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Invited review

Janus kinase (JAK) inhibitors in the treatment of inflammatory and neoplastic diseases

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ABSTRACT

The Janus kinase (JAK) family of non-receptor protein-tyrosine kinases consists of JAK1, JAK2, JAK3, and TYK2 (tyrosine kinase-2). Each of these proteins contains a JAK homology pseudokinase (JH2) domain that regulates the adjacent protein kinase domain (JH1). JAK1/2 and TYK2 are ubiquitously expressed whereas JAK3 is found predominantly in hematopoietic cells. The Janus kinase family is regulated by numerous cytokines including interleukins, interferons, and hormones such as erythropoietin, thrombopoietin, and growth hormone. Ligand binding to cytokine and hormone receptors leads to the activation of associated Janus kinases, which then mediate the phosphorylation of the receptors. The SH2 domain of STATs (signal transducers and activators of transcription) binds to the receptor phosphotyrosines thereby promoting STAT phosphorylation by the Janus kinases and consequent activation. STAT dimers are translocated to the nucleus where they participate in the regulation of the expression of thousands of proteins. JAK-STAT dysregulation results in autoimmune disorders such as rheumatoid arthritis, ulcerative colitis, and Crohn disease. JAK-STAT dysregulation also plays a role in the pathogenesis of myelofibrosis, polycythemia vera, and other myeloproliferative illnesses. An activating JAK2 V617F mutation occurs in 95% of people with polycythemia vera and in a lower percentage of people with other neoplasms. JAK1/3 signaling participates in the pathogenesis of inflammatory afflictions while JAK1/2 signaling participates in the development of several malignancies including leukemias and lymphomas as well as myeloproliferative neoplasms. Tofacitinib is a pan-JAK inhibitor that is approved by the FDA for the treatment of rheumatoid arthritis and ruxolitinib is a JAK1/2 inhibitor that is approved for the treatment of polycythemia vera and myelofibrosis.

Contents

Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; AS, activation segment; CS or C-spine, catalytic spine; CL, catalytic loop; HΦ or Φ, hydrophobic; IFN, interferon; IL, interleukin; JAK1/2, Janus kinases 1 and 2; MW, molecular weight; NSCLC, non-small cell lung cancer; PKA, protein kinase A; RA, rheumatoid arthritis; RS or R-spine, regulatory spine; Sh1, shell residue 1; TNF, tumor necrosis factor.

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1. Introduction to JAK-STAT signaling

The Janus kinase (JAK) family of protein-tyrosine kinases is made up of four members: JAK1, JAK2, JAK3, and TYK2 (Tyrosine kinase 2) [1]. The Janus kinases share seven distinct JAK homology (JH1-JH7) domains. These proteins possess an inactive pseudokinase domain (JH2) adjacent to an active carboxyterminal protein kinase domain (JH1). The pseudokinase domain ordinarily inhibits the functional protein kinase domain. Janus is a two-faced (looking forwards and backwards) Roman God whose name was applied to this enzyme family owing to the presence of the two protein kinase domains within a single polypeptide chain. JAK was earlier conceived as Just Another Kinase [2]. JAK1/2 and TYK2 are ubiquitously expressed whereas JAK3 is confined to hematopoietic, myeloid, and lymphoid cells [3]. Mature blood cells have a limited life span and are thus continuously renewed in an intricate multi-step process. The Janus kinases play an important role in normal hematopoiesis; accordingly, Janus kinase dysregulation can result in a variety of hematological illnesses. These enzymes also function in a wide variety of processes including post-natal growth, metabolism, and satiety.

Manning et al. identified 478 classical and 40 non-classical or atypical human protein kinase family genes (total 518) that correspond to nearly 2% of the human genome [4]. Based upon the identity of the phosphorylated —OH group, these enzymes are catalogued as protein-serine/threonine kinases (385 members), protein-tyrosine kinases (90), or tyrosine-kinase like proteins (43). A small group of proteins including MEK1 and MEK2, which catalyze the phosphorylation of both threonine and tyrosine residues on target proteins, are catalogued as dual specificity kinases. Additionally, there are 106 protein kinases existing as pseudogenes within the human genome. Of the 90 protein-tyrosine kinases, 58 are transmembrane receptors and 32 are cytosolic non-receptor kinases, including the Janus kinase family. This enzyme family catalyzes the following reaction:

$$\mathrm{MgATP^{1-} + protein\text{-}tyrosine - O : H \rightarrow protein\text{-}tyrosine - O : PO_3{}^{2-} + MgADP + H^+}$$

Note that it is not the phosphate ($OPO_3{}^{2-}$) group, but rather it is the phosphoryl group ($PO_3{}^{2-}$) that is transferred from ATP to a tyrosine residue within the protein substrate. Divalent cations such as Mg^{2+} or Mn^{2+} are required for the reaction, although Mg^{2+} is the physiological ion owing to its significantly greater intracellular content.

The JAK-STAT (signal transducer and activator of transcription) pathway transduces extracellular signals from a variety of cytokines, growth factors, and hormones to the nucleus and is responsible for the expression of thousands of protein-encoding genes [5]. Each of the Janus kinase proteins binds to the juxtamembrane region of specific cytokine receptors. The genes specifying each high-affinity cytokine receptor homo- or hetero-dimer are listed in Table 1. Receptors for Type I cytokines share a common amino acid motif (WSxWS) in the extracellular portion adjacent to the cell membrane. Receptors for Type II cytokines are similar to those for type I cytokines except that these receptors lack the WSxWS signature. The cytokine receptors consist of an extracellular domain, a transmembrane segment, and an intracellular domain that lacks catalytic activity, but which binds to specific Janus kinases.

Several steps are involved in the conversion of an extracellular signal into a transcriptional response. First, ligand binding mediates structural changes in the cytokine receptors that result in protein-tyrosine kinase activation following phosphorylation of two tyrosine residues within the activation segment of the JH1 domains as catalyzed by a partner Janus kinase enzyme. This intermolecular reaction is referred to as phosphorylation in *trans*; an intramolecular reaction is referred to as phosphorylation in *cis*. Following activation, the JH1 domain catalyzes the phosphorylation of tyrosine residues within the cytokine receptor that attracts the SH2 domain of STATs. The JH1 domain then catalyzes the phosphorylation of the STAT molecules themselves. Phosphorylated STATS form dimers that are translocated into the nucleus where they mediate the transcription of target genes (Fig. 1). Alternatively, STATs may preexist as dimers and phosphorylation may produce a conformational change resulting in activation [7].

Table 1
Cytokine and growth factor stimulation of JAK-STAT signaling[a].

Cytokine/hormone[b]	Genes of human receptor subunits	Downstream JAKs	Downstream STATs	Selected functions
Type I, common γ-chain (gene *IL2RG*) cytokines				
IL-2	*IL2RA/B-IL2RG*	JAK1/3	STAT3/5	Regulates T cell, B cell, and NK cell activities
IL-4	*IL4RA-IL2RG*	JAK1/3	STAT6	Induces differentiation of helper T cells; anti-inflammatory action on T cells and monocytes
IL-7	*IL7RA-IL2RG*	JAK1/3	STAT3/5	T cell development and homeostasis
IL-9	*IL9R-IL2RG*	JAK1/3	STAT1/3/5	T cell growth and differentiation
IL-15	*IL15RA-IL2RG*	JAK1/3	STAT3/5	Promotes T cell activation and proliferation
IL-21	*IL21R-IL2RG*	JAK1/3	STAT1/3/5	Down regulates NK cell activation
Type I/common β-chain (gene *CSF2RB*) cytokines				
GM-CSF	*CSF2RA-CSF2RB*	JAK2	STAT3/5	Growth of macrophages and granulocytes; stimulation and differentiation of stem cells; used to reverse neutropenia after chemotherapy
IL-3	*IL3RA/B-CSF2RB*	JAK2	STAT3/5/6	Differentiation of stem cells; proliferation of all cells in the myeloid lineage
IL-5	*IL5R-CSF2RB*	JAK2	STAT3/5/6	Stimulates B cell growth and immunoglobulin secretion
Type I, gp130 (gene *IL6RB* = *IL6ST*) cytokines				
IL-6	*IL6RA/B-IL6RB*	JAK1/2, TYK2	STAT1/3	Prototypic pro-inflammatory cytokine increases acute-phase protein production
IL-11	*IL11RA-IL6RB*	JAK1/2, TYK2	STAT3	Induces megakaryocyte colony formation and maturation
IL-27	*IL27RA-IL6RB*	JAK1/2, TYK2	STAT1/2/3/4/5	Regulation of B and T cell activity
Type I, heterodimeric cytokines				
IL-12 (35 kD/40 kD)	*IL12RB1-IL12RB2*	JAK1, TYK2	STAT4	Induces T_H1 T helper cell formation
IL-23	*IL23R-IL12RB1*	JAK1, TYK2	STAT3/4	Pro-inflammatory cytokine
Type I, hormone-like cytokines				
Erythropoietin	*EPOR-EPOR*	JAK2	STAT5	Control of red blood cell production; used to treat anemia
Thrombopoietin	*TPOR-TPOR*	JAK2	STAT1/3/5	Differentiation of megakaryocytes and platelets
G-CSF	*CSF3R-CSF3R*	JAK2	STAT5	Production of stem cells and granulocytes; used to treat neutropenia
Growth hormone	*GHR-GHR*	JAK2	STAT3/5a	Regulates post-natal body growth
Leptin	*LEPR-LEPR*	JAK2	STAT3/5a	Coordination of energy metabolism; increases satiety
Type II, IFN family cytokines				
IFN-α/β	*IFNAR1-IFNAR2*	JAK1, TYK2	STAT1/2/4	Promotes antiviral activity; stimulates T cell, macrophage, and NK cell activity; used to treat multiple sclerosis
IFN-γ	*IFNGR1-IFNGR2*	JAK1, TYK2	STAT1	Regulates macrophage and NK cell activation; used to treat chronic granulomatous disease and osteopetrosis
IL-28	*IFNLR1-IL10RB*	JAK1, TYK2	STAT1/2/3/4/5	Enhances immunity against infection
IL-29	*IFNLR1-IL10RB*	JAK1, TYK2	STAT1/2/3/4/5	Enhances immunity against infection
Type II, IL-10 family cytokines				
IL-10	*IL10RA-IL10RB*	JAK2, TYK2	STAT3	Anti-inflammatory actions
IL-19	*IL20RA-IL20RB*	JAK1/2, TYK2	STAT3	B-cell activation
IL-20	*IL20RA-IL20RB*	JAK1/2, TYK2	STAT3	Regulates differentiation and proliferation of keratinocytes during inflammation
IL-22	*IL22RA1-IL10RB*	JAK1/2, TYK2	STAT1/3/5	Targets non-hematopoietic cells such as hepatocytes, keratinocytes, lung, and intestinal epithelial cells
IL-24	*IL20RA-IL20RB*	JAK1/2, TYK2	STAT1/3	Targets skin, lung, and reproductive cells
IL-26	*IL10RB-IL20RA*	JAK1/2, TYK2	STAT1/3	Enhances IL-10 secretion from monocytes and IL-8 secretion from macrophages
Growth factors				
EGF	*EGFR-EGFR*	JAK1	STAT1//3/5a	Cell proliferation
PDGF	*PDGFRA/B-PDGFRA/B*	JAK1/2	STAT1/2/3/5/6	Stem cell proliferation

[a] Adapted from Refs. [5,6].
[b] GM-CSF, granulocyte-macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor.

Note that the Type I IL-2 or IL-4 receptors along with the common γ-chain bind to JAK1 and JAK3 while the Type I receptors with the common β-chain bind to JAK2 (Table 1). The Type I receptors using gp130 (glycoprotein 130) and those that are activated by heterodimeric cytokines preferentially bind to JAK1/2 and TYK2. The Type I receptors that are activated by the hormone-like cytokines bind to and activate JAK2. The Type II receptors for interferons bind to JAK1 and TYK2 whereas those for the IL-10 family of cytokines bind to JAK1/2 and TYK2. Accordingly, specific Janus kinases bind to the appropriate receptor subunits thereby imparting signaling

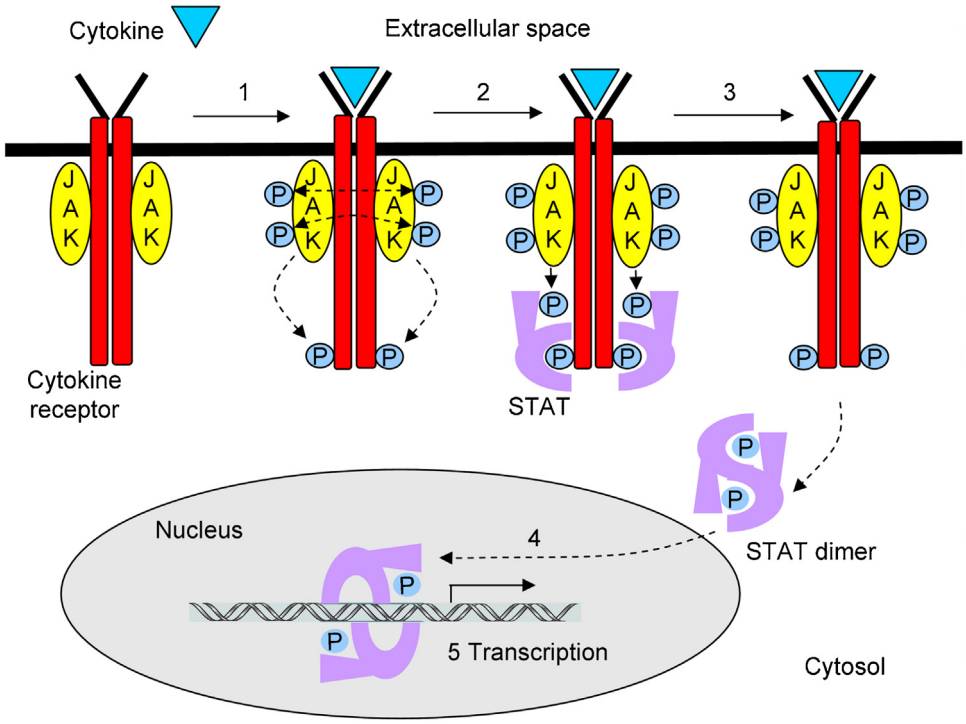

Fig. 1. The JAK-STAT signal transduction pathway.

specificity. The IL-10 receptor is a tetramer consisting of two *IL10RA* and two *IL10RB* encoded subunits while most of the other receptors consist of two subunits as listed in Table 1. Janus kinase activation by EGFR and PDGF is downstream from the activated receptor and may involve other protein kinases such as Src [8].

There are seven STAT genes in humans: *STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B,* and *STAT6* (Table 2). Each STAT protein contains six domains that are illustrated in Fig. 2A including a DNA binding domain, an SH2 domain, and a transcriptional activation domain (TAD), first described for STAT1 [9]. The transcriptional activation domain contains a tyrosine residue that is phosphorylated in a reaction catalyzed by an upstream Janus kinase. Following phosphorylation, the STAT forms a homo- or heterodimer with another phosphorylated STAT that is mediated by the binding of a phosphotyrosine (pY) to its partner's SH2 domain (Fig. 2B). The dimer travels to the nucleus where it binds to target DNA sequences alone or in combination with other transcription factors that either enhance or repress DNA transcription.

STAT1/2/4/6 have restricted roles in signaling. STAT1 participates in interferon, interleukin-9 (IL-9), thrombopoietin, and IL-10 family signaling while STAT2 is chiefly involved in interferon signaling (Table 1). STAT4 plays a role in signaling by the Type I heterodimeric cytokines (IL-12/23) and Type II interferon family cytokines (IFN-α/β, IL-28/29). STAT6 participates in IL-3/4/5 and PDGF signaling. STAT3 and STAT5a/b have broader functions. STAT3 participates in the signaling initiated by many of the Type I, II, and IL-10 families of cytokines while STAT5a/b participate in signaling initiated by Type I cytokines with the common γ-chain or common β-chain receptor subunits and the hormone-like cytokines. The information provided in Table 1 is qualitative in nature and the precise pathway from ligand and receptor to a specific Janus kinase and a specific STAT depends upon the cellular and physiological context. Moreover, more than two dozen cytokines including IL-8, transforming growth factor-β, and RANTES interact with receptors that do not signal directly through the JAK-STAT pathway [6].

The activity of all protein kinases is stringently regulated owing to their overall importance in multiple signaling pathways [10]. Various intracellular phosphoprotein phosphatases including SHP1, SHP2, PTP1B, and TCPTP mediate the dephosphorylation and inactivation of Janus kinases [7]. CD45 is a receptor phosphoprotein phosphatase that is able to catalyze the dephosphorylation of the activation segment phosphotyrosines of all four Janus kinases [11]. The SOCS (suppressor of cytokine signaling) family of eight proteins also negatively regulates cytokine and Janus kinase signaling [7]. The SOCS proteins are ubiquitin ligases that promote the proteasomal degradation of activated Janus kinase-associated cytokine receptors. Moreover, SOCS1 and SOCS3 can bind to the protein-substrate binding grove and sterically inhibit the catalytic activity of JAK1/2 and TYK2, but not JAK3.

2. Janus kinase biochemistry

2.1. Janus kinase family architecture

The Janus kinases contain about 1100 amino acid residues and they contain seven JAK homology modules: JH7-JH1 as we go from the N-terminus to the C-terminus. These have subsequently been reorganized into four functional domains [12]. The JH7-JH6 module corresponds to a FERM domain (F for 4.1 protein, E for ezrin, R for radixin and M for moesin), which is a ubiquitous protein module of ≈350 amino-acids and is involved in targeting proteins to the plasma membrane. The JH5-JH3 module corresponds to an atypical SH2 domain that is unable to bind protein-tyrosine phosphate. The JH3 domain is followed by a JH2 pseudokinase domain and the carboxyterminal JH1 segment represents the catalytically active protein-tyrosine kinase domain (Fig. 2C).

The Janus kinases contain a small amino-terminal lobe and large carboxyterminal lobe that contains several conserved α-helices and β-strands, first described by Knighton et al. for PKA in 1991 [13,14]. The small lobe is dominated by a twisted five-stranded antiparallel β-sheet (β1–β5) [15]. It also contains an important regulatory αC-helix (Fig. 3A and B). The small lobe of JAK1 contains a conserved glycine-rich (GEGHFG) ATP-phosphate-binding loop, which occurs between the β1- and β2-strands. The canonical sequence of

Table 2
Human STAT properties.

Gene	Isoform	Amino acids	MW, kDa	Uniprot ID	pY	Upstream enzyme
STAT1	α	750	87	P42224	701	JAK1, TYK2
STAT1	β	712	83	P42224	701	JAK1, TYK2
STAT2	I	851	98	P52630	690	JAK1/2, TYK2
STAT2	II	847	97	P52630	686	JAK1/2, TYK2
STAT3	I	770	88	P40763	705	JAK1/2, TYK2
STAT3	II	769	88	P40763	704	JAK1/2, TYK2
STAT3	III	722	83	P40763	705	JAK1/2, TYK2
STAT4	I	748	86	Q14765	693	JAK1, TYK2
STAT5	a	794	91	P42229	694	JAK1/2, TYK2
STAT5	b	787	90	P51692	682	JAK1/2, TYK2
STAT6	I	847	94	P42226	641	JAK1/2/3
STAT6	II	673	74	P42226	467	JAK1/2/3
STAT6	III	737	82	P42226	531	JAK1/2/3

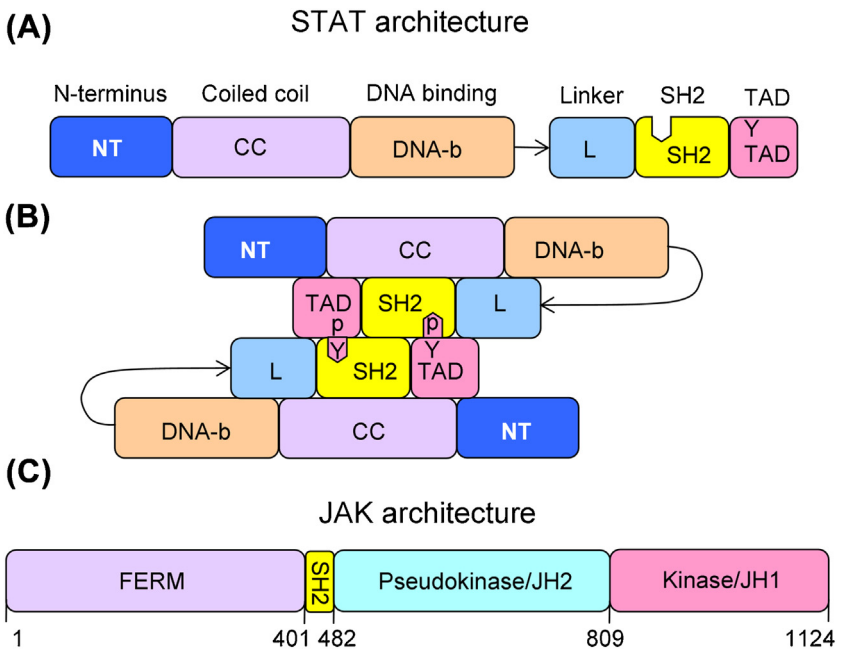

Fig. 2. Domain architecture of the STAT and JAK families. (A) STAT domains. TAD, transcriptional activation domain. (B) Interaction of the STAT domains involved in dimer formation. (C) JAK architecture. The amino acid residue numbers correspond to human JAK3. pY, phosphotyrosine. A and B were adapted with permission from Ref. [8].

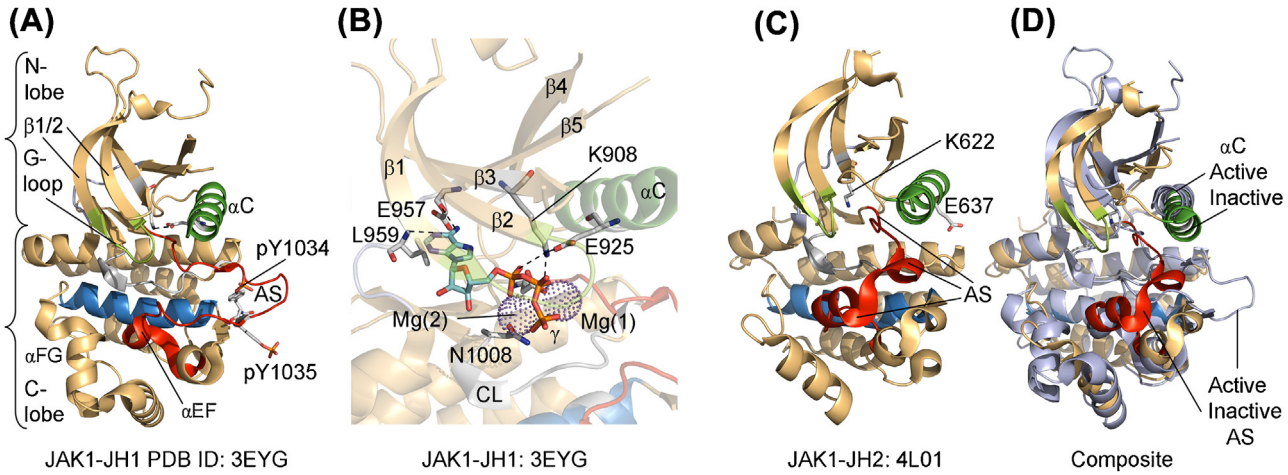

Fig. 3. (A) Structure of the active JAK1 JH1 protein kinase domain. (B) Model of ATP binding to JAK1. (C) Structure of the inactive JAK1 JH2 pseudokinase domain. (D) Superposition of active and inactive protein kinase domains illustrating the differences in position of the αC helix and the activation segment (AS). Figs. 3–6, 9, and 10 were prepared using the PyMOL Molecular Graphics System Version 1.5.0.4 Schrödinger, LLC.

this loop in protein kinases is GxGxΦG, where x refers to any amino acid and Φ is a hydrophobic residue, usually phenylalanine or tyrosine. The β1 and β2-strands occur above the adenine component of ATP. The β3-strand typically contains an Ala-Xxx-Lys sequence, the lysine of which (JAK1 K908) couples the α- and β-phosphates of ATP to the αC-helix. A conserved glutamate occurs near the center of the αC-helix (JAK1 E925) in protein kinases. The presence of a salt-bridge between the β3-lysine and the αC-glutamate is a prerequisite for the formation of the activate state, which corresponds to an "αC-in" conformation (Fig. 3A). In contrast the Lys622 and Glu637 of the pseudokinase of the JAK1 JH2 domain fail to make contact, which corresponds to an "αC-out" conformation (Fig. 3C). Moreover, the G-loop in the JH2 domain consists of a shortened GRG sequence between the β1- and β2-strands. The αC-in conformation is necessary, but not sufficient, for the expression of full protein kinase activity.

The large lobe of the JAK1 protein kinase domain is mainly α-helical with six conserved segments (αD-αI) that occur in all protein kinases [15]. It also contains four short conserved β-strands (β6-β9). The primary structures of the β-strands occur between those of the αE- and αF-helices and include residues just before the catalytic loop (β6), after the catalytic loop and below the adenine ring of ATP (β7 and β8), and within the activation segment (β9). The activation segment of the JAK1 JH1 active state forms an open structure that allows protein/peptide binding (Fig. 3A). The activation segment in the dormant JH2 domain forms a closed, compact structure that blocks protein/peptide binding (Fig. 3C). The active JAK1 protein kinase domain and the pseudokinase domain contain an additional helix (αEF) near the end of the activation segment and this additional helix occurs in most protein kinases.

In an important analysis, Hanks and Hunter identified 12 subdomains (I–VIa, VIb–XI) with canonical sequences that make up the indispensable catalytic residues of protein kinases [16]. The following four amino acids, which are part of a K/E/D/D core, exemplify the mechanistic properties of JAK1. An invariant β3-strand lysine (K908, the K of K/E/D/D) forms ionic bonds with the αC-glutamate (E925, the E of K/E/D/D) and also the α- and β-phosphate groups of ATP (Fig. 3B). The catalytic loop surrounding the actual site of phosphoryl transfer consists of HRDLAARN in most protein-tyrosine kinases including JAK1/3 and TYK2; the sequence HRDLATRN occurs in JAK2. The JAK1 JH2 pseudokinase domain contains HGNVCTKN. The catalytic aspartate (D1003) in JAK1, which is the first D of K/E/D/D serves as a base that accepts a proton from the tyrosyl —OH group (Fig. 4). This residue in the pseudokinase domains of the Janus kinases is an asparagine, which cannot function as a base thereby contributing to the catalytic dormancy of the pseudokinase domains. The AAR sequence in the catalytic loop represents a receptor protein-tyrosine kinase signature and RAA represents a non-receptor protein-tyrosine kinase; the occurrence of AAR in the JAK non-receptor protein kinase is thus anomalous.

The second aspartate of the K/E/D/D signature of JAK1, D1021, is the first residue of the activation segment. The activation segment of nearly all protein kinases including those of the Janus kinases begins with DFG (Asp-Phe-Gly) and ends with APE (Ala-Pro-Glu). DFG-Asp1021 binds Mg^{2+}(1), which in turn coordinates the α- β- and γ-phosphates of ATP. The HRDxxxxN (N1008) binds Mg^{2+}(2), which in turn coordinates the α- and γ-phosphates of ATP (Fig. 3B). The exocyclic 6-amino nitrogen of ATP characteristically forms a hydrogen bond with the carbonyl backbone residue of the first hinge residue (E957 of JAK1 JH1 domain) that connects the small and large lobes of the protein kinase domain and the *N*1 nitrogen of the adenine base forms a second hydrogen bond with the N—H group of third hinge residue (L595) (Fig. 3B). As discussed later, most small-molecule inhibitors of the Janus kinases that are steady-state ATP competitive inhibitors also make hydrogen bonds with the backbone residues of the connecting hinge. Fig. 3D is the

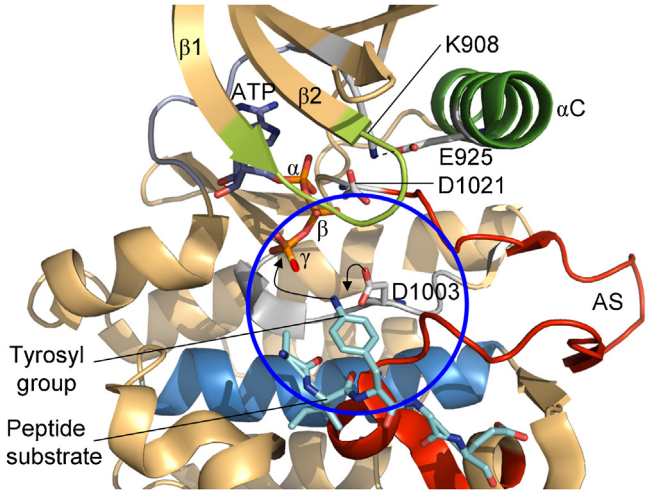

Fig. 4. Mechanism of the JAK1 protein kinase reaction illustrating the role of the catalytic aspartate (D1003). The phenolic oxygen of the tyrosyl residue attacks the γ-phosphate of ATP. JAK1 is derived from PDB ID: 3EYG; ATP and the peptide substrate are from PDB ID: 2GS6 (human EGFR). The carbon atoms of the peptide substrate are sky blue.

superposition of the active JAK1 JH1 domain and the dormant JAK1 JH2 domain, which depicts the relative positions of the αC-helix and the activation segments. The large lobe characteristically binds the peptide/protein substrates near the end of the activation segment as shown in Fig. 4.

The activation segment influences both protein-substrate binding and catalytic efficiency [17]. This segment in Janus kinases contains two phosphorylatable tyrosines (Fig. 3A). The segment is located close in the three-dimensional sense to the amino-terminus of the αC-helix and the conserved HRD portion of the catalytic loop. The interaction of these components is hydrophobic in nature. As is the case for most protein kinases [18], phosphorylation of a residue within the activation segment (tyrosine for the Janus kinases) converts an inactive JAK JH1 domain to an active one [19]. The activation segment of the Janus kinases and other protein kinases is further stabilized by hydrophobic bonds between an amino acid two residues N-terminal to the HRD-H of the catalytic loop and seven residues C-terminal to the DFG-D within the activation segment. Functionally important human Janus kinase JH1 residues are listed in Table 3.

2.2. Active JH1 and inactive JH2 hydrophobic spines

Kornev et al. analyzed the structures of active and inactive conformations of 23 protein kinases and they uncovered the role of important residues by a local spatial pattern alignment algorithm [20,21]. Their analysis led to a classification of eight hydrophobic residues as a catalytic or C-spine and four hydrophobic residues that constitute a regulatory or R-spine. Each of the two spines consists of amino acids occurring in both the small N-terminal lobe and the large C-terminal lobe. The regulatory spine contains a residue from the αC-helix and from the activation segment, both of which are important in determining active and dormant states. The adenine portion of ATP is one component of the catalytic spine. The R-spine positions the protein substrate and the C-spine positions ATP thereby enabling catalysis. Note that the structure of the spines differs between the active JH1 and dormant JH2 domains. Moreover, the correct alignment of both spines is necessary for the assembly of an active protein kinase domain as described for the cyclin-dependent protein kinases, Src, EGFR, ERK1/2, and MEK1/2 [22–27].

Table 3
Important residues in the human Janus kinases[a].

	JAK1	JAK2	JAK3	TYK2	Inferred function	Hanks no.[b]
N-lobe						
Glycine-rich loop: GxGxΦG	^{882}GEGHFG^{887}	^{856}GKGNFG^{861}	^{829}GKGNFG^{834}	^{904}GEGHFG^{909}	Anchors ATP β-phosphate	I
β3-K (K of K/E/D/D)	K908	K882	K855	K930	Forms ion pair with ATP α- and β-phosphates	II
αC-E (E of K/E/D/D)	E925	E898	E871	E947	Forms ion pair with β3-K	III
Hinge residues	^{957}EFLPG^{961}	^{931}YKPYG^{936}	^{903}EYLPSG^{908}	^{979}EYVPLG^{984}	Connects N- and C-lobes	V
C-lobe						
αE-AS loop and AS HΦ-interaction	Y999-A1027	Y972-V1000	C945-L973	Y1019-A1047	Stabilizes AS	VIb-VII
Catalytic loop HRD (first D of K/E/D/D)	D1003	D976	D949	D1023	Catalytic base (abstracts proton)	VIb
Catalytic loop Asn (N)	N1008	N981	N954	N1028	Chelates Mg^{2+}(2)	VIb
Activation segment	1021–1051	994–1024	967–997	1041–1071	Positions protein substrate	VII-VIII
AS DFG (second D of K/E/D/D)	D1021	D994	D967	D1041	Chelates Mg^{2+}(1)	VII
AS phosphorylation site	Y1034/Y1035	Y1007/Y1008	Y980/Y981	Y1054/Y1055	Stabilizes the AS after phosphorylation	VIII
APE, end of AS	1049–1051	1022–1024	995–997	1069–1071	Interacts with the αHI loop and stabilizes the AS	VIII
UniProt KB ID	P23458	O60674	P52333	P29597		

[a] AS, activation segment.
[b] From Ref. [16].

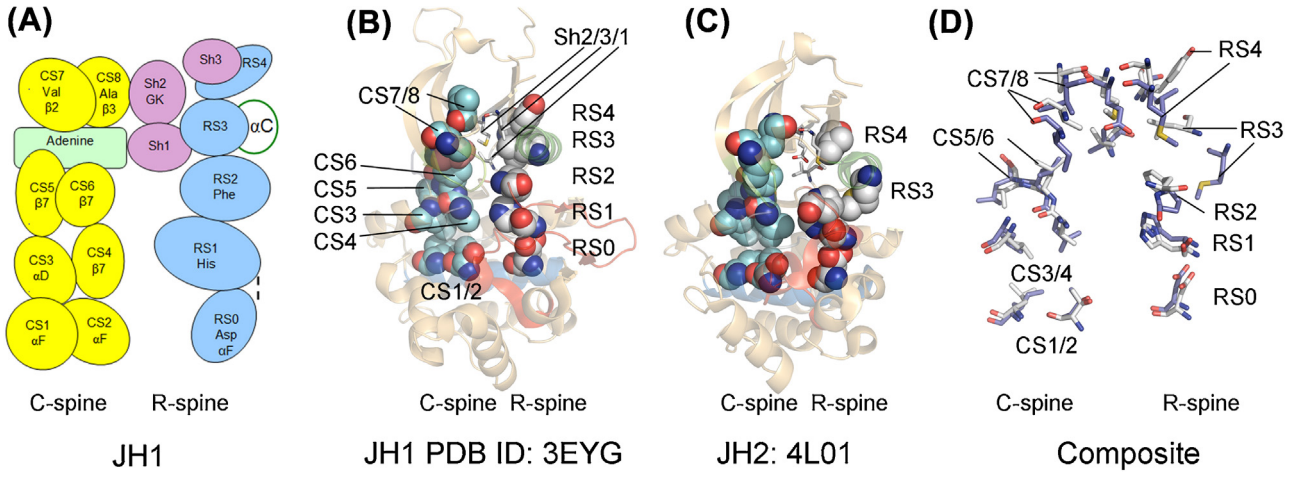

Fig. 5. (A) Frontal view and numbering system of the C- and R-spines. (B) Spines from the active JH1 domain of JAK1. (C) Spines from the inactive JH2 domain of JAK1. (D) Superposition of active and inactive spines. The carbon atoms of the spine residues in an active conformation are gray and those from an inactive conformation are blue.

The canonical protein kinase R-spine consists of the HRD-His of the catalytic loop, the phenylalanine of the activation segment DFG, a residue near the C-terminal end of the αC-helix (four residues carboxyterminal to the conserved αC-glutamate), and a residue at the beginning of the β4-strand. The backbone of the HRD-His is anchored to the very hydrophobic αF-helix by a hydrogen bond to a conserved aspartate residue. Going from the aspartate within the αF-helix up to the top residue of the spine within the β4-strand, Meharena et al. named the R-spine residues as follows: RS0, RS1, RS2, RS3, and RS4 (Fig. 5A and B) [17]. The regulatory spine of the active JH1 protein kinase domain is nearly linear while that of the JH2 pseudokinase domain is broken with RS3 displaced (Fig. 5B and C).

The protein kinase catalytic spines are made up of residues from both the small N-lobes and large C-lobes; the C-spine is completed by the adenine base of ATP (Fig. 5A) [17,21]. The two residues of the small lobe of protein kinase domains that bind to the adenine component of ATP include a valine residue at the beginning of the β2-strand (CS7) and an alanine from the conserved AxK of the β3-strand (CS8). Additionally, a hydrophobic residue from the β7-strand (CS6) juxtaposes with the adenine base. This CS6 residue is flanked by two hydrophobic residues named CS4 and CS5 that bind to the CS3 residue near the beginning of the αD-helix. Finally, CS3 and CS4 interact with the CS1 and CS2 residues of the αF-helix to produce a completed C-spine (Fig. 5A and B) [27]. It is important to notice that the hydrophobic αF-helix anchors both the C-spine and R-spine. Moreover, the spines play an important function in positioning the protein kinase catalytic residues in their active state. When comparing the locations of the spinal residues, the greatest divergence in the structures of the JH1 and JH2 domains involve RS3 and RS4 along with CS7 and CS8 (Fig. 5D).

Using site-directed mutagenesis, Meharena et al. identified three residues in murine PKA that stabilize the R-spine that they labeled Sh1, Sh2, and Sh3, where Sh refers to shell [17]. The Sh2 residue corresponds to the gatekeeper residue. The name gatekeeper signifies the role that this residue plays in controlling access to the back cleft. The back cleft is sometimes called the back pocket or hydrophobic pocket II (HPII). The residues that constitute the spines were identified by their positions in active and inactive enzymes based upon their X-ray crystallographic structures [20,21]. This contrasts with the identification of the DFG, APE, or HRD amino acid signatures based upon their primary structures [16]. Table 4 provides a summary of the spine and shell residues of the JAK JH1 and JH2 domains. Small molecule protein kinase therapeutic antagonists generally interact with residues that make up the C-spine and sometimes the R-spine and shell residues [27].

2.3. Mechanism of Janus kinase activation

Protein kinases are stringently regulated and have not evolved to continuously catalyze the phosphorylation of thousands of molecules per minute like hexokinase, a general metabolic enzyme.

Table 4
Spine and shell residues of murine PKA and human Janus kinase and pseudokinase (Ψ) domains.

	Symbol	PKA[a]	JAK1 Ψ	JAK1	JAK2 Ψ	JAK2	JAK3 Ψ	JAK3	TYK2 Ψ	TYK2
Regulatory spine										
β4-strand (N-lobe)	RS4	L106	L653	Y940	N612	Y913	L587	Y886	V673	Y962
C-helix (N-lobe)	RS3	L95	M641	L929	M600	L902	L575	L875	L661	L951
Activation loop F of DFG (C-lobe)	RS2	F185	P740	F1022	P700	F995	P672	F968	P760	F1042
Catalytic loop His/Tyr (C-lobe)	RS1	Y164	H712	H1001	H671	H974	H645	H947	H732	H1021
F-helix (C-lobe)	RS0	D220	D775	D1063	D735	D1036	D707	D1009	D796	D1083
R-shell										
Two residues upstream from the gatekeeper	Sh3	M118	M665	L954	L624	L927	M598	L900	M685	L976
Gatekeeper, end of β5-strand	Sh2	M120	E667	M956	Q626	M929	Q600	M902	T687	M978
αC-β4 loop	Sh1	V104	V651	V938	V610	V911	V585	V884	A671	I960
Catalytic spine										
β2-strand (N-lobe)	CS8	V57	I597	V889	I559	V863	I535	V863	V603	V911
β3-AxK motif (N-lobe)	CS7	A70	I620	A906	L579	A880	L554	A853	V640	A928
β7-strand (C-lobe)	CS6	L173	L721	L1010	L680	L983	L654	L956	L741	L1030
β7-strand (C-lobe)	CS5	I174	L722	V1011	L681	V984	L655	V057	L742	L1031
β7-strand (C-lobe)	CS4	L172	L720	V1009	I679	I982	V653	I955	I740	V1029
D-helix (C-lobe)	CS3	M128	L675	L964	L634	L937	I608	L910	L695	L986
F-helix (C-lobe)	CS2	L227	I782	T1070	T742	V1043	T714	S1012	I807	T1090
F-helix (C-lobe)	CS1	M231	I786	L1074	I746	L1047	V718	V1016	T803	L1094

[a] From Refs. [17,20,21].

For example, when a Janus kinase is activated by its cytokine receptor, the chief phosphorylated product is the enzyme itself mediated by the trans-phosphorylation of one Janus kinase protein by another. Phosphorylation of the cytokine receptor and STATs also occurs. The concentration of protein kinases and their substrates are nearly equivalent and high turnover numbers are not required. The mechanisms for the interconversion or switching of dormant and active protein kinases vary with the particular enzyme and are generally quite intricate as seen for the Janus kinases.

The interaction of a cytokine ligand with its receptor triggers a change in the receptor conformation that leads to a repositioning of the associated Janus kinase proteins. This rearrangement promotes the *trans*-phosphorylation of activation segment tyrosine residues. Feng et al. prepared Y1007F and Y1008F activation segment single and double mutants of JAK2 [28]. They found that the Y1007F and the double mutant were kinase impaired whereas the Y1008F mutant was active *in vitro*. They also found that the wild type and Y1008F mutants were active in erythropoietin signaling in human fibrosarcoma γ2 cells in culture whereas the Y1007F mutant was not. These studies indicate that phosphorylation of only the first tyrosine residue in the activation segment is necessary for JAK2 enzyme activation.

Zhou et al. prepared Y980F and Y981F activation segment mutants of JAK3 [29]. They demonstrated that both of the activation segment tyrosines can be phosphorylated. They measured the protein kinase activity of various mutants in COS-7 cell immunoprecipitates expressing the proteins. They observed that the Y980F mutant exhibited about 30% of the activity as the wild-type enzyme. In contrast, the Y981F mutant had about 230% of the activity of the wild-type protein while the Y980F and Y981F double mutant had about the same activity as the wild-type enzyme. These results suggest that Y981 *per se* in JAK2 is somehow inhibitory.

Gauzzi et al. studied the role of the activation segment tyrosine residues on TYK2 activity in mutant human fibrosarcoma cell immunoprecipitates that do not express this protein [30]. They demonstrated that the Y1054F/Y1055F double mutant had decreased catalytic activity when compared with the wild-type enzyme. Moreover, the extent of autophosphorylation of the double mutant was markedly decreased. These investigators did not perform experiments with Y1054F or Y1055F single mutants so that we cannot establish with certainty whether TYK2 behaves like JAK2 and JAK3, which indicate that phosphorylation of the first tyrosine residue of the activation segment results in an increase in catalytic activity.

Saharinen et al. reported that the activity of a construct containing only the JAK2 JH1 domain was 50 times more active in catalyzing the phosphorylation of an exogenous peptide substrate that a JH2-JH1 construct [31]. Thus, the JH2 domain inhibits the activity of the JH1 protein kinase domain. As noted later, an activating JAK2 V617F mutation occurs in several myeloproliferative neoplasms and this mutation occurs within the JH2 domain. Lupardus et al. determined the X-ray crystal structure of the TYK2 JH1-JH2 construct [32]. Each of their domains has the characteristic protein kinase fold (Fig. 6A). The JH1 domain has an active structure with the αC-in conformation and with the activation segment in its open conformation despite being non-phosphorylated. The JH2 domain has an inactive structure with the αC-out conformation and with a compact or closed activation segment.

The interaction between the TYK2 pseudokinase and kinase domains is mediated by the N-lobes of each domain (Fig. 6B). The pseudokinase interface surface is primarily composed of residues of the αC-β4 loop and the N-terminal exon 12 segment (TYK2 residues 579–591). Important contacts in the JH1 domain include I900 of the β1-strand, L913 of the β2-strand, M926 of the β3-strand, and Y980 within the hinge. The X-ray crystal structural analysis failed to identify the location of a 17-amino acid pseudokinase-kinase interdomain linker. The JH2 inhibition of JH1 catalytic activity may result from the immobilization of the JH1 N-lobe. In this scheme, the blockade of catalytic activity involves an intraprotein or cis-inhibitory mechanism (Fig. 7A).

In contrast, Waters et al. proposed an interprotein or trans-inhibitory mechanism [33]. Accordingly, the activation segment of a pseudokinase interacts with the active site catalytic domains of a JH1 partner domain and thereby blocks its activity (Fig. 7B). Cytokine receptor activation induces a conformational shift that promotes the *trans*-phosphorylation of the activation segment of the Janus kinase partner leading to enzyme activation. In support of the *trans* mechanism, Lu et al. found that the constitutively activated V617F mutant of JAK2 does not manifest its activity unless the erythropoietin receptor is present [34]. Brooks et al. studied the activation of JAK2 by the growth hormone receptor using fluorescence resonance energy transfer (FRET) to monitor positioning of the JAK2 binding motif in the receptor dimer [35]. They reported that activation of the receptor dimer induced a separation of its JAK2 binding motifs, driven by a ligand-induced transition from a

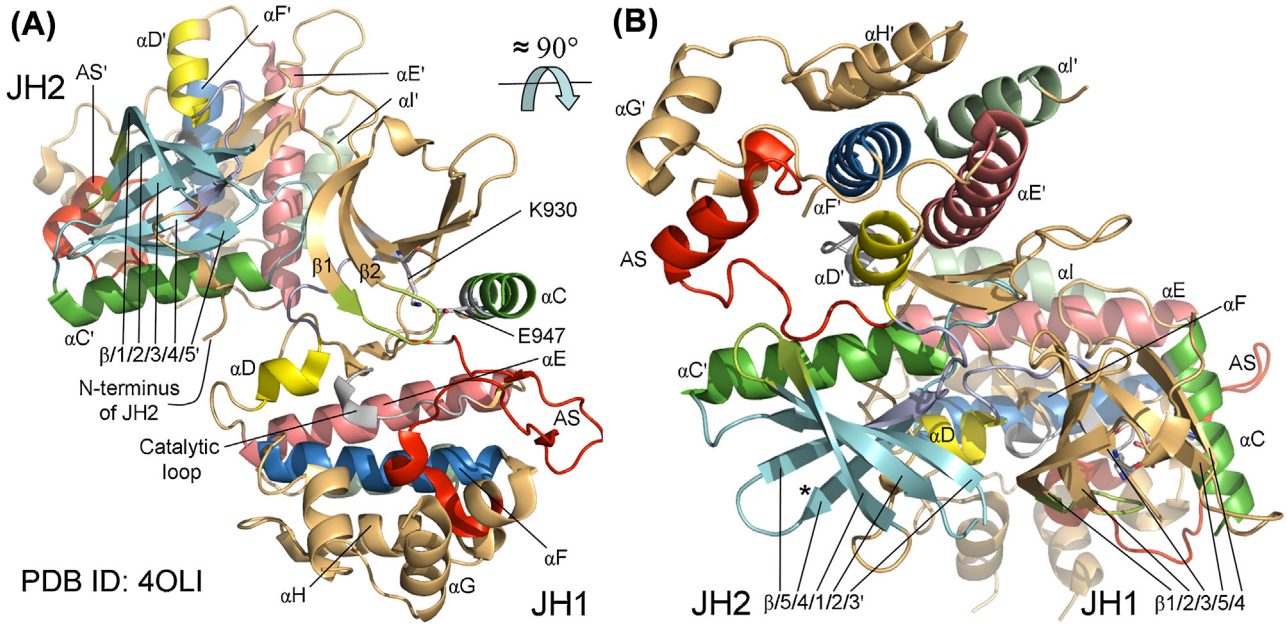

Fig. 6. (A) Frontal view of the TYK2 JH1 domain apposed to the inactive JH2 domain. (B) Top view of the JH1 domain depicting the interaction of the N-lobes. Components of JH2 are depicted with a prime mark. The asterisk at the end of the β4′-strand indicates the location of the JAK2 V617F activating mutation; in the case of TYK2 this residue is V678.

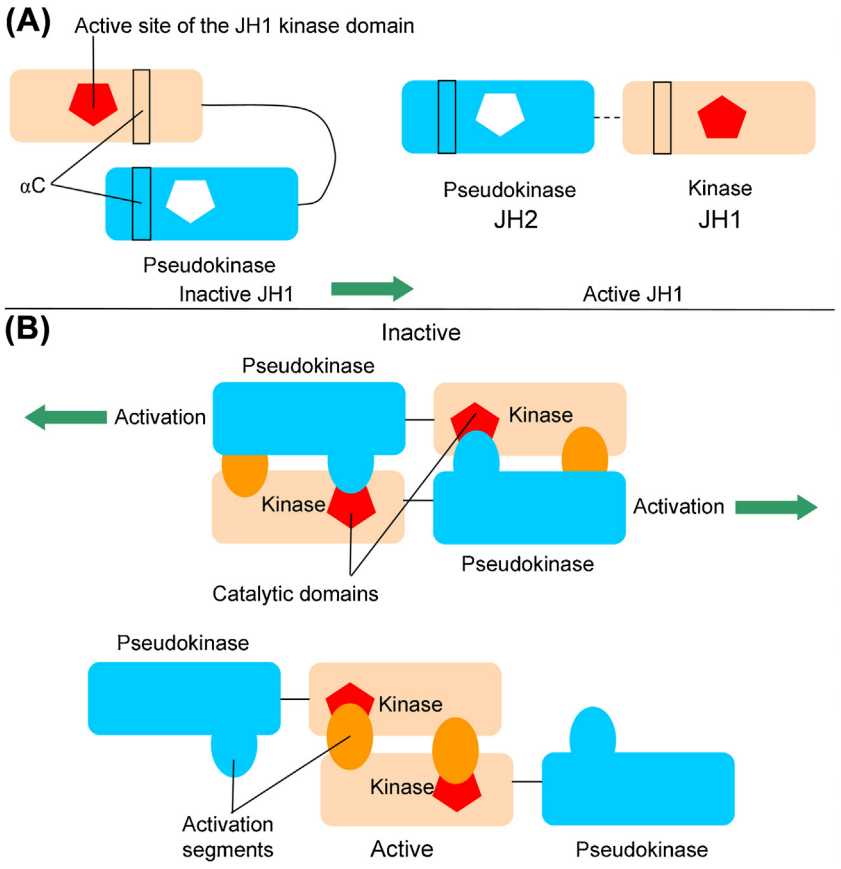

Fig. 7. (A) JAK intramolecular inactivation in *cis*. (B) JAK intermolecular inactivation in *trans*.

parallel transmembrane helix pair to a crossover arrangement. This separation leads to the removal of the pseudokinase domain from the kinase domain of the partner JAK2 and pairing of the two kinase domains, thereby facilitating *trans*-activation. In either the *cis*- or *trans*-mechanisms, immobilization of the small lobe by any means would decrease enzyme activity owing to the requirement that this lobe must be flexible in order to bind ATP, catalyze the reaction, and then release ADP.

Besides activation segment phosphorylation, other activating and inhibiting phosphorylation events participate in Janus kinase regulation. Little is known about the phosphorylation of JAK1/3 and TYK2 outside of the activation segment. However, there is considerable data on the phosphorylation of JAK2. This protein is constitutively phosphorylated on Ser523 [36,37]. Besides the phosphorylation of Tyr1007 and Tyr1008 within the activation segment, phosphorylation of the following tyrosines increases JAK2 protein kinase activity: Tyr637 (within the αD' helix of JH2), Tyr813 (in the last α-helix of JH2), Tyr868 (at the end of the β2-strand of the JH1 domain), and Tyr966 near the end of the αE helix, and Tyr972 (before the catalytic loop) [38,39]. In contrast, phosphorylation of Ser523 (within the SH2 domain) and tyrosines 119, 221, 317 (each within the FERM domain), Tyr570 (within the β2′-β3′ JH2 loop), and Tyr913 (the RS4 residue at the beginning of the β4 loop of the JH1 domain) decrease JAK2 activity.

Adding to the complexity of JAK2 phosphorylation, Ungureanu et al. reported that the pseudokinase domain of JAK2 is a dual-specificity protein kinase that catalyzes the phosphorylation of two negative regulatory sites in this protein: Ser523 and Tyr570 [40]. These investigators demonstrated that the rate of JAK2 JH2 autophosphorylation is about 10% that of the JH1 domain and showed that the β3′-strand K581A mutant was kinase dead. Ser523 phosphorylation occurs before that of Tyr570. They found that Ser523 is constitutively phosphorylated in unstimulated cells and phosphorylation of Tyr570 and other sites occurs after cytokine stimulation. Moreover, Ser523 autophosphorylation appears to be an intramolecular reaction (in *cis*). Thus, under basal conditions Ser523 phosphorylation serves as a negative regulator of JAK2 protein kinase activity. Thus far it is uncertain whether the other Janus kinases behave similarly.

3. Selected Janus kinase disease targets

3.1. Inflammatory disorders

3.1.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common disorder of unknown etiology affecting about 1% of the adult population [41] with a female: male ratio of about 3:1 [42,43]. Uncontrolled active rheumatoid arthritis causes joint damage, disability, decreased quality of life, and cardiovascular and other co-morbidities [41]. The illness is characterized by synovitis of multiple small joints, pain, and morning stiffness. The metacarpophalangeal joints (between the wrist and fingers) are commonly affected. Radiographic changes are diagnostic. Serological findings in RA include the presence of rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA). The replacement of the ketimine group (=NH) by a ketone group (=O) in the amino acid arginine in proteins yields a protein-citrulline. The immune system often attacks such proteins leading to autoimmune disorders such as RA. The anti-citrullinated protein antibody level serves as a valuable biomarker for the diagnosis of this illness. The erythrocyte sedimentation rate and plasma C-reactive protein levels, which are signs of inflammation, are usually elevated. The pathogenic mechanisms for the development of rheumatoid arthritis result from a complex interplay of immunological, environmental, and genetic factors that produce dysregulation of the immune system and a breakdown of self-tolerance.

Disease-modifying antirheumatic drugs (DMARDs), the key therapeutic agents, along with low doses of prednisone reduce synovitis and systemic inflammation and improve function. The leading DMARD is methotrexate, which can be combined with other drugs such as sulfasalazine and leflunomide. Methotrexate is a folate antagonist that inhibits nucleotide biosynthesis; the drug also inhibits enzymes involved in purine metabolism resulting in the accumulation of adenosine thereby leading to inhibition of T cell and B cell activity. Leflunomide is a pyrimidine synthesis inhibitor that blocks dihydroorotate dehydrogenase. The mechanism of anti-inflammatory activity of sulfasalazine is unclear.

Biological agents are used when arthritis is uncontrolled or toxic effects arise with DMARDs [41]. The tumor necrosis factor-α inhibitor infliximab was the first biological agent approved for inflammatory disease, followed by adalimumab, etanercept, abatacept, rituximab, and tocilizumab. Adalimumab (a human construct) and infliximab (a mouse-human chimeric construct) are two monoclonal antibodies directed against TNF-α while etanercept is a protein that functions as a decoy receptor that binds TNF-α. Abatacept is a fusion protein composed of the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA-4 (a protein receptor that down-regulates the immune response). Rituximab is a chimeric mouse-human monoclonal antibody against CD20 that destroys B cells, which express CD20. Tocilizumab is a chimeric mouse-human monoclonal antibody directed against the soluble and membrane forms of the IL-6 receptor. There are at least 20 additional agents called biosimilars that are either approved or are in clinical trials for the treatment of inflammatory disorders [44].

3.1.2. Canine dermatitis

Canine dermatitis is an inflammatory process that affects the skin of the face, axillae, and distal extremities of dogs [45,46]. Depending on the seriousness of the signs of the dermatitis and the attitudes of the owners, the symptomatic treatment and interventional therapy for environmental allergies (allergen avoidance and allergen-specific desensitization) may be implemented [47]. Symptomatic treatment includes the use of various anti-inflammatory agents including glucocorticoids (systemically or topically) and cyclosporine. Other treatment modalities of lower or less proven efficacies include antihistamines, feline interferon-ω, and pentoxifylline (a phosphodiesterase inhibitor that reduces inflammation).

3.1.3. Psoriasis

Psoriasis is a common illness that affects 2–4% of the population and it occurs in men and women with an equal frequency. It is a chronic autoimmune disease that occurs at any age and is typified by patches of abnormal skin [48]. These patches are red, scaly, and they produce itching. They vary in size from small localized lesions to those that completely cover the body. Patients suffer more during the winter and exacerbations can arise after the use of medications such as β-blockers or NSAIDs (non-steroidal anti-inflammatory drugs). The underlying pathogenesis involves an immune reaction to dermal and epidermal cells.

Although there is no cure for psoriasis, various treatments can provide symptomatic relief including such therapies as steroid ointments, vitamin D3 ointments, ultraviolet light, and methotrexate. About three-quarters of cases can be managed with topical ointments alone. People with the disease have an increased risk of cardiovascular problems, Crohn disease, lymphomas, and psoriatic arthritis. Psoriatic arthritis occurs in up to 30% of individuals with the disease.

Psoriasis is characterized by the rapid growth of the epidermis. Accordingly, epidermal cells are not replaced in the usual 28–30 days, but rather they are replaced every 3–5 days. These changes arise from the premature maturation of keratinocytes that result from an inflammatory cascade in the dermis that is produced by various immune cells including macrophages, T cells, and dendritic cells. These various cells travel from the dermis to the epidermis and secret cytokines such as interleukin-1β (IL-1β), IL-6, IL-22,

along with tumor necrosis factor-α (TNF-α). It appears that these cytokines induce the growth and proliferation of keratinocytes.

3.1.4. Ulcerative colitis

Ulcerative colitis is a chronic relapsing form of inflammatory bowel disease that causes inflammation and ulcers in the colon [49]. The illness usually occurs in young adults with symptoms ranging from mild to severe. The hallmark symptom of active disease is bloody diarrhea. The incidence of newly diagnosed ulcerative colitis ranges from 1 to 20 people per 100,000. Ulcerative colitis can be treated with a number of medications, including 5-aminosalicylic acid (ASA) pro-drugs such as sulfasalazine and mesalazine, which inhibit the synthesis of inflammatory cytokines.

Immunosuppressive corticosteroids such as prednisone are used for short-term, but not long-term treatment [49]. Other immunosuppressive medications such as azathioprine, which is metabolized to 6-mercaptopurine thereby inhibiting cellular replication and purine synthesis, are also used. Biological agents such as infliximab and adalimumab are given only if people cannot achieve remission with 5-aminosalicylic acid and corticosteroids. Biologic treatments such as the TNF inhibitors infliximab, adalimumab, golimumab (a fully human antibody directed against TNF-α), and vedolizumab (a humanized monoclonal antibody directed against $\alpha_{4\beta7}$ integrin) are also used to treat patients with ulcerative colitis who are no longer responding to corticosteroids. Usually these medications are used only after other options have been exhausted (i.e., the patient has received and not responded favorably to high-dose corticosteroids and immunomodulators such as azathioprine and mesalazine). In contrast to Crohn disease as described next, methotrexate is ineffective in the treatment of ulcerative colitis. Surgical removal of the colon (colectemy) can cure ulcerative colitis. This is usually performed only in patients with severe symptomatology that is unresponsive to medical management.

3.1.5. Crohn disease

In contrast to ulcerative colitis which affects only the colon, Crohn disease is an inflammatory bowel disease (IBD) that may affect any part of the gastrointestinal tract [50]. It is an uncommon disorder with a prevalence of about 30 per 100,000 people in North America and Europe while it is even less common in Africa and Asia. Typical signs and symptoms include abdominal pain, diarrhea, and fever along with weight loss. The pathogenesis of Crohn disease involves a combination of bacterial, genetic, environmental, and immunological factors. It is a chronic disorder resulting from an immune response to the gastrointestinal tract that may be directed at microbial antigens. Because the immune system is not reacting to indigenous body cells, Crohn disease is not an autoimmune disease *per se*. Despite considerable work, the mechanistic basis of Crohn disease is puzzling.

There are no medications or surgical procedures that can cure Crohn disease [50]. Moreover, surgery is contraindicated owing to the development of fistulas. Treatment options can only reduce symptoms, maintain remission, and prevent relapse. Medications used to treat the symptoms of Crohn disease include 5-aminosalicylic acid (5-ASA) formulations, prednisone, immunomodulators such as azathioprine, methotrexate, infliximab, adalimumab, and vedolizumab. Corticosteroids are used in severe attacks of Crohn disease. Biological therapies are medications used to avoid long-term steroid use and to decrease inflammation.

3.2. Neoplastic disorders

3.2.1. Myelofibrosis

Myelofibrosis is a rare idiopathic disorder that results from the proliferation of hematopoietic stem cells in the bone marrow and results in the replacement of these cells with scar tissue [51]. Primary myelofibrosis occurs spontaneously whereas secondary myelofibrosis develops after polycythemia vera or after thrombocythemia. Besides bone marrow fibrosis, primary myelofibrosis is a chronic illness characterized by ineffective red cell production, excess production of dysplastic megakaryocytes, extramedullary hematopoiesis, systemic inflammation with excess circulating levels of proinflammatory cytokines, and shortened life expectancy. The occurrence of extramedullary hematopoiesis in the spleen is commonly observed.

The median age at the time of the initial diagnosis of primary myelofibrosis is about 64 years [52] and the signs and symptoms include fatigue, early satiety, insomnia, abdominal discomfort, night sweats, abdominal pain, dizziness, cough, bone pain, itching, weight loss, headache, and fever [51]. Laboratory examination typically reveals anemia with tear-drop shaped erythrocytes, nucleated erythrocytes, and immature myeloid cells as a consequence of extramedullary hematopoiesis. About half of the patients with primary myelofibrosis posses a JAK2 V615F mutation, about 30% possess a calreticulin mutation, and about 10% possess a mutation in the thrombopoietin receptor (*MPL*); these mutations are mutually exclusive [53]. These genetic changes are considered to be the principal drivers of neoplastic myeloproliferation [51]. The V617F mutation appears to make hematopoietic cells more sensitive to growth factors that need JAK2 for signal transduction, which include erythropoietin and thrombopoietin (Table 1). Fibroblasts producing the scar tissue in this disorder are polyclonal and not part of a neoplastic clone. Median life expectancy for primary myelofibrosis is about six years.

Myelofibrosis has been treated with immunomodulatory agents such as thalidomide, lenalidomide, or pomalidomide. Hydroxyurea (a ribonucleotide reductase inhibitor), melphalan (a DNA-alkylating agent), busulfan (an alkylating agent), and cladribine (an adenosine deaminase inhibitor) have been used for the treatment of the splenomegaly (enlarged spleen) of primary myelofibrosis with only minimal success. Other treatment modalities for myelofibrosis include transfusion for anemia or radiation therapy for painful sites of extramedullary hematopoiesis. Stem cell transplantation in selected patients is curative, but is infrequently used owing to the lack of suitable donors.

3.2.2. Polycythemia vera

Polycythemia vera is a clonal neoplastic stem cell disorder characterized by excessive red blood cell production; this condition may also result in the overproduction of white blood cells and platelets [54]. The overproduction of red cells is independent of erythropoietin and the serum levels of this agent are low. Patients with polycythemia vera may be asymptomatic and the disease is discovered on routine blood analysis. The median age a diagnosis is about 61 years and the incidence is about 22 per 100,000 population [55]. The signs and symptoms include those listed above for primary myelofibrosis. The JAK2 V615 F mutation occurs in about 95% of these people. This condition has the capacity to transform into myelofibrosis 10–20 years after diagnosis or into acute myelogenous leukemia in 5–20 years after diagnosis. The overall median life expectancy is about 13.5 years [51]. The goal of therapy is to decrease the hematocrit to less than 45%. This is most commonly achieved by periodic phlebotomies. Moreover, hydroxyurea is a commonly used medication for cytoreductive therapy. This agent inhibits ribonucleotide reductase by scavenging tyrosyl free radicals that are involved in the production of deoxyribonucleoside diphosphates (dADP, dGDP, dCDP, and dUDP). Low-dose aspirin is used to reduce the risk of vascular events.

3.2.3. Primary thrombocythemia

Primary thrombocythemia (thrombocytosis) is a clonal disorder of unknown etiology involving a multipotent hematopoietic progenitor cell manifested clinically by the overproduction of blood platelets without erythrocyte overproduction [56]. The median age at diagnosis is in the 50–60 year range and the incidence is about 24 per 100,000 people [57]. Most patients are asymptomatic and elevated platelets are observed on routine hematological analysis. The hematocrit is normal, but there may be a mild elevation of leukocytes. Splenomegaly occurs in about one-quarter of the patients. Although the JAK2 V617F mutation occurs in about half of the people with primary thrombocythemia and myelofibrosis and in 95% of patients with polycythemia vera, Spivak et al. argue that this mutation cannot be the sole explanation for the pathogenesis of these rare disorders [58]. This affliction transforms into myelofibrosis in 8–16 years after the onset of thrombocythemia.

4. Janus kinase mutations in human illnesses

Somatic mutations in *JAK1* are found in the SH2 domain and the small and large lobes of the pseudokinase domain that result in AML, ALL, breast ductal carcinoma, and NSCLC [32]. The incidence of activating mutations in T-cell ALL ranges from 6 to 27% and the incidence in B-cell ALL is about 1.5% while that of T-cell prolymphocytic leukemia ranges from 8 to 12% [59]. More than three dozen *JAK2* mutations in exon 12 (^{535}MVFHKIRNEDLIF^{547}) result in polycythemia vera while mutations in the small lobe of the pseudokinase domain have been reported in ALL and polycythemia vera [32]. The majority of JAK2 mutations in lymphoid lineage neoplasms involve R683; this residue is at the end of the β7′-strand below the ATP-binding site of JH2 and potentially interacts with the β2–β3 loop of the small lobe of JH1. Mutations of the *JAK3* pseudokinase N-lobe have been reported in acute megakaryoblastic leukemia (15%) while large lobe mutations occur in breast ductal carcinomas and ALL. *TYK2* mutations have been described in about 21% of T-cell ALL [59]. TYK2 deficiencies are associated with susceptibility to viral and bacterial infections and several *TYK2* polymorphisms show a strong linkage to autoimmune afflictions such as multiple sclerosis, systemic lupus erythematosus, Crohn disease, primary biliary cirrhosis, and type I diabetes. Moreover, *TYK2* polymorphisms have also been linked to AML.

Rare chromosomal rearrangements linking the JH1 domain-coding portion of the *JAK2* gene to the oligomerization domain of either TEL, BCR, PCM1, Pax5, or ETV6 have been reported in atypical chronic myelogenous leukemia [59]. The fusion proteins oligomerize and promote the transphosphorylation of the activation segments resulting in JAK2 JH1 activation and subsequent downstream signaling including the STAT, phosphatidylinositol-3 kinase, or MAP kinase pathways. Moreover, the *JAK2* locus is amplified in 30–50% of Hodgkin lymphomas and in some B-cell lymphomas.

5. Selected Janus kinase inhibitors in clinical trials

5.1. First-generation drugs

5.1.1. Tofacitinib

Tofacitinib bears a pyrrolo[2,3-*d*]pyrimidine scaffold (Fig. 8A). It is an orally effective drug that was approved by the FDA for the treatment of rheumatoid arthritis, an autoimmune disorder, in 2012 (www.brimr.org/PKI/PKIs.htm). Tofacitinib inhibits JAK1/2 with sub-nanomolar EC_{50} values; it is also a potent (EC_{50} less than 5 nM) inhibitor of JAK3 and TYK2 (Table 5). Van Vollenhoven et al. reported on a randomized phase III clinical trial involving 717 patients who were receiving methotrexate who were randomly assigned to cohorts receiving 5 mg of tofacitinib twice daily, 10 mg of tofacitinib twice daily, 40 mg of adalimumab by injection once every 2 weeks, or placebo [42]. After 6 months, positive outcomes were higher among patients receiving 5 mg or 10 mg of tofacitinib (52% and 53%, respectively) and among those receiving adalimumab (47%) than among those receiving placebo (28%). Positive outcomes were defined as a 20% reduction in the number of tender and swollen joints along with improvements in three of five other standard assessment parameters.

Table 5
Janus kinase inhibitor EC_{50} values (nM)[a].

Drug	JAK1	JAK2	JAK3	TYK2
First generation				
Tofacitinib[b]	0.16	0.58	1.6	4.8
Oclacitinib[c]	10	18	99	84
Baricitinib[d]	4	6.6	259	21.1
Ruxolitinib[d]	0.09	0.036	2	0.4
Second generation				
Decernotinib[e]	11	13	2	11
Peficitinib[f]	3.9	5.0	0.71	4.8
Filgotinib[g]	10	2.8	81	11.6
Fedratinib[b]	18	1.1	?	?
Momelotinib[d]	11	18	155	17
Lestaurtinib[d]	8.8	3.7	2.3	15
Gandotinib[h]	25	3	60	?
Pacritinib[i]	1280	23	520	50

[a] EC_{50} (effective concentration) refers to IC_{50}, K_d, or K_i.
[b] Ref. [60].
[c] Ref. [61].
[d] pubchem.ncbi.nlm.nih.gov.
[e] Ref. [62].
[f] Ref. [63].
[g] Ref. [64].
[h] Ref. [65].
[i] Ref. [66].

Adverse events occurred more frequently with tofacitinib and adalimumab than with placebo [42]. Herpes zoster and cellulitus infections occurred in the two tofacitinib groups, but none in the antibody or placebo groups. Tofacitinib was associated with an increase in both low-density and high-density lipoprotein cholesterol levels and with a reduction of blood neutrophils. A small decrease in hemoglobin (1–3 g/dl) occurred in about 8% of patients in each of the four groups. Owing to the inhibition of erythropoietin receptor signaling by tofacitinib, this lack of an adverse response is somewhat surprising. The authors concluded that tofacitinib was significantly superior to placebo and was numerically similar to adalimumab in efficacy. Based upon this clinical trial, tofacitinib was approved for the treatment of rheumatoid arthritis in 2012.

Lee et al. reported on a randomized clinical trial involving 958 patients comparing first-line treatment of methotrexate with either 5 mg or 10 mg doses of tofacitinib (twice daily) in previously untreated patients [43]. About 26% of the 5 mg group and 38% of the 10 mg group had positive outcomes compared with 12% of the methotrexate cohort. Positive outcomes were defined as a 70% reduction in the number of tender and swollen joints along with five additional criteria including decreased erythrocyte sedimentation rates, C-reactive protein levels, pain, assessment by the patient, and assessment by the physician. The most common adverse events included infections (herpes zoster) and gastrointestinal symptoms. Herpes zoster occurred in 13 of 373 patients in the 5 mg group, 18 of 397 in the 10 mg group, and 2 of 186 in the methotrexate group. Decreases in neutrophils and increases in serum creatinine were observed in all groups. Low-density lipoprotein levels increased about 20% in the tofacitinib groups and 4% in the methotrexate group while increases in the high-density lipoprotein levels increased by about 17% in the tofacitinib groups and 7% in the methotrexate group. Tofacitinib is in numerous

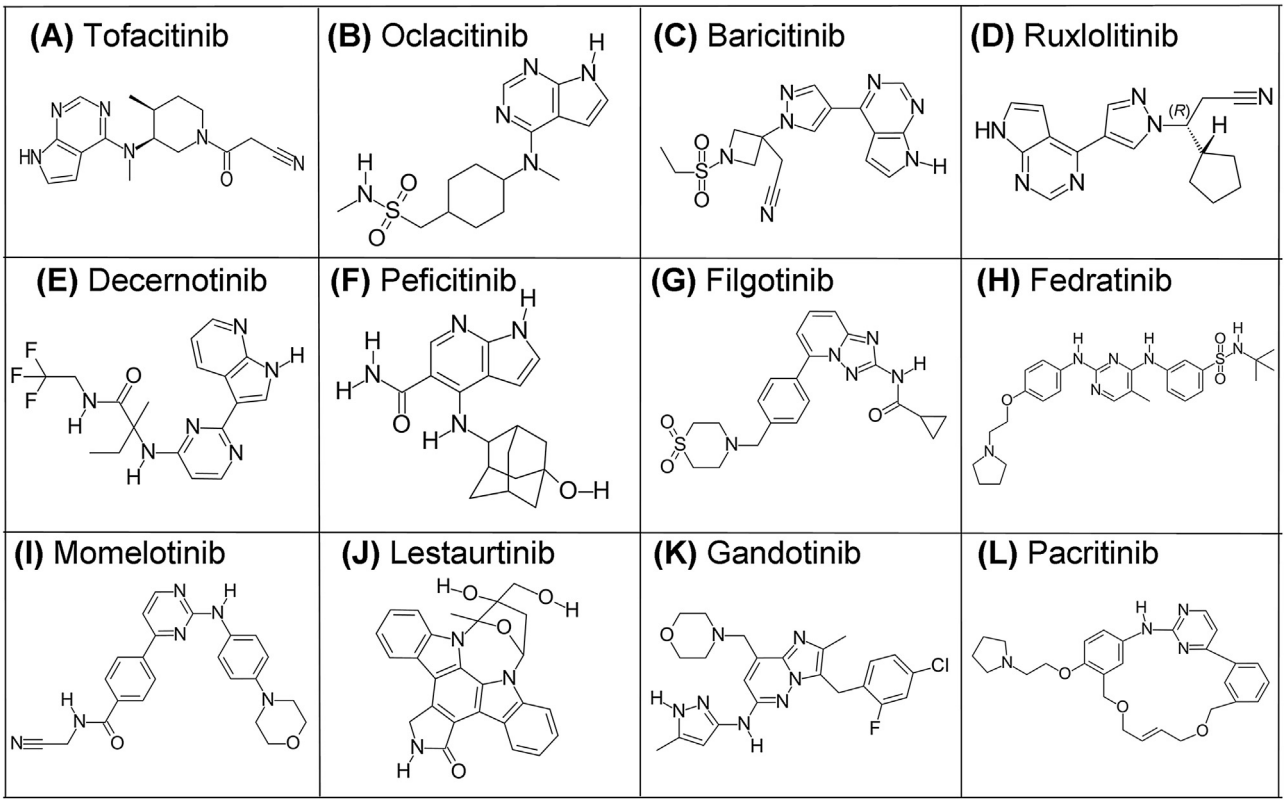

Fig. 8. Structures of selected JAK antagonists.

Table 6
Properties of small molecule Janus kinase inhibitors in clinical trials[a,b].

Name, code, trade name®	Targets	PubChem CID	Formula	MW	D/A[c]	cLogP[d]	Indications and clinical trials
First generation							
Tofacitinib, CP690550, Xeljanz®	JAK1/2/3	9926791	$C_{16}H_{20}N_6O$	312.37	1/7	1.076	RA[e], psoriasis, alopacia areata, atopic eczema, spondyloarthritis, systemic lupus, ulcerative colitis, acute host-vs.-graft disease
Oclacitinib, OF03394197, Apoquel®	JAK1/2	44631938	$C_{15}H_{23}N_5O_2S$	337.44	2/6	1.528	Canine allergic dermatitis[f]
Baricitinib, INCB28050, LY3009104	JAK1/2	44205240	$C_{16}H_{17}N_7O_2S$	371.42	1/7	0.330	RA, psoriasis, autoinflammatory disease
Ruxolitinib, INC424, Jakafi®	JAK1/2	25126798	$C_{17}H_{18}N_6$	306.37	1/6	1.967	Myelofibrosis[g], polycythemia vera[g], ALL, AML, CLL, CML, NSCLC, breast, colorectal, head and neck, prostate, and pancreatic cancers, RA, psoriasis
Second generation							
Decernotinib, VX509	JAK3	59422203	$C_{18}H_{19}F_3N_6O$	392.38	3/8	2.021	RA
Peficitinib, ASP015 K	JAK3	57928403	$C_{18}H_{22}N_4O_2$	326.39	4/4	2.046	Psoriasis, RA, ulcerative colitis
Filgotinib, GLPG0634	JAK1/2	49831257	$C_{21}H_{23}N_5O_3S$	425.50	1/6	1.958	RA, Crohn disease
Fedratinib, SAR302503, TG101348	JAK2	16722836	$C_{27}H_{36}N_6O_3S$	524.68	3/9	4.934	Myelofibrosis, polycythemia vera, primary thrombocythemia
Momelitinib, Cyt387	JAK1/2	25062766	$C_{23}H_{22}N_6O_2$	414.46	2/7	2.352	Myelofibrosis, polycythemia vera, NSCLC, pancreatic carcinoma
Lestaurtinib, CEP-701	JAK2, FLT3, TRKA/B/C	126565	$C_{26}H_{21}N_3O_4$	439.36	3/4	2.816	Myelofibrosis, psoriasis, polycythemia vera, ALL, AML, prostate cancer, multiple myeloma, neuroblastoma, Hodgkin lymphoma
Gandotinib, LY2784544	JAK2	46213929	$C_{23}H_{25}ClFN_7O$	469.94	2/7	3.661	Myeloproliferative disorders
Pacritinib, SB1518	JAK2	46216796	$C_{28}H_{32}N_4O_3$	472.58	1/7	4.499	Myelodysplastic syndromes, myelofibrosis, AML, CLL, NSCLC, colorectal cancer

[a] clinicaltrials.gov.
[b] ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; NSCLC, non-small cell lung cancer; RA, rheumatoid arthritis.
[c] No. of hydrogen bond donors/acceptors.
[d] Calculated log of the partition coefficient as determined by MedChem Designer® v.1.0.1.15.
[e] FDA-approved treatment in 2012.
[f] FDA-approved treatment in 2013.
[g] FDA-approved treatment in 2011.

clinical trials in patients with various inflammatory disorders as indicated in Table 6.

X-ray crystal structures of complexes of tofacitinib with the Janus kinases have been reported [1,67]. Tofacitinib binds to the active conformation of the protein kinase domains of these proteins and is therefore a type I inhibitor [27]. Tofacitinib binds within the adenine pocket of these proteins (Fig. 9A). The *N*7 N—H of the pyrrolo[2,3-*d*]pyrimidine scaffold forms one hydrogen bond with the hinge residue carbonyl group of Glu957 and the *N*1 nitrogen of the scaffold forms a second hydrogen bond with the N—H group of Leu959 in JAK1 (Glu 930 and Leu932 in JAK2; PDB ID: 3FUP and Glu903 and Leu905 in JAK3; PDB ID: 3LXK). A hydrogen bond is observed between (i) β3-K908 and αC-E925 and (ii) β3-K908 and DFG-D1021 (Fig. 9A). The terminal cyanoacetyl handle extends into the front cleft underneath the G-rich loop and the methyl group of the piperidine ring is also found within the front cleft. On the whole, tofacitinib makes hydrophobic contacts with the β1-strand L881 before the G-rich loop, V889 (CS7), A906 (CS8), and M956, F958, and L959 of the hinge, and L1010 (CS6). The drug makes van der Waals contact with β3-K908, DFG-D1021, and R1007 and N1008 within the catalytic loop. The drug makes similar hydrophobic and van der Waals contacts with JAK2/3.

In TYK2, the *N*7 N—H of the pyrrolo[2,3-*d*]pyrimidine core forms one hydrogen bond with the hinge residue carbonyl group of Glu979, the *N*1 nitrogen of the scaffold forms a second hydrogen bond with the N—H group of Val981, and a third hydrogen bond forms between the cyano group and Gly906 in the G-rich loop. A hydrogen bond is observed between (i) β3-K930 and αC-E947, (ii) β3-K930 and DFG-D1041, and (iii) HRD-H1021 and DFG-D1041 (Fig. 9B). Additionally, tofacitinib makes hydrophobic contacts with the β1-strand L903 before the G-rich loop, V911 in the β2 strand, A928 (CS8), and Leu1030 (CS6). The drug makes van der Waals contact with Tyr980 of the hinge, N1028 at the end of the catalytic loop, and DFG-D1041. Although tofacitinib makes one more hydrogen bond with TYK2 than JAK1, its binding affinity is an order of magnitude less probably because of fewer favorable hydrophobic interactions.

5.1.2. Oclacitinib

Oclacitinib is an orally effective cyclohexylamino pyrrolo[2,3-*d*]pyrimidine derivative (Fig. 8B) that was approved by the FDA in 2013 for the control of pruritus associated with allergic dermatitis and for the control of atopic dermatitis in dogs. The drug targets human JAK1/2 (Table 5). Gonzales et al. found that oclacitinib was much less effective in blocking JAK2 activity in human cells in culture with IC_{50} values near 1400 nM compared with 18 nM *in vitro,* thereby indicating that findings with purified enzymes may not represent activities in the cellular or whole animal context [61]. Differences in potency of one or two orders of magnitude between values obtained with purified proteins and with cellular assays should be considered in all cases. The human and canine proteins exhibit about 95% homology.

Cosgrove et al. reported on a randomized clinical study evaluating oclacitinib vs. placebo [68]. They reported that the drug provided rapid and safe control of atopic dermatitis with improvements in skin lesions. This same group performed a double-blinded and randomized clinical trial comparing the efficacy and safety of oral oclacitinib and oral cyclosporine for the control of atopic dermatitis in client-owned dogs [69]. They concluded that both drugs were effective, but that oclacitinib brought about a faster resolution of symptoms. Most adverse events with oclacitinib included vomiting and diarrhea early in the course of treatment and these symptoms were resolved following standard practices. There is no indication that oclacitinib is being tested in human clinical trials.

No X-ray structural studies of oclacitinib bound to any Janus kinase have been reported. To obtain an idea on the possible interaction of this drug with JAK1, the Schrödinger Glide Suite (2016-1 release) was used to dock the drug into human JAK1 (with initially bound tofacitinib, PDB ID: 3EYG) [70]. The pyrrolopyrimidine core in the resulting model makes hydrogen bonds with the carbonyl oxygen of E957 and with the N—H group of L959, both within the hinge (Fig. 9C). The drug makes hydrophobic contacts with L881 immediately before the G-rich loop, V889 immediately after the G-rich loop, A906 (CS7), V938 (Sh1), M956 within the hinge, and L1010 (CS6). These interactions parallel those observed in the model of ruxolitinib binding to JAK2 as described later.

5.1.3. Baricitinib

Baricitinib is an orally effective pyrrolo[2,3-*d*]pyrimidine derivative like tofacitinib, oclacitinib, and ruxolitinib (Fig. 8C). This congener is undergoing clinical trials for RA and psoriasis. Keystone et al. performed a phase II study involving 301 patients that were randomized 2:1:1:1:1 to receive once daily doses of placebo or 1, 2, 4 or 8 mg of baricitinib for 12 weeks [71]. The patients included in this study failed to respond to prior methotrexate treatment. The primary endpoint was the proportion of patients in the combined 4 and 8 mg groups achieving a standard response at 12 weeks as defined by the American College of Rheumatology. They reported that more patients in the combined baricitinib 4 and 8 mg groups compared with placebo achieved such a response (76% vs. 41%). Serious infections developed in three patients receiving baricitinib. Dose-dependent decreases in hemoglobin were observed with baricitinib. They found that baricitinib improved the signs and symptoms of RA in these people who did not respond to methotrexate. Moreover, baricitinib was well tolerated with no unexpected safety findings after 24 weeks of treatment.

Genovese et al. performed a phase III study involving 527 patients with refractory rheumatoid arthritis with an inadequate response to or unacceptable side effects associated with one or more TNF inhibitors as described in Section 3.1.1, other disease-modifying antirheumatic drugs (DMARDs), or both [72]. They randomly assigned the patients in a 1:1:1 ratio to baricitinib at a dose of 2 or 4 mg daily or placebo for 24 weeks. They used standard responses as defined by the American College of Rheumatology and other standard criteria in their assessment of efficacy. They reported that significantly more patients receiving baricitinib at the 4-mg dose than those receiving placebo had a positive response at 12 weeks (55% *vs.* 27%). The rates of serious adverse events were 4%, 10%, and 7% in the three groups, respectively. Two non-melanoma skin cancers and two major adverse cardiovascular events, including a fatal stroke, occurred in the higher-dose group. Baricitinib was associated with a small reduction in neutrophils and increases in serum creatinine and low-density lipoprotein cholesterol levels. They concluded that patients with RA and a previously inadequate response to biologic DMARDs exhibited significant clinical improvement at 12 weeks.

Papp et al. reported on the results of a phase II clinical trial on the effectiveness of baricitinib in patients with moderate-to-severe psoriasis. Patients were randomized (n = 271) to receive placebo or oral baricitinib at 2, 4, 8 or 10 mg once daily for 12 weeks [73]. Dose adjustment for 12 additional weeks was based on improvements in the Psoriasis Area and Severity Index (PASI) score. The primary end point was PASI 75% (PASI-75) after 12 weeks for North American patients (n = 238); secondary end points were safety and efficacy measures. After 12 weeks, they found that more North American patients in the 8-mg (43%) and 10-mg (54%) baricitinib groups achieved a PASI-75 score than in the placebo group (17%). All baricitinib-treated groups had greater mean changes from baseline in their PASI scores at 12 weeks. More than 81% of PASI-75 responders maintained their scores through 24 weeks. Study discontinuations due to adverse events were 0%, 0%, 2.8%, 6.3% and 5.8% for placebo and 2-, 4-, 8- and 10-mg baricitinib

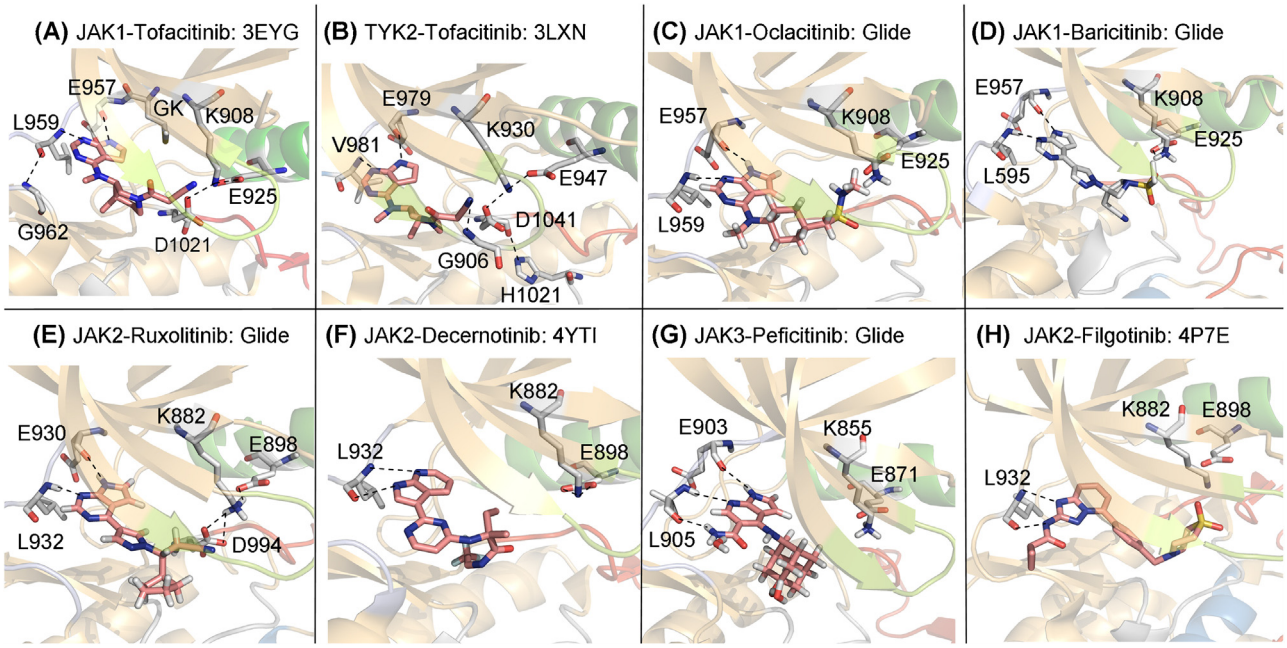

Fig. 9. Binding of selected drugs to the JAK family. Polar bonds are depicted as dashed lines. The PDB IDs used to prepare the structures are given. Otherwise the Schrödinger Glide program (Version 2016-1) was used to generate the poses of the ligand docking sites [70].

groups, respectively. No opportunistic infections were observed in any treatment group. Dose-dependent changes in laboratory values were observed. These included small decreases in neutrophil and hemoglobin levels and small increases in creatinine and low- and high-density lipoproteins. Lymphocyte levels increased during the first week of treatment and returned to normal levels after the second week. They concluded that patients with moderate-to-severe psoriasis treated with baricitinib for 12 weeks achieved significant improvements in their PASI-75 scores.

No X-ray structural studies of baricitinib bound to the Janus kinases have been reported. To obtain an idea on the possible interaction of this drug with JAK1, the Schrödinger Glide Suite (2016-1 release) was used to dock the drug into JAK1 (with initially bound tofacitinib, PDB ID: 3EYG) [70]. The pyrrolopyrimidine core in the resulting pose makes hydrogen bonds with the carbonyl oxygen of E957 and with the N—H group of L959, both within the hinge (Fig. 9D). The drug makes hydrophobic contacts with L881 immediately before the G-rich loop, V889 immediately after the G-rich loop, A906 (CS7), V938 (Sh1), M956 within the hinge, and L1010 (CS6). It also makes van der Waals contact with DFG-D1021. Thus far the clinical trails of baricitinib in the treatment of RA and of psoriasis look promising.

5.1.4. Ruxolitinib

Ruxolitinib, like the three previous drugs, is a pyrrolo[2,3-*d*]pyrimidine derivative (Fig. 8D) and is approved for the treatment of myelofibrosis and polycythemia vera (www.brimr.org/PKI/PKIs.htm). Ruxolitinib is an orally effective JAK1/2 inhibitor; its IC_{50} values for these proteins are in the sub-nanomolar range while those for JAK3 and TYK2 are in the low nanomolar range (Table 5). Verstovsek et al. examined the efficacy of ruxolitinib vs. placebo in the treatment of 309 randomized intermediate- and high-risk groups of patients with myelofibrosis in the COMFORT-I clinical trial [74]. The primary endpoint was the proportion of patients with a decrease in spleen volume (determined by magnetic resonance imaging) of 35% or more at 24 weeks. Secondary endpoints included the durability of response, changes in symptom burden, and overall survival. About 42% of the ruxolitinib group (n = 155) achieved the primary response vs. 0.7% of the placebo group (n = 154). About 46% of the ruxolitinib group experienced symptom improvement compared with 5% who received placebo. They reported that 13 deaths occurred in the group receiving drug compared with 24 in the control group. Anemia occurred in 96% and thrombocytopenia occurred in 70% of the drug-treated group compared with 87% and 31% of the control group. However, these adverse events did not lead to the discontinuance of the drug. Fatigue, diarrhea, peripheral edema were the most common non-hematologic adverse responses. In a parallel study, Harrison reported that ruxolitinib was equally effective in patients with or without the V617F JAK2 mutation [75]. As a result of these studies, the FDA approved the use of ruxolitinib in the treatment of primary myelofibrosis in 2012.

In a randomized study, Verstovsek et al. evaluated ruxolitinib vs. best available therapy for efficacy/safety in hydroxyurea-resistant or intolerant patients with polycythemia vera in the 80-week follow-up of the RESPONSE clinical trial [76]. The best available therapeutic options included interferon, pegylated interferon, pipobroman (an alkylating agent), anagrelide (a phosphodiesterase inhibitor), or immunomodulators (lenalidomide, thalidomide). They reported that the hematocrit normalized in 60% *vs.* 19% and a 35% or more reduction in spleen volume occurred in 40% *vs.* 0.9% of patients taking ruxolitinib *vs.* the control group. New or worsening hematologic laboratory abnormalities in ruxolitinib-treated patients were primarily grade 1 or 2 decreases in hemoglobin, lymphocytes, and platelets (where grade 4 is most serious). The thromboembolic event rate per 100 patient-years was 1.8 with ruxolitinib treatment *vs.* 8.2 with best available therapy. These data support ruxolitinib as an effective long-term treatment option for hydroxyurea-resistant or intolerant patients with polycythemia vera. The most common non-hematologic adverse events each occurring in about 15% of patients were back pain, cough, diarrhea, and fever. Ruxolitinib was approved for the treatment of polycythemia vera in 2014 and currently is in several clinical trials in patients with hematologic and solid tumors along with inflammatory disorders as listed in Table 6.

No X-ray structural studies of ruxolitinib bound to the Janus kinases have been reported. To obtain an idea on the possible interaction of this drug with JAK2, the Schrödinger Glide Suite (2016-1

release) was used to dock ruxolitinib into JAK2 (with initially bound tofacitinib, PDB ID: 3FUP) [70]. In the pose of the resulting model, the N—H group from the core forms a hydrogen bond with E930 carbonyl group of the hinge and the N—H group from L932 of the hinge forms a hydrogen bond with a nitrogen of the fused pyrimidine ring of the drug (Fig. 9E). The β3-K882 makes polar contacts with αC-E988 and with DFG-D994. The drug makes hydrophobic contact with L855 before the G-rich loop, V863 in the β2-strand, A880 (CS7) in the β3-strand, V911 (Sh1) in the αC-β4 back loop, M929 before the hinge, and L983 (CS6). The protein is in its active conformation thus making ruxolitinib a type I inhibitor of JAK2 [27]. The computer-derived interaction of ruxolitinib with JAK1/3 and TYK2 is similar to that described here for JAK2.

Ruxolitinib is also a type I inhibitor of Src (PDB:ID 4U5J) [27]. However, the disposition of the pyrrolo[2,3-*d*]pyrimidine core is flipped about 180° with the same side of the core interacting with the hinge when compared with the binding to Src; we refer to the experimentally determined Src pose as orientation I and the *in silico* version with JAK2 as orientation II. Ruxolitinib is a type I inhibitor of Src that binds within the cleft of its active conformation and it forms two hydrogen bonds with hinge residue M341 [27]. The drug also makes hydrophobic contacts with several residues including CS6, CS7, and CS8.

5.2. Second-generation drugs

5.2.1. Decernotinib

Decernotinib is an orally effective pyrrolo[2,3-*b*]pyridine derivative (Fig. 8E) that is in clinical trials for the treatment of RA. In a pre-clinical study, Mahajan et al. evaluated the potency and selectivity of decernotinib against JAK3 kinase activity and inhibition of JAK3-mediated signaling *in vitro* and JAK3-dependent physiologic processes *in vivo* [77]. They found that decernotinib potently inhibits JAK3 in enzyme assays (K_i = 2.5 nM) and cellular assays dependent on JAK3 activity (IC_{50} range, 50–170 nM), with limited or no measurable potency against other Janus kinase isotypes or non-JAK enzymes. These data again indicate that potencies based upon purified proteins can differ markedly from cellular or whole animal measurements. Decernotinib resulted in dose-dependent reduction in ankle swelling in a rat collagen-induced arthritis model. In a mouse model of oxazolone-induced delayed-type hypersensitivity, decernotinib reduced the T cell-mediated inflammatory response in skin. These investigators concluded that the data support evaluation of decernotinib for treatment of patients with autoimmune and inflammatory problems such as rheumatoid arthritis.

Genovese et al. reported on a double-blinded, randomized, phase II clinical trial in patients with RA receiving decernotinib at doses of 100 mg (n = 11), 200 mg (n = 10), 300 mg (n = 10), or placebo (n = 12) once daily for 12 weeks [78]. They determined the American College of Rheumatology score (ACR20; improvement of ≥20%) and disease activity score (DAS) using C-reactive protein (CRP) and the RA MRI (magnetic resonance imaging) scoring (RAMRIS) systems. They found that the ACR20 response at 12 weeks was 63%, 60%, and 60% in the decernotinib 100-mg, 200-mg and 300-mg groups, respectively, compared with 25% in the placebo group. They also reported that the DAS-CRP scores decreased in a dose-dependent fashion. Decreases in RAMRIS synovitis scores were significantly different from placebo for all of the decernotinib-dose cohorts and for the RAMRIS osteitis scores for the decernotinib–300 mg cohort. They reported that treatment was generally well tolerated. These investigators also found that decernotinib combined with methotrexate improved the signs and symptoms of RA after 12 and 24 weeks when compared with the placebo group.

Decernotinib binds to the active conformation of JAK2 and is therefore a type I inhibitor [27]. The pyridine nitrogen forms a hydrogen bond with the N—H group of the hinge L932 while the pyrrolo N—H group hydrogen bonds with the L932 carbonyl oxygen (Fig. 9F). The drug makes hydrophobic contacts with L855 before the G-rich loop, V863 after the G-rich loop, A880 (CS7), V911 (Sh1), I982 (CS4), L983 (CS6), V984 (CS5), and N981 of the catalytic loop (HRDLATRN). The drug makes van der Waals contact with DFG-D994 and Y931 of the hinge. The decernotinib clinical trials for the treatment of RA thus far appear promising.

5.2.2. Peficitinib

Peficitinib is an orally effective pyrrolo[2,3-*b*]pyridine derivative (Fig. 8F) that is in clinical trials for the treatment of psoriasis, RA, and ulcerative colitis. Takeuchio et al. evaluated the efficacy, safety and dose response of peficitinib as monotherapy in Japanese patients with moderate to severe rheumatoid arthritis [79]. In a 12-week, double-blind study, 281 adult patients with active RA and not on concomitant disease-modifying antirheumatic drug (DMARD) therapy were randomized equally to once-daily placebo or peficitinib at a dosage of 25, 50, 100 or 150 mg. The primary endpoint was American College of Rheumatology (ACR) 20 response in the peficitinib treatment groups vs. placebo after 12 weeks. The mean age of the cohort was 53 years and one quarter had previously received anti-TNF therapy.

Peficitinib treatment prescribed at 50, 100 and 150 mg amounts each showed statistically significantly higher ACR20 response rates compared to the placebo and response rates increased up to the 150 mg dosage. Adverse events included neutropenia, headache, and abdominal pain. The treatment-emergent adverse events occurring more frequently in the peficitinib group compared with the placebo group included diarrhea, nasopharyngitis, and increased serum creatine phosphokinase activity. No cases of serious infections were reported. Herpes zoster occurred in four patients (two each in the peficitinib 25 and 100 mg cohorts). The authors concluded that treatment with peficitinib as monotherapy for 12 weeks in Japanese patients with moderate to severe RA is efficacious and showed an acceptable safety profile.

No X-ray structural studies of peficitinib bound to the Janus kinases have been reported. To obtain an idea on the possible interaction of this drug with JAK3, the Schrödinger Glide Suite (2016-1 release) was used to dock the drug into JAK3 (with initially bound tofacitinib, PDB ID: 3LXK) [70]. The pose in the computed model indicates that the pyrrolo N—H group makes a hydrogen bond with the carbonyl group of E903 of the hinge and the pyridine nitrogen hydrogen bonds with the N—H group of L905 while the exocyclic N—H group hydrogen bonds with the carbonyl group of L905, also within the hinge (Fig. 9G). In this model, the drug makes hydrophobic contacts with L828 immediately before the G-rich loop, V835 immediately after the G-rich loop, A853 (CS7), V884 (Sh1), M902 within the hinge, and I955 (CS4) and L956 (CS6). It also makes van der Waals contact with DFG-D967. Clinical studies with this drug are in the early stages.

5.2.3. Filgotinib

Filgotinib is an orally effective triazolopyridine congener (Fig. 8G) that is in clinical trials for the treatment of RA and Crohn disease. Filgotinib is an orally effective JAK1 (EC_{50} of 10 nM), JAK2 (2.8 nM), and TYK2 (11 nM) inhibitor; it is a less effective JAK3 inhibitor (81 nM) [64]. Clinical trials are in early stages thus far establishing a daily dose of 200 mg [80]; no studies reporting clinical efficacy have been forthcoming.

X-ray crystal structures of complexes of figlotinib with JAK2 have been reported [64]. The drug binds to the active conformation of the protein kinase domain and is therefore a type I inhibitor [27]. Figlotinib binds within the adenine pocket of these proteins. The *N*1 of the triazolopyridine scaffold forms one hydrogen bond with the hinge residue N—H group of L932 of the hinge and the exocyclic N—H of the drug forms a hydrogen bond with the carbonyl

group of L932 (Fig. 9H). On the whole, figlotinib makes hydrophobic contacts with the β1-strand L885 before the G-rich loop, V863 (CS7), A886 (CS8), and M929 and Y931 of the hinge, and L983 (CS6). The drug also makes van der Waals contact with β3-K882. There are no available crystal structures for the next five drugs and the glide and the induced-fit docking programs [70,81] failed to yield satisfactory models.

5.2.4. Fedratinib

Fedratinib is an anilinopyrimidine derivative (Fig. 8H) that inhibits JAK2 and is in clinical trials for the treatment of myelofibrosis (Table 6). Pardanani et al. performed a phase I trial with fedratinib in 59 patients with high- or intermediate-risk primary myelofibrosis or myelofibrosis developing after polycythemia vera or essential thrombocythemia [82]. The maximum-tolerated dose was 680 mg daily and dose-limiting toxicity was a reversible asymptomatic increase in the serum amylase level. Forty-three patients (73%) continued treatment beyond six cycles; the median cumulative exposure to fedratinib was 380 days. Grade 3–4 incidence rates of adverse events included nausea (3%), vomiting (3%), diarrhea (10%), anemia (35%), and thrombocytopenia (24%). Fedratinib treatment had a modest effect on serum cytokine levels, but more than half of the patients achieved rapid and durable improvement in their constitutional symptoms including early satiety, night sweats, fatigue, pruritus, and cough. After six and 12 cycles of treatment, 39% and 47% of the patients, respectively, exhibited a decreased spleen size. The majority of patients with leukocytosis or thrombocytosis at the initial phase of the study (n = 28 and n = 10, respectively) achieved normalization of their blood counts after six (57% and 90%) and 12 (56% and 88%) cycles. A significant decrease in the JAK2 V617F allele burden was observed at six months in the 51 mutation-positive patients and the decrease lasted for more that a year. The authors concluded that fedratinib is well tolerated and produces a significant reduction in the disease burden and a durable clinical benefit in patients with myelofibrosis.

5.2.5. Momelotinib

Momelotinib is an anilinopyrimidine derivative (Fig. 8I) that is in clinical trials for the treatment of myeloproliferative neoplasms as well as NSCLC and pancreatic cancer (Table 6). In pre-clinical studies, Tyner et al. found that between 0.5 and 1.5 μM momelotinib produced growth suppression and apoptosis in JAK2-dependent human and murine hematopoietic cell lines, while non-hematopoietic cell lines were unaffected [83]. Based upon work with an experimental murine myeloproliferative system, Tyner et al. found that momelotinib restored physiologic levels of inflammatory cytokines and it also normalized the hematocrit, white blood cell counts, and spleen size [83]. Although these responses included a reduction of the JAK2 V617F allele burden, the JAK2 V617F mutant-containing cells persisted. Consistent with the results of clinical trails with JAK2 inhibitors, myeloproliferative growth resumes following cessation of treatment.

Pardanani et al. conducted a Phase I/II clinical trial of momelotinib in patients with high- or intermediate-risk primary or post-polycythemia vera/essential thrombocythemia myelofibrosis [84]. In the dose-escalation phase ($n = 21$), the maximum-tolerated oral dose was 300 mg daily based on reversible grade 3 headaches and an increase in serum lipase activity. Anemia and spleen responses, based upon standardized criteria, were 59% in a cohort receiving 150 mg daily. Among 33 patients who received blood transfusions in the month prior to study entry, 70% achieved a minimum 12-week period without transfusions. Most patients experienced an improvement of their constitutional symptoms. Grade 3 or 4 adverse reactions included thrombocytopenia (32%), hyperlipasemia (5%), elevated liver transaminases (3%), and headache (3%). The plasma levels of more than a dozen cytokines decreased in a large percentage of patients. They reported that momelotinib is well tolerated and produces significant improvements in the signs and symptoms including anemia and spleen size in myelofibrosis patients.

5.2.6. Lestaurtinib

Lestaurtinib is an orally available indolocarbazole (Fig. 8J) that is in clinical trials for the treatment of psoriasis and a variety of neoplastic disorders (Table 6). This drug is a potent inhibitor of the Janus kinases (Table 5) as well as FLT3, TRKA, and PRK1 (pubchem.ncbi.nlm.nih.gov). FLT3 is a transmembrane receptor protein-tyrosine kinase that is stimulated by FLT3LG and regulates the growth, proliferation, differentiation, and survival of hematopoietic progenitor and dendritic cells. TRKA is a transmembrane receptor protein-tyrosine kinase that is stimulated by nerve growth factor and regulates the growth, proliferation, differentiation, and survival of neurons in both the central and peripheral nervous systems. PRK1 is a protein kinase C-related cytosolic serine/threonine-protein kinase that is activated by Rho proteins and is involved in the regulation of transcription, cell motility, and tumor cell invasion.

Santos et al. conducted a phase II clinical study of lestaurtinib in 22 JAK2 V617F mutant-positive myelofibrosis patients (80 mg orally twice daily) [85]. The response rate was 27%. Median time to response was three months and the response lasted more than 14 months. These investigators found that there was no improvement in bone marrow fibrosis nor was there any improvement in the JAK2 V617F allele burden. However, they found that lestaurtinib decreased the phosphorylated STAT3 levels. They reported that eight patients (36%) encountered various grade 3 or 4 toxicities and 6 (27%) required a reduction in dosage. The main side effects that resulted from lestaurtinib treatment included myelosuppression with anemia and thrombocytopenia as well as gastrointestinal symptoms including nausea, vomiting, and diarrhea. Based upon the results of this clinical trial, the authors concluded that lestaurtinib produced modest beneficial responses in myelofibrosis.

5.2.7. Gandotinib

Gandotinib is an imidazopyridazine derivative (Fig. 8K) that inhibits JAK2 and is in clinical trials for myeloproliferative disorders. Results from a phase I/II trial with 19 subjects demonstrated a decrease in spleen size in 22% of the patients with myeloproliferative neoplasms [86]. The JAK2 V716F allele burden was unaffected. Main adverse events included anemia, diarrhea, electrolyte imbalance, and nausea.

5.2.8. Pacritinib

Pacritinib is a macrocyclic JAK2 inhibitor (Fig. 8L, Table 5) that is in clinical trials for the treatment of myelofibrosis, leukemia, and solid tumors (Table 6). Chow et al. reported that the drug reduced spleen size and ameliorated bone pain, fatigue, fever, itching, night sweats, and weight loss [87]. Common adverse events included nausea, vomiting, and diarrhea, while the development of severe hematologic toxicities including anemia and thrombocytopenia was limited. However, the FDA placed all ongoing clinical trials with pacritinib on hold in February 2016 owing to possibility of the increased danger of intracranial hemorrhages and cardiac events.

6. Non-conserved amino acid residues near the Janus kinase ATP-binding sites

The amino acid residues that make up the Janus kinase ATP-binding site and the surrounding region are highly conserved thereby making it difficult to design inhibitors that are specific for a particular family member. Non-conserved residues are listed in

Table 7
Non-conserved residues near the ATP-binding site of Janus kinases.

Location[a]	JAK1	JAK2	JAK3	TYK2
β1-strand	Arg879	Gln853	Ser826	Arg901
G-rich loop	His885	Asn859	Asn832	His907
Hinge 1	Phe958	Tyr931	Tyr904	Tyr980
Hinge 2	Ser961	Tyr934	Ser907	Leu983
Hinge 3	Ser963	Ser936	Cys909	Ser985
αDE loop	Pro969	Gln942	Gln915	Pro991
Catalytic loop	Ala1006	Thr979	Ala952	Ala1026
After catalytic loop	Val1009	Ile982	Ile955	Val1029
Before DFG	Gly1020	Gly993	Ala966	Gly1040

[a] Depicted in Fig. 10.

Table 7 and their locations within the JH1 domains are depicted in Fig. 10. Of importance is the Cys909 in JAK3 that serves as a potential target for the covalent attachment of an inhibitor. Drugs that possess an α,β-unsaturated carbonyl group undergo a Michael reaction that involves the addition of a nucleophile (the –SH of cysteine) to the double bond to form a covalent Michael adduct. Non-covalent interactions position the drug in a productive orientation within the ATP-binding pocket that allows the covalent modification to proceed. Three FDA-approved drugs use this inhibitory mechanism including afatinib (targeting EGFR in the treatment of NSCLC), ibrutinib (targeting BTK in mantle cell lymphoma), and osimertinib (targeting EGFR T970M mutants in NSCLC) (www.brimr.org/PKI/PKIs.htm).

The gatekeeper residue in the four Janus kinases is the rather bulky methionine. This residue serves as a molecular gate that controls access to a hydrophobic pocket or back cavity adjacent to the adenine binding site [88,89]. Its shape and size are primarily controlled by the nature of the gatekeeper residue, which is the last residue of the β5-strand for the Janus kinases or the first residue of the hinge connecting the small and large lobes, depending upon the particular enzyme. When the gatekeeper is small, the hydrophobic pocket expands toward the β5-strand and the αC-helix and large antagonists can be accommodated. When the gatekeeper residue has a bulky side chain, as in the cases of the Janus kinases, the back cavity is small and delimited by the β4-strand and by the gatekeeper side chain itself. This limits the size of potential Janus kinase antagonists owing to the gatekeeper methionine blockade of the back pocket. In agreement with this supposition, none of the drugs described in Section 5 extend toward the β5-strand or the αC-helix.

7. Epilogue

Of all of the human protein kinase proteins, only five contain both a pseudokinase and an active kinase: JAK1/2/3, TYK2, and GCN2 (a serine/threonine protein kinase) (Supplementary material in Ref. [4]). The latter enzyme is encoded by the *EIF2AK4* gene and catalyzes the phosphorylation of protein synthesis elongation factor-2. MSK1/2, obscurin, RSK1/2/3/4, and SPEG are protein-serine/threonine kinases that contain two active catalytic domains. The MSK and RSK enzymes catalyze the phosphorylation of ribosomal proteins and SPEG is a striated muscle preferentially expressed gene that participates in muscle cell differentiation. Obscurin is the product of the *OBSCN* gene and consists of 7968 amino acid residues with a molecular mass of 868 kDa! This enzyme participates in myofibrillogenesis.

There are 32 small molecule protein kinase inhibitors approved by the FDA for the treatment of human illnesses (www.brimr.org/PKI/PKIs.htm) and one drug (oclacitinib) that is approved for the treatment of canine dermatitis. Whereas the cost of the drugs for human sicknesses is around $10,000 per month in the United States and is steadily increasing, the cost of the canine drug is about $60 per month. As noted previously, the high cost of small molecule inhibitors produces financial toxicity [22], a concern that is not addressed in clinical trials and only becomes apparent when the patient is billed. Even with health insurance, the cost of co-payments is often prohibitive.

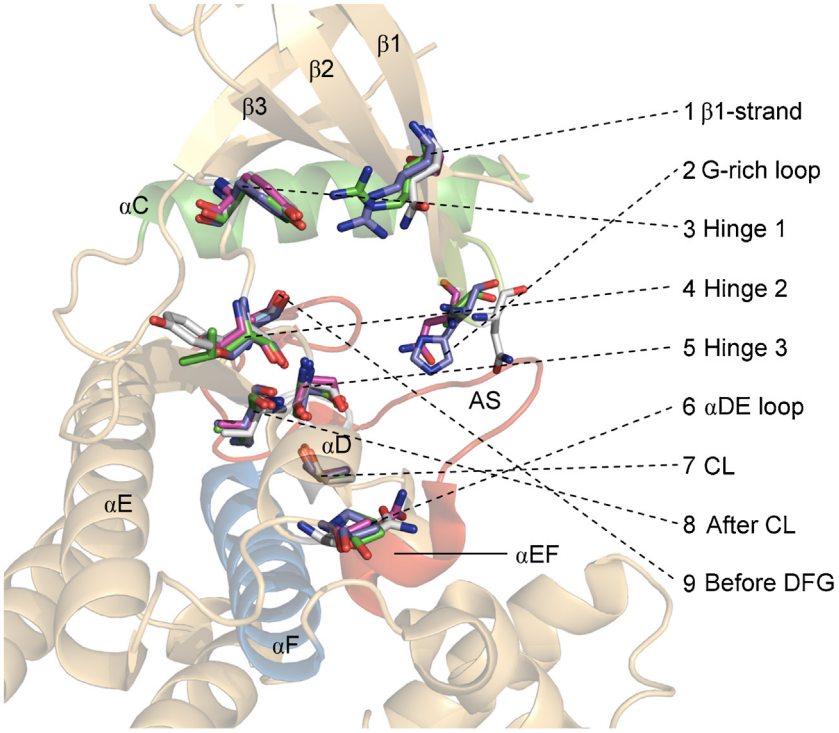

Fig. 10. Non-conserved residues in the ATP-binding site listed in the amino to carboxyl direction. The carbon atoms have the following colors: JAK1, blue; JAK2, gray; JAK3, magenta; TYK2, green. The figure is a superposition of JAK1/2/3 and TYK2 PDB IDs 3EYG/3FUP/3LXK and 3LXN, respectively.

There are more than 200 different small molecule protein kinase inhibitors in clinical trials worldwide. Although one aim in the development of such inhibitors has been to target a single enzyme, it has turned out that many, if not most, of the selective inhibitors have been found at later stages to inadvertently inhibit multiple targets. It may be counterintuitive, but the majority of the approved protein kinase antagonists are multikinase inhibitors. Moreover, their therapeutic effectiveness may be related to the simultaneous inhibition of more than a single target. Accordingly, we have the question of whether magic shotguns are to be preferred over magic bullets [90].

Nearly all of the initial approved small molecule protein kinase inhibitors were directed at various malignancies. The exceptions include: sirolimus (an mTOR inhibitor) that was approved for the prevention of renal graft-vs.-host disease in 1999, tofacitinib (a pan-JAK inhibitor) that was approved for the treatment of RA in 2012, and nintedanib (a fibroblast growth factor receptor multikinase inhibitor) that was approved for the treatment of idiopathic pulmonary fibrosis in 2014. As the number of disease targets increases, we can expect advances in clinical efficacy and subsequent approval of new drugs targeting additional protein kinases as well as additional illnesses such as autoimmune afflictions (as described in this paper), hypertension, and Parkinson disease [91].

Conflict of interest

The author is unaware of any affiliations, memberships, or financial holdings that might be perceived as affecting the objectivity of this review.

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