 and F595. The side chain position of E596 and F537 were artificially modeled on the structure.

COVALENT INHIBITORS

Another class of inhibitors has been designed by structure-guided integration of an electrophilic moiety into compounds already possessing certain binding affinity for the kinase (Table 2, Figure 2 and Supplementary Table S2). These covalent inhibitors are usually developed to target a nucleophilic cysteine lying in the active site of the kinase, where it will irreversibly bind through the formation of a covalent bond.[66] Although cysteine residues are not conserved in kinase active sites, some kinases serendipitously possess this residue in or around their ATP-binding site. In fact, there are 211 kinases that have at least one cysteine residue in their ATP-binding site disseminated in 27 positions.[67]

JAK3 kinase domain possesses a unique cysteine in its active site, C909, which has been exploited by covalent inhibitor for specific targeting.[68,69] This cysteine is shared by 11 kinases such as EGFR, HER2, HER3 or BTK, and was previously used for covalent inhibitors development guided against EGFR (Neratinib, HIK--272),[70] which would easily justify its clinical development. Although this cysteine is not conserved in the kinase domains of the other JAK family members, the pseudokinase domains of JAK1-2 and TYK2 share a cysteine around the ATP-binding site in the pseudo-catalytic loop (at position 675 in JAK2; Figure 4). Of note, this cysteine is also conserved in three other pseudokinases SgK223/pragmin, SCYL1 and SCYL3,[67] also able to bind ATP. The co-crystal structure of TYK2 JH2 with a small-molecule inhibitor[63] showed the proximity of the inhibitor piperidine group with the cysteine 736 (homologous to C675 in JAK2), suggesting that an electrophilic moiety added at this position would potentially react with the cysteine (Figure 4). The accessibility to the cysteine thiol group lying in JAKs JH2 should be examined. Finally, a combination of JH2 covalent inhibitors and JH1 classical inhibitors could possibly achieve higher specificity and potency. Hitting the target kinase from two angles might show better potency, as it was already demonstrated for BCR-ABL with the combination of the allosteric inhibitor GCN2 and the ATP competitor Nilotinib.[71]

TUNING JAK2 SIGNALING FROM THE OUTSIDE

Cytokine receptors serve as scaffolds for JAK2 molecules and thus, are necessary for signaling. Receptors and JAKs are believed to form functional units (Figure 5). JAK2 V617F-driven transformation necessarily requires homodimeric cytokine receptors such as EpoR or TpoR.[72,73] Orientation-specific signaling has been demonstrated for TpoR[74,75] and EpoR,[76] where inactive and active conformations of the dimeric receptors could be determined. However, in contrast to JAK2 WT, JAK2 V617F can signal regardless of the orientation imposed by the upstream receptor.[48] This assumes that larger changes of the receptor dimer topology might be required to efficiently reduce activated JAK2 signaling. The team of KC Garcia used diabodies (small recombinant bispecific antibody-derived fragments) to neutralize EpoR/JAK2 V617F ligand-independent signaling.[77] These substitute ligands can reorient the receptor–JAK2 complex geometry in an inactive conformation, presumably by imposing a physical separation between the two JAK2 molecules large enough to prevent trans-phosphorylation of the cytosolic kinases.

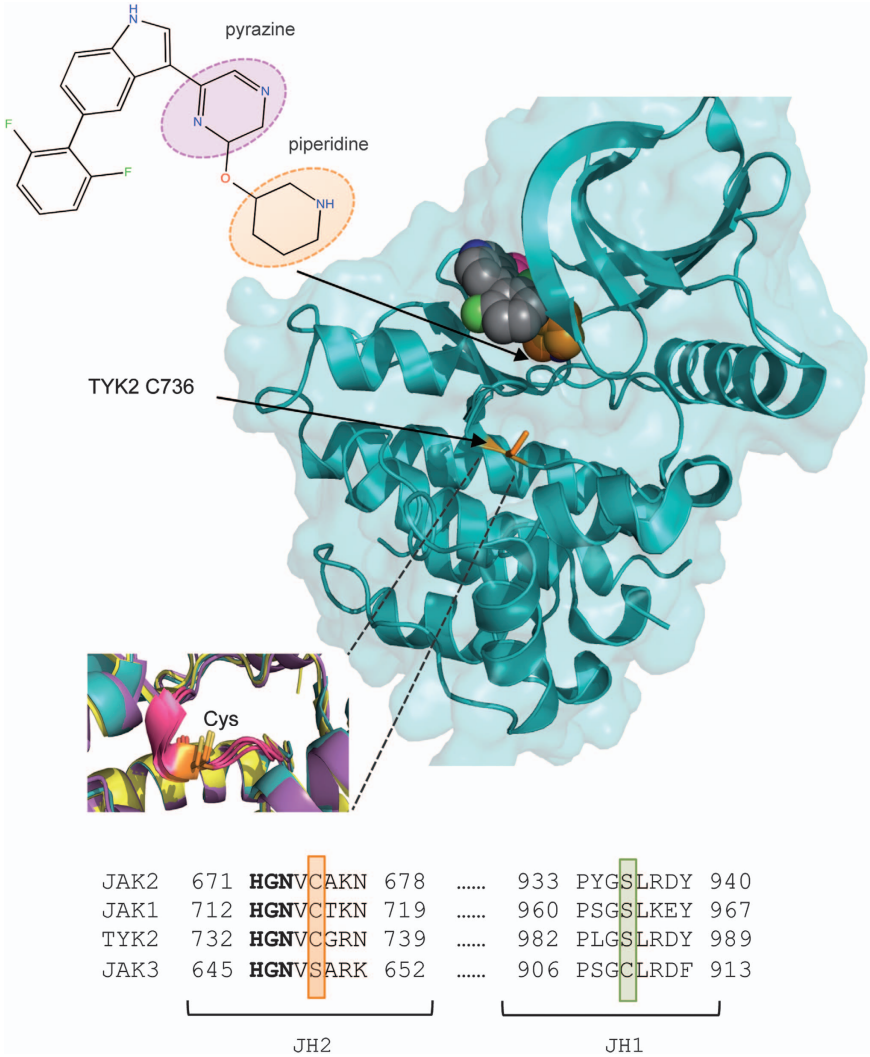


Figure 4. Possible strategy for the development of covalent allosteric inhibitors. Shown in the main panel is the co-crystal structure of TYK2-JH2 (cyan ribbon and surface) bound to a ligand that contains pyrazine (pink) and piperidine (orange) moieties linked by an ether group (PDB: 5C01[63]). A cysteine residue colored as orange stick, C736, is located in the ATP-binding region of TYK2-JH2 not far from the piperidine moiety of the compound. Modification of the piperidine group can potentially lead to covalent binding to C736. This cysteine residue is conserved in the pseudokinase domains of JAK1 and JAK2 but not JAK3; see sequence alignment. The small square represents the structural alignment of TYK2-JH2, JAK2-JH2 and JAK1-JH2 that highlights the conserved position of this cysteine residue. While JAK3 does not carry this cysteine in JH2, it possess a unique cysteine in its JH1 active site used for the development of specific JAK3 covalent inhibitors.

Aside from the inter-subunit distance within dimers, it appears that JAK2 V617F is free to signal whatever receptor dimeric geometry is imposed to EpoR.[48] Thus, rotation alone will not suffice, in contrast to inducing separation of dimers.[77]

TARGETING CYTOKINE RECEPTOR–JAK2 INTERACTION

Receptor–JAK2 interactions are crucial for cytokine signaling, in that cytokine binding to the extracellular receptor domains transmits a conformational change to the cytosolic domain, and to the JAK2 molecules that are attached to this region. The two JAK2 molecules, if correctly oriented, will *trans*-phosphorylate each other to become fully active and then phosphorylate tyrosine residues of the receptor tails that will provide binding sites for STATs and other signaling molecules. JAK2 contains in its N-terminal end a FERM domain (four-point-one/ezrin/radixin/moesin) and a pseudo SH2 domain (Figure 3). This FERM-SH2 segment mediates non-covalent interactions with the receptor cytosolic tail.[78,79] Cytokine receptors possess specific motifs, the membrane proximal Pro-rich box1 and the downstream hydrophobic box2, which are required for these interactions. The FERM domain is also crucial for discriminating between the large repertoires of cell surface cytokine receptors.[80–83] Interestingly, the FERM domain was shown to positively regulate JAK2 V617F,[84] while negatively regulating JAK2 WT.[85] Thus, targeting the specific receptor-JAK2 interface has always been a tantalizing approach. Lack of structural information has hindered this quest.

Due to the advances made by the group of P. Lupardus, the first structural understanding of JAK-cytokine receptor recognition could be provided, where tandem FERM and SH2 domains participate in the binding interface with the receptor.[78,79] In a first study, the solved structure of TYK2 FERM-SH2 domain in complex with the box2 of IFNR1 (IFNα receptor 1) revealed a central and unexpected role of the atypical JAK SH2 domain in receptor interaction.[79] The SH2 domain binds to the receptor around a glutamate residue in box2 that mimics the phosphotyrosine-binding site for classical SH2 domains. In a second study, structures of JAK1 FERM-SH2 in complex with

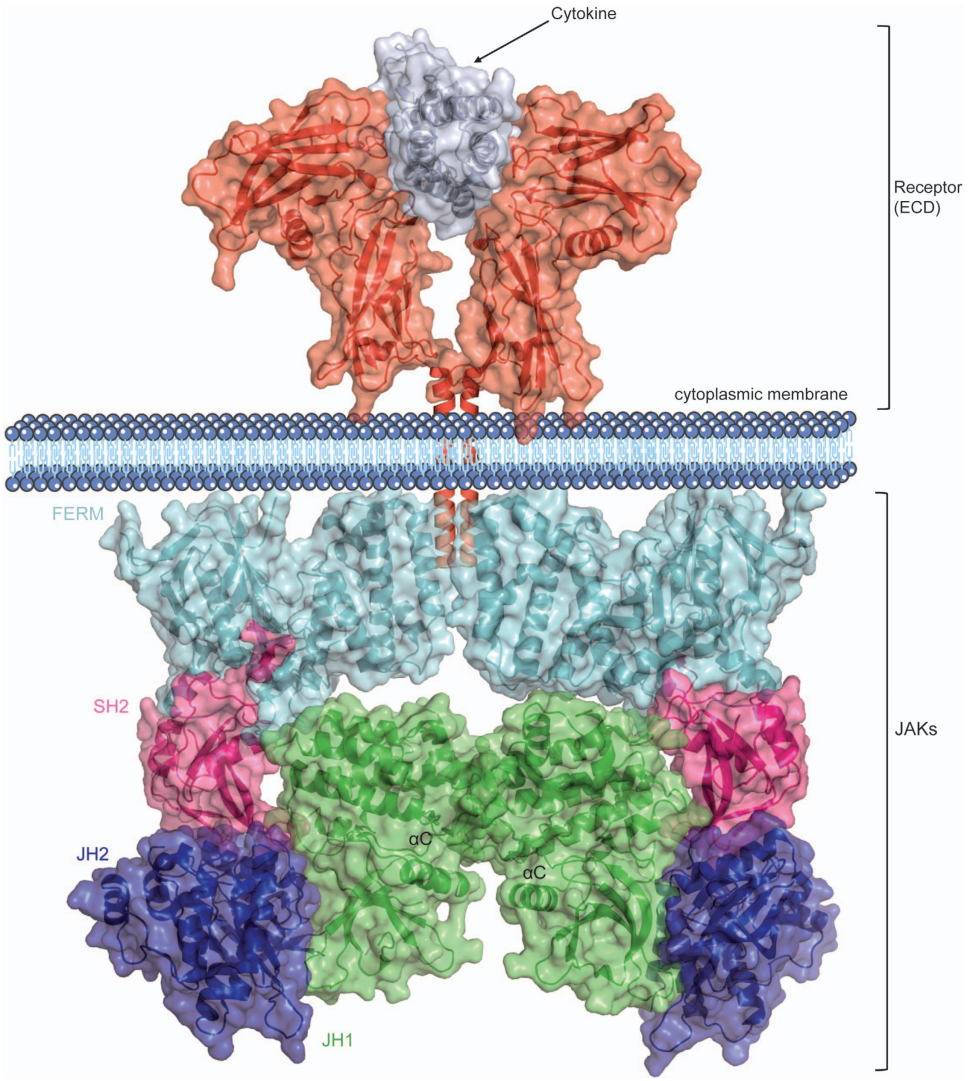


Figure 5. Model of a cytokine receptor-JAK complex. The cytosolic domains of cytokine receptors and the two appended JAKs form functional units that together, control and trigger signaling. Shown here is a model of the EpoR-JAK2 complex, where the two JAK2 molecules are represented to scale the size of the receptor. Above the plasma membrane the crystal structure of extracellular domains of EpoR dimer bound to Epo (PDB: 1EER,[94]) is represented. Below the plasma membrane shown are ribbon diagrams of the crystal structure of JAK2-FERM-SH2 (PDB: 4Z32,[86] arbitrarily assembled to the JH2-JH1 coordinates derived from molecular dynamic simulations using JH1 (PDB: 3KRR) and JH2 (PDB: 4FVQ).[55] The transmembrane domain of EpoR is shown (PDB: 2MXB[95]). The molecular architecture of the intracellular domain of EpoR is not known and is not represented here.

peptides derived from either IFN-λ or IL-10 receptors were solved. JAK1 principally binds to these receptors through a well-defined box1, while box2 only stabilizes this interaction.[78] Recently, the structure of JAK2 FERM-SH2 was solved and identified potential receptor-binding sites that might contribute to the specificity of receptor engagement.[86] The compatible topology between the two structural models of TYK2 and JAK1 offers now a concrete basis for the simultaneous binding of box1 and box2 of one receptor to one JAK molecule.

These advances pave the way for the generation of small molecules or therapeutics that would induce disruption of receptor-JAK association in a specific manner. Indeed, exploring targetable pockets around the defined interface between the FERM-SH2 domain and the cytosolic receptor portions, avoiding conserved/identical regions, could be an avenue for achieving inhibition of specific receptor-JAK complexes. In addition these structures explain why point mutations in the FERM domain exerted such spectacular inhibitory effects on JAK function: mutations in Y114 in JAK2, or equivalents in other JAKs,[5,81,87] likely misfolded the entire JAK FERM domain.

CONCLUSION

Current JAK2 inhibitors are type-I ATP competitors targeting the ATP-pocket of the JH1 kinase. Using the major current structural and biochemical advances, a number of new strategies are available to obtain novel, more potent and more specific inhibitors, and to overcome some of the major challenges of JAK2 inhibition: i) to achieve potent catalytic inhibition without off-target effects; ii) to reach a high degree of selectivity for JAK2 against the other JAKs; iii) to specifically or preferentially target mutated forms against JAK2 WT, thus directly targeting the MPN clone and sparing normal hematopoiesis; iv) to specifically disrupt JAK2 function in defined cytokine receptor complexes. We believe that this selectivity could be achieved by developing allosteric inhibitors targeting sites outside of the active site. JAK2 activity is

controlled through diverse levels of regulation across its different protein domains and through the receptors, which offers several possible drug-targetable sites. Taking advantage of the non-catalytic domains of kinases, which play other roles and are less conserved within the kinome, may offer attractive avenues to improve specificity.

Since JAK2 activation by the pseudokinase V617F mutation seems to be mediated through a community of specific residues across JAK2,[51] exploiting these interactions has the potential to yield drugs with specificity against mutant JAK2 exclusively. All the results for JAK2 V617F could be validated also for JAK1 and TYK2 homologous mutants, suggesting results obtained with JAK2 should be transferrable to activating mutants of JAK1 and TYK2.

The therapeutic potential of JAK2 inhibitors has emerged throughout the past decade in MPNs,[88] autoimmune diseases,[89] and possibly solid tumors.[90,91] In that sense JAKs are in a situation comparable to EGF receptors several years ago for a variety of pathologies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Drs Andrew K. Shiau, Leila Varghese and Thomas Radimerski for critical comments. We acknowledge FRIA PhD and Salus Sanguinis fellowships to EL. Support to SNC was from Ludwig Institute for Cancer Research, the Fondation contre le cancer (Grant Number F/2004/266), from Actions de Recherche Concertées of the communauté Française de Belgique (Grant Number ARC10/15-027) and the PAI program Belgian Medical Genetics Initiative (Grant Number IAP-P7/43).

REFERENCES

1 Vainchenker W, Constantinescu SN. JAK/STAT signaling in hematological malignancies. *Oncogene* 2013; **32**: 2601–2613.
2 Briscoe J, Rogers NC, Witthuhn BA, Watling D, Harpur AG, Wilks AF *et al.* Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. *EMBO J* 1996; **15**: 799–809.
3 Drachman JG, Millett KM, Kaushansky K. Thrombopoietin signal transduction requires functional JAK2, not TYK2. *J Biol Chem* 1999; **274**: 13480–13484.
4 Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O *et al.* JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* 1993; **74**: 227–236.
5 Royer Y, Staerk J, Costuleanu M, Courtoy PJ, Constantinescu SN. Janus kinases affect thrombopoietin receptor cell surface localization and stability. *J Biol Chem* 2005; **280**: 27251–27261.
6 Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S *et al.* Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* 2005; **365**: 1054–1061.
7 James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C *et al.* A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* 2005; **434**: 1144–1148.
8 Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR *et al.* A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med* 2005; **352**: 1779–1790.
9 Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ *et al.* Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* 2005; **7**: 387–397.
10 Pietra D, Brisci A, Rumi E, Boggi S, Elena C, Pietrelli A *et al.* Deep sequencing reveals double mutations in cis of MPL exon 10 in myeloproliferative neoplasms. *Haematologica* 2011; **96**: 607–611.
11 Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC *et al.* Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med* 2013; **369**: 2391–2405.
12 Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD *et al.* Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med* 2013; **369**: 2379–2390.
13 Chachoua I, Pecquet C, El-Khoury M, Nivarthi H, Albu RI, Marty C *et al.* Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants. *Blood* 2016; **127**: 1325–1335.
14 Rampal R, Al-Shahrour F, Abdel-Wahab O, Patel JP, Brunel JP, Mermel CH *et al.* Integrated genomic analysis illustrates the central role of JAK-STAT pathway activation in myeloproliferative neoplasm pathogenesis. *Blood* 2014; **123**: e123–e133.
15 Passamonti F, Caramazza D, Maffioli M. JAK inhibitor in CALR-mutant myelofibrosis. *N Engl J Med* 2014; **370**: 1168–1169.
16 Guglielmelli P, Biamonte F, Rotunno G, Artusi V, Artuso L, Bernardis I *et al.* Impact of mutational status on outcomes in myelofibrosis patients treated with ruxolitinib in the COMFORT-II study. *Blood* 2014; **123**: 2157–2160.
17 Badrinarayan P, Sastry GN. Rational approaches towards lead optimization of kinase inhibitors: the issue of specificity. *Curr Pharm Des* 2013; **19**: 4714–4738.
18 Harrison C, Kiladjian JJ, Al-Ali HK, Gisslinger H, Waltzman R, Stalbovskaya V *et al.* JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. *N Engl J Med* 2012; **366**: 787–798.
19 Verstovsek S, Mesa RA, Gotlib J, Levy RS, Gupta V, DiPersio JF *et al.* A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. *N Engl J Med* 2012; **366**: 799–807.
20 Vannucchi AM. Ruxolitinib versus standard therapy for the treatment of polycythemia vera. *N Engl J Med* 2015; **372**: 1670–1671.
21 Verstovsek S, Kantarjian H, Mesa RA, Pardanani AD, Cortes-Franco J, Thomas DA *et al.* Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. *N Engl J Med* 2010; **363**: 1117–1127.
22 Meyer SC, Keller MD, Woods BA, LaFave LM, Bastian L, Kleppe M *et al.* Genetic studies reveal an unexpected negative regulatory role for Jak2 in thrombopoiesis. *Blood* 2014; **124**: 2280–2284.
23 Schepers H, Wierenga AT, Vellenga E, Schuringa JJ. STAT5-mediated self-renewal of normal hematopoietic and leukemic stem cells. *JAKSTAT* 2012; **1**: 13–22.
24 Wierenga AT, Schepers H, Moore MA, Vellenga E, Schuringa JJ. STAT5-induced self-renewal and impaired myelopoiesis of human hematopoietic stem/progenitor cells involves down-modulation of C/EBPalpha. *Blood* 2006; **107**: 4326–4333.
25 Zhang Q, Zhang Y, Diamond S, Boer J, Harris JJ, Li Y *et al.* The Janus kinase 2 inhibitor fedratinib inhibits thiamine uptake: a putative mechanism for the onset of Wernicke's encephalopathy. *Drug Metab Dispos* 2014; **42**: 1656–1662.
26 Nakaya Y, Shide K, Naito H, Niwa T, Horio T, Miyake J *et al.* Effect of NS-018, a selective JAK2V617F inhibitor, in a murine model of myelofibrosis. *Blood Cancer J* 2014; **4**: e174.
27 Ma L, Clayton JR, Walgren RA, Zhao B, Evans RJ, Smith MC *et al.* Discovery and characterization of LY2784544, a small-molecule tyrosine kinase inhibitor of JAK2V617F. *Blood Cancer J* 2013; **3**: e109.
28 Andraos R, Qian Z, Bonenfant D, Rubert J, Vangrevelinghe E, Scheufler C *et al.* Modulation of activation-loop phosphorylation by JAK inhibitors is binding mode dependent. *Cancer Discov* 2012; **2**: 512–523.
29 Feng J, Witthuhn BA, Matsuda T, Kohlhuber F, Kerr IM, Ihle JN. Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. *Mol Cell Biol* 1997; **17**: 2497–2501.
30 Pratilas CA, Taylor BS, Ye Q, Viale A, Sander C, Solit DB *et al.* (V600E)BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. *Proc Natl Acad Sci USA* 2009; **106**: 4519–4524.
31 Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R *et al.* RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* 2010; **464**: 431–435.
32 Koppikar P, Bhagwat N, Kilpivaara O, Manshouri T, Adli M, Hricik T *et al.* Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. *Nature* 2012; **489**: 155–159.
33 Meyer SC, Keller MD, Chiu S, Koppikar P, Guryanova OA, Rapaport F *et al.* CHZ868, a type II JAK2 inhibitor, reverses type I JAK inhibitor persistence and demonstrates efficacy in myeloproliferative neoplasms. *Cancer Cell* 2015; **28**: 15–28.
34 Marty C, Saint-Martin C, Pecquet C, Grosjean S, Saliba J, Mouton C *et al.* Germline JAK2 mutations in the kinase domain are responsible for hereditary thrombocytosis and are resistant to JAK2 and HSP90 inhibitors. *Blood* 2014; **123**: 1372–1383.
35 Liu Y, Gray NS. Rational design of inhibitors that bind to inactive kinase conformations. *Nat Chem Biol* 2006; **2**: 358–364.
36 Nagar B, Bornmann WG, Pellicena P, Schindler T, Veach DR, Miller WT *et al.* Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res* 2002; **62**: 4236–4243.
37 Wu SC, Li LS, Kopp N, Montero J, Chapuy B, Yoda A *et al.* Activity of the type II JAK2 inhibitor CHZ868 in B cell acute lymphoblastic leukemia. *Cancer Cell* 2015; **28**: 29–41.
38 Manley PW, Bruggen J, Floersheimer A, Furet P, Jensen MR, Mestan J *et al.* Chimia 2008; 62.

39 Ma DL, Chan DS, Wei G, Zhong HJ, Yang H, Leung LT *et al.* Virtual screening and optimization of type II inhibitors of JAK2 from a natural product library. *Chem Commun (Camb)* 2014; **50**: 13885–13888.
40 Losdyck E, Hornakova T, Springuel L, Degryse S, Gielen O, Cools J *et al.* Distinct acute lymphoblastic leukemia (ALL)-associated Janus kinase 3 (JAK3) mutants exhibit different cytokine-receptor requirements and JAK inhibitor specificities. *J Biol Chem* 2015; **290**: 29022–29034.
41 Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* 2009; **9**: 28–39.
42 Cowan-Jacob SW, Mobitz H, Fabbro D. Structural biology contributions to tyrosine kinase drug discovery. *Curr Opin Cell Biol* 2009; **21**: 280–287.
43 Cowan-Jacob SW, Jahnke W, Knapp S. Novel approaches for targeting kinases: allosteric inhibition, allosteric activation and pseudokinases. *Future Med Chem* 2014; **6**: 541–561.
44 Adrian FJ, Ding Q, Sim T, Velentza A, Sloan C, Liu Y *et al.* Allosteric inhibitors of Bcr-abl-dependent cell proliferation. *Nat Chem Biol* 2006; **2**: 95–102.
45 Jatiani SS, Cosenza SC, Reddy MV, Ha JH, Baker SJ, Samanta AK *et al.* A Non-ATP-competitive dual inhibitor of JAK2 and BCR-ABL kinases: elucidation of a novel therapeutic spectrum based on substrate competitive inhibition. *Genes Cancer* 2010; **1**: 331–345.
46 Lipka DB, Hoffmann LS, Heidel F, Markova B, Blum MC, Breitenbuecher F *et al.* LS104, a non-ATP-competitive small-molecule inhibitor of JAK2, is potently inducing apoptosis in JAK2V617F-positive cells. *Mol Cancer Ther* 2008; **7**: 1176–1184.
47 Samanta AK, Chakraborty SN, Wang Y, Schlette E, Reddy EP, Arlinghaus RB. Destabilization of Bcr-Abl/Jak2 network by a Jak2/Abl kinase inhibitor ON044580 overcomes drug resistance in blast crisis chronic myelogenous leukemia (CML). *Genes Cancer* 2010; **1**: 346–359.
48 Dusa A, Mouton C, Pecquet C, Herman M, Constantinescu SN. JAK2 V617F constitutive activation requires JH2 residue F595: a pseudokinase domain target for specific inhibitors. *PLoS One* 2010; **5**: e11157.
49 Toms AV, Deshpande A, McNally R, Jeong Y, Rogers JM, Kim CU *et al.* Structure of a pseudokinase-domain switch that controls oncogenic activation of Jak kinases. *Nat Struct Mol Biol*. 2013; **20**: 1221–1223.
50 Hammaren HM, Ungureanu D, Grisouard J, Skoda RC, Hubbard SR, Silvennoinen O. ATP binding to the pseudokinase domain of JAK2 is critical for pathogenic activation. *Proc Natl Acad Sci USA* 2015; **112**: 4642–4647.
51 Leroy E, Dusa A, Colau D, Motamedi A, Cahu X, Mouton C *et al.* Uncoupling JAK2 V617F activation from cytokine-induced signalling by modulation of JH2 alphaC helix. *Biochem J* 2016; **473**: 1579–1591.
52 Saharinen P, Silvennoinen O. The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction. *J Biol Chem* 2002; **277**: 47954–47963.
53 Yeh TC, Dondi E, Uze G, Pellegrini S. A dual role for the kinase-like domain of the tyrosine kinase Tyk2 in interferon-alpha signaling. *Proc Natl Acad Sci USA*. 2000; **97**: 8991–8996.
54 Chen M, Cheng A, Candotti F, Zhou YJ, Hymel A, Fasth A *et al.* Complex effects of naturally occurring mutations in the JAK3 pseudokinase domain: evidence for interactions between the kinase and pseudokinase domains. *Mol Cell Biol* 2000; **20**: 947–956.
55 Shan Y, Gnanasambandan K, Ungureanu D, Kim ET, Hammaren H, Yamashita K *et al.* Molecular basis for pseudokinase-dependent autoinhibition of JAK2 tyrosine kinase. *Nat Struct Mol Biol* 2014; **21**: 579–584.
56 Lupardus PJ, Ultsch M, Wallweber H, Bir Kohli P, Johnson AR, Eigenbrot C. Structure of the pseudokinase-kinase domains from protein kinase TYK2 reveals a mechanism for Janus kinase (JAK) autoinhibition. *Proc Natl Acad Sci USA* 2014; **111**: 8025–8030.
57 Bandaranayake RM, Ungureanu D, Shan Y, Shaw DE, Silvennoinen O, Hubbard SR. Crystal structures of the JAK2 pseudokinase domain and the pathogenic mutant V617F. *Nat Struct Mol Biol* 2012; **19**: 754–759.
58 Ungureanu D, Wu J, Pekkala T, Niranjan Y, Young C, Jensen ON *et al.* The pseudokinase domain of JAK2 is a dual-specificity protein kinase that negatively regulates cytokine signaling. *Nat Struct Mol Biol* 2011; **18**: 971–976.
59 Hasan S, Lacout C, Marty C, Cuingnet M, Solary E, Vainchenker W *et al.* JAK2V617F expression in mice amplifies early hematopoietic cells and gives them a competitive advantage that is hampered by IFNalpha. *Blood* 2013; **122**: 1464–1477.
60 Bhagwat N, Koppikar P, Keller M, Marubayashi S, Shank K, Rampal R *et al.* Improved targeting of JAK2 leads to increased therapeutic efficacy in myeloproliferative neoplasms. *Blood* 2014; **123**: 2075–2083.
61 Tokarski JS, Zupa-Fernandez A, Tredup JA, Pike K, Chang C, Xie D *et al.* Tyrosine kinase 2-mediated signal transduction in T lymphocytes is blocked by pharmacological stabilization of its pseudokinase domain. *J Biol Chem* 2015; **290**: 11061–11074.
62 Davis MI, Hunt JP, Herrgard S, Ciceri P, Wodicka LM, Pallares G *et al.* Comprehensive analysis of kinase inhibitor selectivity. *Nat Biotechnol* 2011; **29**: 1046–1051.
63 Min X, Ungureanu D, Maxwell S, Hammaren H, Thibault S, Hillert EK *et al.* Structural and functional characterization of the JH2 pseudokinase domain of JAK family tyrosine kinase 2 (TYK2). *J Biol Chem* 2015; **290**: 27261–27270.
64 Dusa A, Staerk J, Elliott J, Pecquet C, Poirel HA, Johnston JA *et al.* Substitution of pseudokinase domain residue Val-617 by large non-polar amino acids causes activation of JAK2. *J Biol Chem* 2008; **283**: 12941–12948.
65 Zhao L, Dong H, Zhang CC, Kinch L, Osawa M, Iacovino M *et al.* A JAK2 interdomain linker relays Epo receptor engagement signals to kinase activation. *J Biol Chem* 2009; **284**: 26988–26998.
66 Liu Q, Sabnis Y, Zhao Z, Zhang T, Buhrlage SJ, Jones LH *et al.* Developing irreversible inhibitors of the protein kinase cysteinome. *Chem Biol* 2013; **20**: 146–159.
67 Leproult E, Barluenga S, Moras D, Wurtz JM, Winssinger N. Cysteine mapping in conformationally distinct kinase nucleotide binding sites: application to the design of selective covalent inhibitors. *J Med Chem* 2011; **54**: 1347–1355.
68 Tan L, Akahane K, McNally R, Reyskens KM, Ficarro SB, Liu S *et al.* Development of selective covalent janus kinase 3 inhibitors. *J Med Chem* 2015; **58**: 6589–6606.
69 Goedken ER, Argiriadi MA, Banach DL, Fiamengo BA, Foley SE, Frank KE *et al.* Tricyclic covalent inhibitors selectively target Jak3 through an active site thiol. *J Biol Chem* 2015; **290**: 4573–4589.
70 Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK *et al.* The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc Natl Acad Sci USA* 2008; **105**: 2070–2075.
71 Zhang J, Adrian FJ, Jahnke W, Cowan-Jacob SW, Li AG, Iacob RE *et al.* Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. *Nature* 2010; **463**: 501–506.
72 Lu X, Levine R, Tong W, Wernig G, Pikman Y, Zarnegar S *et al.* Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation. *Proc Natl Acad Sci USA* 2005; **102**: 18962–18967.
73 Lu X, Huang LJ, Lodish HF. Dimerization by a cytokine receptor is necessary for constitutive activation of JAK2V617F. *J Biol Chem* 2008; **283**: 5258–5266.
74 Staerk J, Defour JP, Pecquet C, Leroy E, Antoine-Poirel H, Brett I *et al.* Orientation-specific signalling by thrombopoietin receptor dimers. *EMBO J* 2011; **30**: 4398–4413.
75 Matthews EE, Thevenin D, Rogers JM, Gotow L, Lira PD, Reiter LA *et al.* Thrombopoietin receptor activation: transmembrane helix dimerization, rotation, and allosteric modulation. *FASEB J* 2011; **25**: 2234–2244.
76 Seubert N, Royer Y, Staerk J, Kubatzky KF, Moucadel V, Krishnakumar S *et al.* Active and inactive orientations of the transmembrane and cytosolic domains of the erythropoietin receptor dimer. *Mol Cell* 2003; **12**: 1239–1250.
77 Moraga I, Wernig G, Wilmes S, Gryshkova V, Richter CP, Hong WJ *et al.* Tuning cytokine receptor signaling by re-orienting dimer geometry with surrogate ligands. *Cell* 2015; **160**: 1196–1208.
78 Ferrao R, Wallweber HJ, Ho H, Tam C, Franke Y, Quinn J *et al.* The structural basis for class II cytokine receptor recognition by JAK1. *Structure* 2016; **24**: 897–905.
79 Wallweber HJ, Tam C, Franke Y, Starovasnik MA, Lupardus PJ. Structural basis of recognition of interferon-alpha receptor by tyrosine kinase 2. *Nat Struct Mol Biol* 2014; **21**: 443–448.
80 Frank SJ, Yi W, Zhao Y, Goldsmith JF, Gilliland G, Jiang J *et al.* Regions of the JAK2 tyrosine kinase required for coupling to the growth hormone receptor. *J Biol Chem* 1995; **270**: 14776–14785.
81 Haan C, Is'harc H, Hermanns HM, Schmitz-Van De Leur H, Kerr IM, Heinrich PC *et al.* Mapping of a region within the N terminus of Jak1 involved in cytokine receptor interaction. *J Biol Chem* 2001; **276**: 37451–37458.
82 Kohlhuber F, Rogers NC, Watling D, Feng J, Guschin D, Briscoe J *et al.* A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses. *Mol Cell Biol* 1997; **17**: 695–706.
83 Zhao Y, Wagner F, Frank SJ, Kraft AS. The amino-terminal portion of the JAK2 protein kinase is necessary for binding and phosphorylation of the granulocyte-macrophage colony-stimulating factor receptor beta c chain. *J Biol Chem* 1995; **270**: 13814–13818.
84 Wernig G, Gonneville JR, Crowley BJ, Rodrigues MS, Reddy MM, Hudon HE *et al.* The Jak2V617F oncogene associated with myeloproliferative diseases requires a functional FERM domain for transformation and for expression of the Myc and Pim proto-oncogenes. *Blood* 2008; **111**: 3751–3759.
85 Zhao L, Ma Y, Seemann J, Huang LJ. A regulating role of the JAK2 FERM domain in hyperactivation of JAK2(V617F). *Biochem J* 2010; **426**: 91–98.
86 McNally R, Toms AV, Eck MJ. Crystal structure of the FERM-SH2 module of human Jak2. *PLoS One* 2016; **11**: e0156218.
87 Cacalano NA, Migone TS, Bazan F, Hanson EP, Chen M, Candotti F *et al.* Autosomal SCID caused by a point mutation in the N-terminus of Jak3: mapping of the Jak3-receptor interaction domain. *EMBO J* 1999; **18**: 1549–1558.

88 O'Sullivan JM, McLornan DP, Harrison CN. Safety considerations when treating myelofibrosis. *Expert Opin Drug Safety* 2016; **15**: 1185–1192.
89 Genovese MC, Kremer J, Zamani O, Ludivico C, Krogulec M, Xie L *et al*. Baricitinib in patients with refractory rheumatoid arthritis. *N Engl J Med* 2016; **374**: 1243–1252.
90 Plimack ER, Lorusso PM, McCoon P, Tang W, Krebs AD, Curt G *et al.* AZD1480: a phase I study of a novel JAK2 inhibitor in solid tumors. *Oncologist* 2013; **18**: 819–820.
91 Seavey MM, Lu LD, Stump KL, Wallace NH, Hockeimer W, O'Kane TM *et al.* Therapeutic efficacy of CEP-33779, a novel selective JAK2 inhibitor, in a mouse model of colitis-induced colorectal cancer. *Mol Cancer Ther* 2012; **11**: 984–993.
92 Baffert F, Regnier CH, De Pover A, Pissot-Soldermann C, Tavares GA, Blasco F *et al.* Potent and selective inhibition of polycythemia by the quinoxaline JAK2 inhibitor NVP-BSK805. *Mol Cancer Ther* 2010; **9**: 1945–1955.
93 Duan Y, Chen L, Chen Y, Fan XG. c-Src binds to the cancer drug Ruxolitinib with an active conformation. *PLoS One* 2014; **9**: e106225.
94 Syed RS, Reid SW, Li C, Cheetham JC, Aoki KH, Liu B *et al.* Efficiency of signalling through cytokine receptors depends critically on receptor orientation. *Nature* 1998; **395**: 511–516.
95 Li Q, Wong YL, Yueqi Lee M, Li Y, Kang C. Solution structure of the transmembrane domain of the mouse erythropoietin receptor in detergent micelles. *Sci Rep* 2015; **5**: 13586.
96 Abdelrahman RA, Begna KH, Al-Kali A, Hogan WJ, Litzow MR, Pardanani A *et al.* Momelotinib treatment-emergent neuropathy: prevalence, risk factors and outcome in 100 patients with myelofibrosis. *Br J Haematol* 2015; **169**: 77–80.
97 Purandare AV, McDevitt TM, Wan H, You D, Penhallow B, Han X *et al.* Characterization of BMS-911543, a functionally selective small-molecule inhibitor of JAK2. *Leukemia* 2012; **26**: 280–288.
98 Hexner EO, Mascarenhas J, Prchal J, Roboz GJ, Baer MR, Ritchie EK *et al.* Phase I dose escalation study of lestaurtinib in patients with myelofibrosis. *Leuk Lymphoma* 2015; **56**: 2543–2551.
99 Verstovsek S, Hoffman R, Mascarenhas J, Soria JC, Bahleda R, McCoon P *et al.* A phase I, open-label, multi-center study of the JAK2 inhibitor AZD1480 in patients with myelofibrosis. *Leuk Res* 2015; **39**: 157–163.
100 Komrokji RS, Seymour JF, Roberts AW, Wadleigh M, To LB, Scherber R *et al.* Results of a phase 2 study of pacritinib (SB1518), a JAK2/JAK2(V617F) inhibitor, in patients with myelofibrosis. *Blood* 2015; **125**: 2649–2655.
101 Verstovsek S, Tam CS, Wadleigh M, Sokol L, Smith CC, Bui LA *et al.* Phase I evaluation of XL019, an oral, potent, and selective JAK2 inhibitor. *Leuk Res* 2014; **38**: 316–322.
102 Ringel F, Kaeda J, Schwarz M, Oberender C, Grille P, Dorken B *et al.* Effects of Jak2 type 1 inhibitors NVP-BSK805 and NVP-BVB808 on Jak2 mutation-positive and Bcr-Abl-positive cell lines. *Acta Haematol* 2014; **132**: 75–86.
103 Geissler K, Jager E, Barna A, Sliwa T, Knobl P, Schwarzinger I *et al. In vitro* and *in vivo* effects of JAK2 inhibition in chronic myelomonocytic leukemia. *Eur J Haematol* 2016; **97**: 562–567.
104 Miyamoto N, Sugita K, Goi K, Inukai T, Lijima K, Tezuka T *et al.* The JAK2 inhibitor AG490 predominantly abrogates the growth of human B-precursor leukemic cells with 11q23 translocation or Philadelphia chromosome. *Leukemia* 2001; **15**: 1758–1768.

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)