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Small molecule drug discovery targeting the JAK-STAT pathway

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ABSTRACT

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway functions as a central hub for transmitting signals from more than 50 cytokines, playing a pivotal role in maintaining hematopoiesis, immune balance, and tissue homeostasis. Dysregulation of this pathway has been implicated in various diseases, including immunodeficiency, autoimmune conditions, hematological disorders, and certain cancers. Proteins within this pathway have emerged as effective therapeutic targets for managing these conditions, with various approaches developed to modulate key nodes in the signaling process, spanning from receptor engagement to transcription factor activation. Following the success of JAK inhibitors such as tofacitinib for RA treatment and ruxolitinib for managing primary myelofibrosis, the pharmaceutical industry has obtained approvals for over 10 small molecule drugs targeting the JAK-STAT pathway and many more are at various stages of clinical trials. In this review, we consolidate key strategies employed in drug discovery efforts targeting this pathway, with the aim of contributing to the collective understanding of small molecule interventions in the context of JAK-STAT signaling. We aspire that our endeavors will contribute to advancing the development of innovative and efficacious treatments for a range of diseases linked to this pathway dysregulation.

1. Introduction

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway mediates membrane-to-nucleus signaling, facilitating rapid gene transcription and serving as a central communication node in mammalian cells [1,2]. This pathway plays a crucial role in maintaining hematopoiesis, immune balance, and tissue homeostasis (Fig. 1A). By regulating the differentiation, activation, and proliferation of immune cells, as well as the synthesis of immune mediators, the JAK-STAT pathway harmonizes the orchestration of immune responses, oversees inflammatory processes, preserves immune equilibrium, and conducts vigilant surveillance against tumor development [3–6]. Complete deficiencies in JAK1, JAK2, STAT3, or STAT5 lead to lethality, while the dysregulation of this pathway can lead to various diseases conditions [6,7]. Genetic variations in genes encoding the JAK-STAT components including cytokines, receptors, JAKs (JAK1, JAK2, JAK3 and TYK2) and STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) have been identified as risk factors for immunodeficiencies, autoimmune diseases, or hematological disorders, and certain cancers [8–10]. Notably, mutations in JAK3 and TYK2 have been associated with primary immunodeficiency, particularly severe combined immune deficiency (SCID), characterized by impaired development or function of lymphocytes [8,11,12]. Gene mutations in the shared γ_c subunit are responsible for X-linked SCID, leading to diminished T-cell and natural killer (NK) cell numbers and function, while B-cell numbers are preserved but functionally impaired [11–14]. Mutations in STAT1 have been identified in patients with disseminated infections caused by Bacille Calmette–Guérin (BCG) or nontuberculous mycobacteria, while

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excessive STAT1 activation leads to exaggerated interferon (IFN)-γ responses and inhibits interleukin (IL)-17 production, leading to the development of autoimmune conditions [12,15]. Polymorphisms in STAT3 have been linked to Crohn's disease (CD), psoriasis and Behçet's disease [16]. Similarly, polymorphisms in STAT4, activated by IL-12 and type I IFNs, have been linked to systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren's syndrome, and variants in STAT6 are associated with elevated IgE levels and atopic dermatitis [12, 17]. Single nucleotide polymorphisms in the TYK2 gene have been strongly linked to SLE, and also associated with other autoimmune conditions including CD, ulcerative colitis (UC), type 1 diabetes mellitus, multiple sclerosis (MS), and RA [12,18,19]. The sustained activation of JAK1 and TYK2, resulting from increased serum IFNs and expression of IFN inducible genes, is considered crucial in the molecular pathogenesis of SLE. Variants in the IL-12 and IL-23 signaling pathway have been linked to disorders such as CD and UC [20,21]. While JAK2 signaling is crucial for maintaining hematopoietic stem cell (HSC) homeostasis, conditional knockout of JAK2 leads to bone marrow failure,

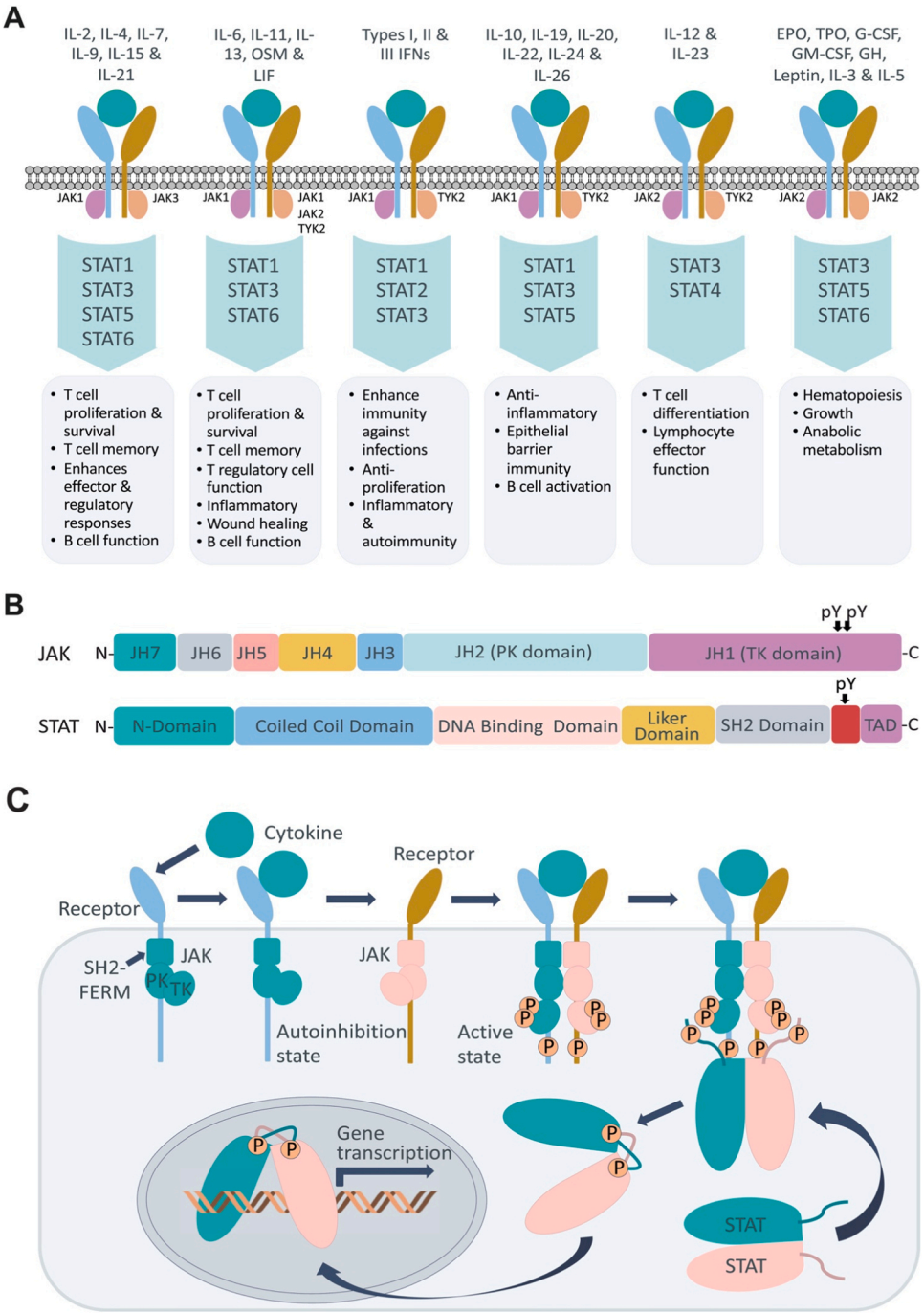

Fig. 1. The main functions and mechanism of the JAK-STAT pathway signaling. (**A**) Over 50 cytokines participate in the JAK-STAT pathway. These cytokines bind to receptor pairs, triggering the phosphorylation of specific STATs and giving rise to a variety of biological effects. **(B)** Domain arrangement of JAKs (top) and STATs (bottom). **(C)** Signal transduction in the JAK-STAT pathway. In the absence of cytokine binding, the receptor is in an autoinhibited state. Upon binding to a cytokine, it forms a pair with another receptor partner, inducing a conformational change in the tyrosine kinase (TK) domain that leads to the release of autoinhibition. Subsequently, JAKs are activated, resulting in the phosphorylation of the intracellular domain of receptors, creating docking sites for STATs. The STATs are recruited, phosphorylated, and then translocated into the nucleus, initiating gene transcription.

increased apoptosis, and loss of quiescence in HSC-enriched cells [22–24]. On the other hand, gain-of-function mutations in JAK2 are specifically associated with myeloproliferative neoplasms (MPNs), including polycythemia vera, essential thrombocythemia, and primary myelofibrosis (PMF). The most prevalent JAK2 mutation, V617F, is detected in more than 95% of polycythemia vera patients and in 32–57% of individuals with essential thrombocythemia or PMF [23–26].

Due to its significant implications for human health and disease, the components of the JAK-STAT pathway have emerged as crucial targets for a range of conditions, including autoimmune diseases, immunodeficiency, hematopoietic disorders, and cancer [27–30]. Several cytokines within this pathway have obtained FDA approval for therapeutic purposes, spanning the treatment of hematopoietic disorders, antiviral interventions, cancer therapy, and other applications. For instance, hematopoietic cytokines such as erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are utilized in the management of anemia-associated conditions, low neutrophil counts, and myeloid reconstitution, respectively [31–34]. The IFN family of cytokines such as IFNα, which have been approved for the management of viral infections, including hepatitis B virus (HBV) and hepatitis C virus (HCV) [35,36]. Beyond their antiviral applications, cytokines hold significant importance in oncology. IFNα, approved in 1986 for the treatment of hairy cell leukemia, has demonstrated versatility and success in various cancers, including chronic myeloid leukemia (CML), follicular lymphoma, melanoma, renal carcinoma, hepatocellular carcinoma (HCC), infantile hemangioma, and Kaposi sarcoma [37–39]. Another significant cytokine in cancer therapy is IL-2, which regulates and activates various immune cells, including T, B, NK, and dendritic cells[40]. High-dose IL-2 has received FDA approval for metastatic kidney cancer in 1992 and metastatic melanoma in 1998 [41,42]. Its therapeutic efficacy is attributed to its ability to stimulate the immune system and enhance immune cell activity, particularly cytotoxic T cells and NK cells, promoting the recognition and elimination of cancer cells [43]. In recent years, cytokine engineering has emerged as a promising approach to modify cytokines, enhancing their pharmacokinetic and pharmacodynamic properties, and demonstrating significant potential in improving therapeutic efficiency, particularly in cancer therapy. Additionally, antibodies targeting cytokines or receptors present another class of medical molecules targeting this pathway [44,45]. Antibodies targeting IL-6 or its receptor, such as Tocilizumab, Siltuximab, Sirukumab, Olokizumab, and Sarilumab, have been approved by the FDA or are undergoing clinical trials for various autoimmune conditions, including RA, CD, ankylosing spondylitis, and Castleman's disease [46–48]. Antibodies binding to IL-23 receptors, such as Ustekinumab, Briakinumab, Tildrakizumab, Guselkumab, LY2525623, AMG139, BI-655066, and LY3074828, have been developed for treating autoimmune disorders like psoriasis, psoriatic arthritis (PsA), and CD [46, 49–51].

In addition to targeting cytokines or their receptors within the JAK-STAT pathway, small molecules that aim at intracellular components downstream of this pathway, including JAKs and STATs, represent another significant class of therapeutics for JAK-STAT-related diseases [8, 16, 52–55]. The successful development of JAK inhibitors such as tofacitinib for RA treatment and ruxolitinib for managing PMF stands as landmarks, highlighting the potential of small molecules in modulating the JAK-STAT pathway [56,57]. Subsequently, many other compounds have gained FDA approval for treating autoimmune conditions such as RA, UC, AA, and AD, or hematopoietic disorders like myelofibrosis and polycythemia, with numerous others progressing through clinical trials (Table 1)[53,55,58]. Notably, following the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) worldwide, small molecule JAK inhibitors have demonstrated significant efficacy in alleviating symptoms and reducing mortality rates among COVID-19 patients experiencing cytokine storm and cytokine release syndrome [59–61].

In this review, we focus on the discovery and development of small molecule drugs targeting the JAK-STAT pathway, providing key practical and conceptual proposals that could contribute to the advancement of safer and more effective therapeutics for autoimmune diseases and JAK-STAT-related hematopoietic tumors in the future.

2. JAK-STAT pathway

The JAK-STAT pathway is structurally simple, consists of four key elements: ligands, receptors, JAKs and STATs. To date, approximately 50 cytokines, and 40 receptors have been identified to engage in this pathway (Fig. 1A)[62,63]. While these cytokines are small proteins with four or five helices that are connected by loops, the receptors are type I transmembrane proteins typically featuring extracellular N-terminal domains consisting of one or more cytokine-binding homology regions (CHRs), crucial for ligand binding, and an unstructured intracellular C-terminal region [63–65]. In contrast, only four JAKs, and seven STATs are involved downstream. The JAKs are composed of seven JAK homology (JH) domains, arranged from the C-terminus to the N-terminus as JH1 to JH7 (Fig. 1B). JH1, also known as the tyrosine kinase (TK) domain, is approximately 270 amino acids in length, primarily responsible for phosphorylating substrate molecules. JH2 is structurally similar to the kinase domain but lacks kinase activity, earning it the name pseudokinase (PK) domain. Despite its lack of enzymatic activity, JH2 plays a crucial role in regulating the activity of the kinase domain [62, 66,67]. JH3 and JH4 in JAKs constitute the Src homology 2 (SH2) domain, while the 'four-point-one, ezrin, radixin, moesin' (FERM) domain is composed of JH5, JH6 and JH7. The SH2 and FERM domains in JAKs work together to facilitate cytokine receptor recognition and binding by JAKs [68,69]. The STATs are approximately 600–850 amino acids in length and comprise the N-terminal domain (NTD), coiled-coil domain, DNA-binding domain, linker region, SH2 domain, and transactivation domain (TAD) from the N- to the C-terminus (Fig. 1B)[70,71].

Without ligand binding, individual receptors diffuse separately on the cell membrane and the JAKs are associate with the intracellular domain of receptors, but maintaining an autoinhibited state (Fig. 1C) [72]. Activation of the pathway commences when cytokines bind to receptors, resulting in the formation of a functional ligand-receptor complex. In this complex, receptor pairs are in close proximity, relieving the autoinhibition state of JAKs. Subsequent activation of JAKs leads to the phosphorylation of the intracellular domain of receptors, creating docking sites for the recruitment of STATs [72–74]. This is followed by the recruitment and phosphorylation of STATs, leading to the formation of STAT dimers that adopt a conformation ready for DNA binding. The phosphorylated STATs then translocate into the nucleus, where they promote gene transcription (Fig. 1C)[62,70,71].

3. Current JAK-STAT inhibitors

As no compensatory pathways are currently known to circumvent the JAK-STAT pathway, the JAKs themselves play an indispensable role in regulating cytokine signaling. Consequently, small molecules that selectively bind to the active site of the TK domain of JAKs have become the cornerstone of drug development endeavors targeting this pathway [29,30,75]. The successful development of JAK inhibitor tofacitinib for RA treatment and ruxolitinib for the management of PMF, which now benefit thousands of patients, stand as landmarks, exemplifying the potential of small molecules in modulating the JAK-STAT pathway [56, 57]. Subsequently, several further compounds have gained approval from the FDA, with numerous others progressing through clinical trials (Table 1)[29,75]. In addition to targeting the TK domain of JAKs, the exploration of small molecules targeting the PK domain of JAKs or the STATs themselves has gained traction in recent years as promising avenues for drug development [76–78].

Table 1
Development progress of JAK inhibitors.

Inhibitor	JAK selectivity (IC_{50}, nM)				Indication	Clinical status
	JAK1	JAK2	JAK3	TYK2		
Tofacitinib	3.2	4.1	1.6	34.0	RA, UC, PsA, AA	Approved (FDA, EMA, PMDA, NMPA)
					psoriasis	
					GVHD, CD, SLE	Phase III
						Phase II
Upadacitinib	8	600	2300	NA	RA, AD, PsA	Approved (FDA, EMA, PMDA, NMPA)
					CD, UC, SLE, HS	
					leukoderma	Phase III
						Phase II
Abrocitinib	29	803	>10,000	1250	AD	Approved (FDA, EMA, PMDA, NMPA)
					hypersensitivity, immunologic derangement	
						Phase III
Ruxolitinib	3.3	2.8	390	18	myelofibrosis, GVHD, polycythemia vera, acute myeloid leukemia	Approved (FDA, EMA, PMDA, NMPA)
					myeloproliferative disorders, AD, essential thrombocythemia	Phase III
					breast cancer	Phase II
Baricitinib	5.9	5.7	420	60	RA	Approved (FDA, EMA, PMDA, NMPA)
					AD, AA, SLE	
					DM, ITP	Phase III
						Phase II
Filgotinib	10	28	810	116	RA	Approved (EMA, PMDA)
					UC, CD, PsA	Phase III
					Sjtoegren syndrome, SLE, uveitis, MN	Phase II
Peficitinib	3.9	5.0	0.7	4.8	RA	Approved (PMDA)
					UC, psoriasis	Phase II
Ritlecitinib	>10,000	>10,000	33.1	>10,000	AA	Approved (FDA)
					leukoderma	Phase III
					RA, Sezary syndrome	Phase II
Oclacitinib	10	18	99	84	For dogs: AD, pruritus	Approved (FDA, EMA)
Fedratinib	NA	3	NA	NA	myelofibrosis	Approved (FDA, EMA)
					hematologic malignancies, polycythemia vera	Phase III
					myeloproliferative disorders, myeloma, acute myeloid leukemia	Phase II
Delgocitinib	2.8	2.6	13	58	AD	Approved (PMDA)
					eczema	Phase III
					AA, SLE	Phase II
Pacritinib	1280	23	520	50	myelofibrosis	Approved (FDA)
					essential thrombocythemia	Phase III
					myeloproliferative disorders, leukemia, HD	Phase II
Deucravacitinib	1(JH2)	NA	NA	0.2 (JH2)	psoriasis	Approved (FDA, EMA, PMDA)
					SLE, PsA, Sjogren-Larsson syndrome	Phase III
					IBD, AID	Phase II
Momelotinib	11	18	155	NA	myelofibrosis,polycythemia vera, PDAC	Approved (FDA)
					essential thrombocythemia, polycythemia	Phase II
Jaktinib	NA	NA	NA	NA	myelofibrosis, AD, spondyloarthritis	Phase III
					psoriasis, pneumonia, myeloproliferative disorders	Phase II
Ivarmacitinib	NA	NA10	NA77	NA420	RA, AD, spondyloarthritis, UC, leukoderma	Phase III
					CD	Phase II
Deuterated Ruxolitinib	NA	NA	NA	NA	AA	Phase III
Povorcitinib	<10	NA	NA	NA	HS	Phase III
					Leukoderma, urticarial, pruritus, asthma	Phase II
Decernotinib	10	10	2.5	10	RA	Phase III
WXFL-10203614	NA	NA	NA	NA	RA	Phase III
Itacitinib	2	63	>2000	795	GVHD	Phase III
					myelofibrosis, NSCLC, myeloproliferative disorders, HD	Phase II
TLL-018	NA	NA	NA	NA	RAIII	Phase III
					Psoriasis, DMD, UC	Phase II
Brepocitinib	17	77	6.9	NA	DM	Phase III
					psoriasis, leukoderma, AA, uveitis, HS, CD, UC	Phase II
Bewintinib	NA	NA	NA	NA	myelofibrosis	Phase III
					GVHD, essential thrombocythemia, polycythemia vera	Phase II
MH-004	NA	NA	NA	NA	AD	Phase III
ATI-77	NA	NA	NA	NA	ADII	Phase II
ATI-501	NA	NA	NA	NA	alopecias	Phase II
ATI-502	NA	NA	NA	NA	alopecias, AA, AD	Phase II
LW-402	NA	NA	NA	NA	AD, RA	Phase II
VC-005	NA	NA	NA	NA	AD, AS	Phase II
Golidocitinib	73	>14,700	>30,000	NA	NSCLC	Phase II
PG-011	NA	NA	NA	NA	AD	Phase II
XH-5102	NA	NA	NA	NA	myeloproliferative disorders	Phase II
Ilginatinib	33	0.72	39	22	myelofibrosis, essential thrombocythemia, polycythemia vera	Phase II
Gandotinib	NA	3	48	44	hematologic malignancies	Phase II
AC-201	NA	NA	NA	NA	SLE, psoriasis	Phase II

(*continued on next page*)

Table 1 (*continued*)

Inhibitor	JAK selectivity (IC_{50}, nM)				Indication	Clinical status
	JAK1	JAK2	JAK3	TYK2		
QY201	NA	NA	NA	NA	AD	Phase II
OST-122	NA	NA	NA	NA	UC	Phase II
CPL-409116	NA	NA	NA	NA	RA	Phase II
AZD-4604	NA	NA	NA	NA	asthma	Phase II
Nezulcitinib	NA	NA	NA	NA	pneumonia	Phase II
KL-130008	NA	NA	NA	NA	AA	Phase II
TD-8236	NA	NA	NA	NA	asthma	Phase II
CJ-15314	NA	NA	NA	NA	SACC	Phase II
TQ-05105	NA	NA	NA	NA	myelofibrosis, GVHD	Phase II
Gusacitinib	46	4	11	8	AD, myelofibrosis	Phase II
SYHX-1901	NA	NA	NA	NA	psoriasis	Phase II
Cerdulatinib	11	6	8	0.5	T-cell lymphoma	Phase III
					Leukoderma, lymphoma, postoperation pain	Phase II
ICP-332	NA	NA	NA	NA	AD, psoriasis	Phase II
VTX-958	NA	NA	NA	NA (JH2)	CD, psoriasis, PsA	Phase II
Zasocitinib	NA	NA	NA	< 0.2 (JH2)	psoriasis, PsA	Phase II
ICP-488	NA	NA	NA	NA (JH2)	psoriasis, AID	Phase II
GLPG-3667	NA	NA	NA	NA	SLE, DM, psoriasis	Phase II
Ropsacitinib	382	74	> 10,000	15	HS, psoriasis, UC	Phase II
ESK-001	NA	NA	NA	NA (JH2)	SLE, uveitis, psoriasis	Phase II

The data presented in this table is extracted from the Pharmacodia database. NA, not applicable; AA, alopecia areata; AD, atopic dermatitis; AS, ankylosing spondylitis; CD, Crohn's disease; DM, dermatomyositis; DMD, duchenne muscular dystrophy; HD, Hodgkin's disease; IBD, inflammatory bowel disease; ITP, idiopathic thrombocytopenic purpura; MN, membranous nephropathy; NSCLC, non-small cell lung cancer; PDAC, pancreatic ductal adenocarcinoma; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SACC, salivary adenoid cystic carcinoma; SLE, systemic lupus erythematosus; TSLP, thymic stromal lymphopoietin; UC, ulcerative colitis.

4. Structural basis of JAK inhibitor binding

Sequence alignment results show that the sequence identity across the length of the two JAKs falls within the range of 35–47%, while the TK domain exhibits greater conservation among JAKs, with sequence identities ranging from 46% to 57%. Crystal structures reveal that the kinase domain of JAKs displays the bilobed architecture—a hallmark shared among the catalytic domains of all protein kinases (Fig. 2A)[79]. The N-terminal lobe of this domain showcases a 6-stranded antiparallel β-sheet, complemented by a singular helix that assumes a pivotal role in catalytic regulation across an array of protein kinases. Positioned within the same N-terminal lobe, the glycine-rich loop, also known as the P-loop, plays an active role in coordinating phosphate groups within ATP. While the C-terminal lobe is predominantly helical with 8 α-helices and two 3/10 helices that harbor two short β-strands within the activation loop. These two lobes are inherently interconnected through a succinct bridge referred to as the "hinge" region. Precisely situated between the two lobes and proximal to the hinge region is the ATP-binding site—a central hub crucial not only for enzymatic activities but also for accommodating inhibitor binding (Fig. 2A)[80]. Crystal structures of tofacitinib in complex with all four JAK isoforms have been solved, providing an avenue for analyzing the inhibition mechanism and for drawing comparisons regarding inhibitor binding (Fig. 2C)[79,80]. Structural alignments unveil that tofacitinib binds to the kinase domain

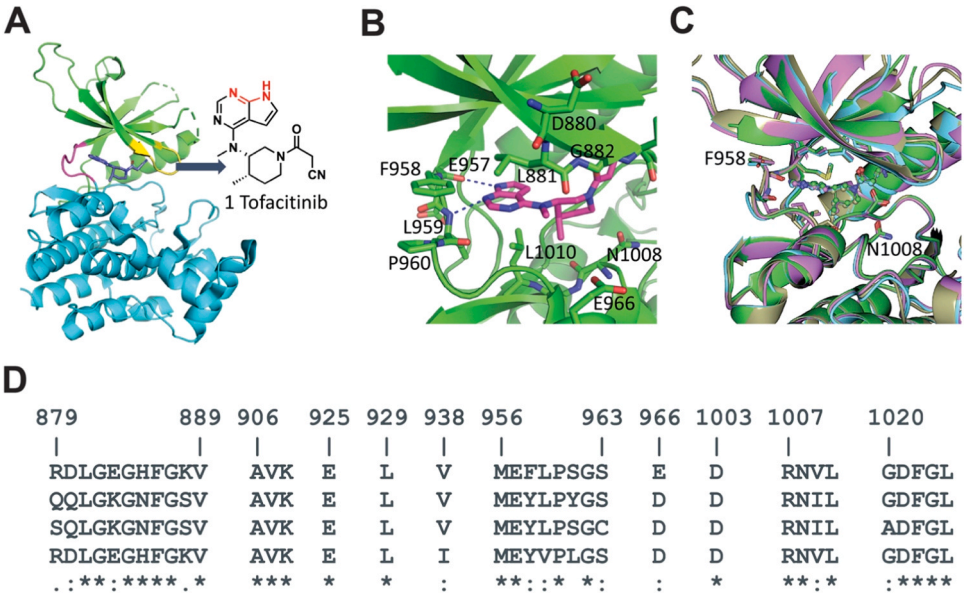

Fig. 2. Structural basis of JAK inhibition. (A) Overall structure of the JAK TK domain, exemplified by JAK1 in complex with the pan-JAK inhibitor tofacitinib (PDB ID: 3EYG). The N-terminal lobe, the C-terminal lobe, P-loop, and hinge region are colored in green, cyan, gold, and magenta, respectively. **(B)** Detailed interaction of the inhibitor with JAK, illustrated by the JAK1-tofacitinib complex. **(C)** Comparison of structures of JAK isoforms in complex with tofacitinib. JAK1, JAK2 (PDB ID: 3FUP), JAK3 (PDB ID: 3LXK), and TYK2 (PDB ID: 3LXN) are represented in green, cyan, gold and magenta, respectively. **(D)** Sequence alignment of active site residues in the four JAKs, arranged from top to bottom as JAK1, JAK2, JAK3, and TYK2, with residue labeled according to JAK1 numbering.

of all four JAK isoforms in a consistent manner (Fig. 2C). The root-mean-square deviation (RMSD) values across the four crystal structures measure less than 1 Å, with ligands from different structures closely overlapping upon superimposition. The pyrrole and pyrimidine groups in tofacitinib, which form hydrogen bonds with the carbonyl of E957 and amino group of L959 (JAK1 numbering, unless otherwise specified) in the hinge region of the protein, are likely pivotal contributors to the binding process. Additionally, the hydrophobic pocket constituted by residues L881, A906, V938, L959, and L1010 can engage in hydrophobic interactions with the inhibitor (Fig. 2B). The binding pockets within the kinase domains of these four JAK isoforms exhibit a considerable degree of similarity, albeit with minor variations (Fig. 2C) [79,80]. This is not surprising, as a comparison of residues within the active site across the four JAKs reveals a significant degree of conservation, except for a few variations in certain residues (Fig. 2D).

5. General strategy for JAK inhibitor design

The crystal structures of tofacitinib:JAK complexes provide an illustrative binding model for JAK inhibitors. We examined several

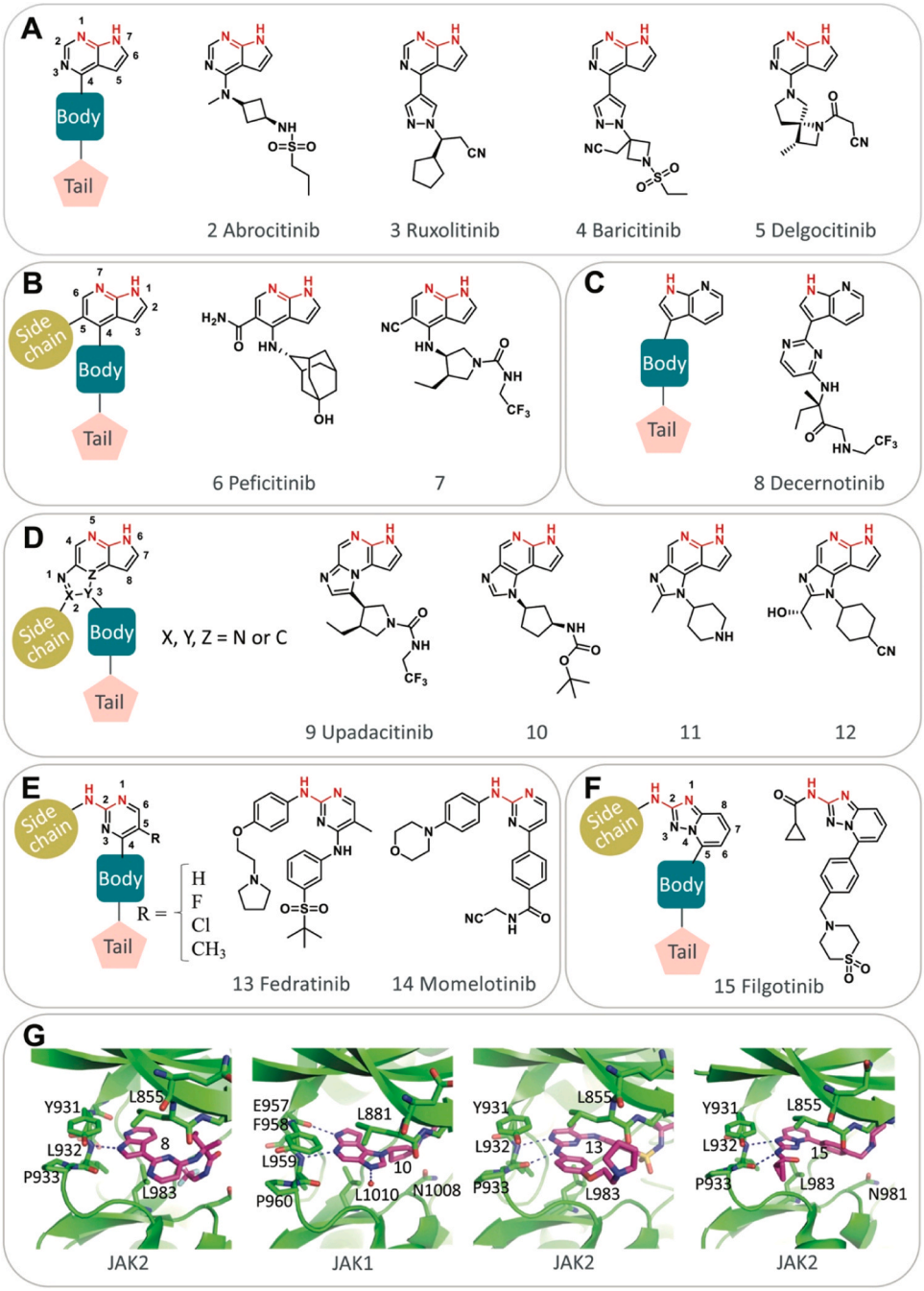

Fig. 3. Representative JAK inhibitors with distinct 'head' groups and their interactions with JAKs. (A) Typical JAK inhibitors featuring a pyrrolopyrimidine head group. **(B)** Typical JAK inhibitors employing a pyrrolopyridine head group. **(C)** JAK inhibitors with a pyrrolopyridine head but an unconventional head-body connection, exemplified by decernotinib. **(D)** Typical JAK inhibitors with a tricyclic compound head. **(E)** Typical JAK inhibitors with a pyrimidine amine compound head. **(F)**Triazolopyridine head JAK inhibitors. **(G)** Binding modes of different types of inhibitors with JAKs. From left to right: decernotinib:JAK2 complex (PDB ID: 4YTI), compound 10:JAK1 complex (representative tricyclic compound head inhibitor, PDB ID: 4E4N), fedratinib:JAK2 complex (typical pyrimidine amine compound head inhibitor, PDB ID: 6VNE), and filgotinib:JAK2 complex (representative triazolopyridine-based inhibitor, PDB ID: 4P7E), respectively.

hundred JAK inhibitors, many of which (but not all) possess distinctive scaffolds, and conceptually divide the inhibitor structure into four distinct segments: the "head", "body", "tail", and "side-chain". The head typically engages with the hinge region through hydrogen bonding; the body is accommodated within the enzyme's hydrophobic pocket; the tail extends from the body to enhance binding affinity; and the side-chain extends from the head, often into the solvent. The design of JAK inhibitors usually starts with creating a hinge-binding head that provides a suitable scaffold for optimizing vectors to enhance potency and selectivity. We categorized these inhibitors into three major groups: bicyclic hinge-binding heads, tricyclic hinge-binding heads, and monocyclic amine hinge-binding heads, based on their structure and interaction with the hinge region (Fig. 3).

Bicyclic compounds, particularly pyrrolopyrimidine and pyrrolopyridine, have been widely employed in the development of JAK inhibitors, as both structures share a common characteristic in binding to the hinge region. Prominent JAK inhibitors, including tofacitinib, ruxolitinib, baricitinib, oclacitinib and abrocitinib, feature a pyrrolopyrimidine head structure (Fig. 3A)[29,30,75]. In the context of the pyrrolopyrimidine head, the N-7 H and N-1 positions form hydrogen bonds with the carbonyl of E957 and the amino group of L959, respectively, as shown in tofacitinib JAK1 complex structure (Fig. 3A) [80]. The nitrogen-atom (N-3) position faces outward towards the solvent, often accompanied by the presence of a water molecule bridging N-3 and E966, which is highly thermodynamically favorable. The carbon atom (C-4) position serves as an attachment site for the molecule's body component. JAK inhibitors featuring the pyrrolopyridine head may exhibit hinge-binding patterns akin to those with a pyrrolopyrimidine head. However, these inhibitors lack the nitrogen atom that corresponds to the N-3 position in pyrrolopyrimidine. Instead, a carbon atom occupies this location, which is thermodynamically unfavorable when facing the solvent. As a result, to optimize binding interactions, this position is typically modified with polar or charged groups such as carboxyl, cyanogroup, acetamide, or pyrrole (Fig. 3B). Peficitinib, a potent inhibitor belonging to this class, has gained approval for treating RA[81,82]. The C-5 position is another point of modification in bicyclic compounds, though it is less common. Decernotinib (VX-509), a potent JAK3 selective inhibitor, serves as a representative example of this type (Fig. 3C)[83]. Decernotinib binds to the enzyme's hinge region in an unconventional manner, with the pyrrole ring facing outward (Fig. 3G), differing from other bicyclic head inhibitors like tofacitinib, ruxolitinib, and baricitinib. In this scenario, only a single hydrogen bond forms between the NH of pyrrole and the amino group of L932 (JAK2 numbering). The inhibitor's body segment is attached to the C-5 position along with the tail, collectively enhancing binding interactions.

Tricyclic pyrrolopyridine derivatives bind to the hinge region of JAKs in a manner similar to tofacitinib's pyrrolopyrimidine (Fig. 3D), with the imidazole, triazole, pyrrole, and pyrazine groups oriented outward toward the solvent. Position 3 is the most common attachment site for the inhibitor body, while position 2 is frequently used for side-chain attachment. Upadacitinib, marketed under the brand name Rinvoq, stands out as a representative inhibitor of this type[84]. While the crystal structure of upadacitinib in complex with JAKs is not available in the Protein Data Bank, we can draw insights from a similar tricyclic hinge-binding head inhibitor, compound 10 (Fig. 3G), which likely shares a similar binding mode with upadacitinib. In this structure, it is observed that the N-5 and N-6 H positions form hydrogen bonds with the amino group of L959 and the carbonyl of E957, respectively. Another hydrogen bond is formed by N-1 position of the ring structure and a water molecule from the solvent. The five-membered body segment is accommodated within the hydrophobic pocket, while the tail resides beneath the P-loop [85].

Monocyclic amine compounds, comprising pyrimidine amines, pyridine amines, pyridazine amines and triazolopyridine, constitute a significant category of hinge-binding units in JAK inhibitors. Within pyrimidine amine compounds, the nitrogen atom within the heterocycle, as well as the exocyclic NH group, form hydrogen bonds with the amino group and carbonyl of L959 in the hinge region, respectively. The carbon atom at the C-4 position serves as the anchoring point for the molecule's body segment. The exocyclic N atom can be attached to various groups, which extend toward the solvent. While the C-5 position is modifiable, its confined space permits modifications with single atoms such as F or Cl, or smaller groups like methyl. JAK inhibitors, such as fedratinib and momelotinib, exemplify this inhibitor type (Fig. 3E). Fedratinib is a JAK2-selective inhibitor that received FDA approval for treating myeloproliferative diseases, including myelofibrosis, in 2019 [86,87]. Momelotinib is a dual JAK1 and JAK2 inhibitor with IC_{50} values of 11 and 18 nM, respectively, which obtained FDA approval for the treatment of myelofibrosis in September 2023 [88,89]. The crystal structure of fedratinib bound to JAK2 underscores its distinct interactions with the hinge region (Fig. 3E)[90]. Notably, it was observed that the N-1 position in the pyrimidine and the exocyclic NH group form hydrogen bonds with the amino and carbonyl groups of L932 (JAK2 numbering), respectively, and the side-chain extending from the exocyclic N atom points towards the solvent, while the body segment linked to C-4 fits snugly into the hydrophobic pocket of JAK2.

In the realm of triazolopyridine-based JAK inhibitors, a somewhat different structure emerges with its bicyclic arrangement; however, it maintains a comparable binding mode to other monocyclic amines at the hinge region. Back in 2012, Dugan and colleagues disclosed a series of 1,2,4-triazolo[1,5-*a*]pyridine derivatives as potent JAK2-selective inhibitors [91]. Two years later, Menet and colleagues reported the discovery of a notable triazolopyridine-based JAK inhibitor filgotinib (Fig. 3F), which has been approved for medical use in both the European Union and Japan [92,93]. Structure elucidation unveiled two hydrogen bond interactions between the triazolopyridine amine moiety and the exocyclic NH group with the carbonyl of L932 (JAK2 numbering) within the hinge region. The phenyl moiety takes occupancy within the enzyme's hydrophobic pocket, while the cyclopropyl group is exposed to the solvent (Fig. 3G).

Once the head is determined, the design of the body typically starts with screening a series of hydrophobic groups, encompassing a spectrum of structures from diverse cyclic formations to aromatic rings or other moieties capable of occupying a binding pocket. The bilobed architecture of JAKs suggests that the pocket's size might undergo slight variations to accommodate the inhibitor optimally. The body segment can span from a compact four-membered ring as seen in abrocitinib to a more extensive adamantane group as observed in peficitinib (Figs. 4A and 4B)[81,94]. It appears that the binding pocket has a preference for accommodating larger-sized groups. Using the same hinge-binding head C-5 pyrazole-substituted pyrrolopyridine, the order of potency follows from cycloheptyl (IC_{50} 119 nM), adamantyl (IC_{50} 209 nM), cyclohexyl (IC_{50} 387 nM), cyclopentyl (IC_{50} 622 nM) to cyclobutyl (IC_{50} 6471 nM) [95]. We measured the distances between the carbon atoms of the cyclobutyl group in abrocitinib and the carbon, nitrogen, or oxygen atoms from its surrounding residues in the JAK1-abrocitinib complex structure. We found that the majority of these distances are greater than 4.0 Å, indicating that they may not be optimized for interaction. Therefore, it is possible that the enhancement of potency could rely on both the characteristics of the head and the tail segments of the molecule. By comparing the structures of JAK1 in complex with abrocitinib and peficitinib, we observed that the larger size of the inhibitor body induced subtle conformational changes. This, in turn, resulted in a tiny enlargement of the hydrophobic pocket (Fig. 4C). Another key feature of the hydrophobic pocket is its pronounced concavity at the C-terminal lobe, which plays a crucial role in boosting the potency during inhibitor design. Crystal structures of tofacitinib in complex with JAKs reveal that the addition of the methyl group more efficiently occupies the concave region of the hydrophobic pocket, potentially leading to an enhancement in their potency (Fig. 4D)[80]. Upadacitinib lacks crystal structures in complex with JAKs; however, the presence of the ethyl group in its structure may serve a similar function to the methyl group found in

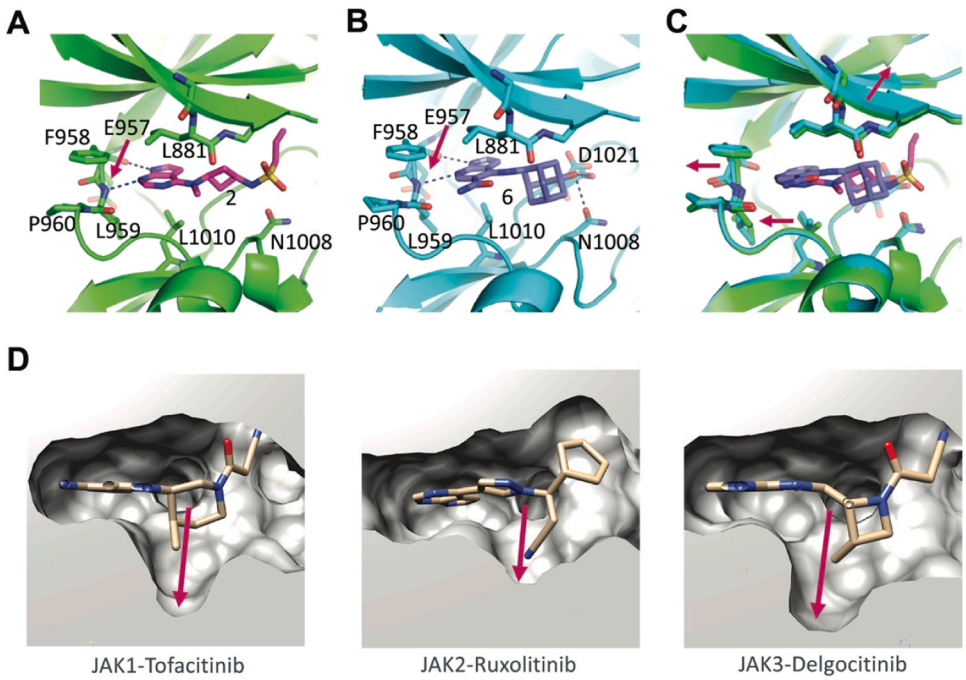

Fig. 4. Accommodation of the 'body' and 'tail' of the inhibitor within the hydrophobic pocket formed between the two lobes of JAKs. (A) Binding of abrocitinib with JAK1, representing a small-sized 'body'. **(B)** Binding of peficitinib with JAK1, representing a large-sized 'body'. **(C)** A comparison of JAK1 in complex with abrocitinib and JAK1 in complex with peficitinib, with red arrows indicating the directions in which JAK1 undergoes a slight enlargement. **(D)** A cutaway view of the JAK hydrophobic pocket, emphasizing the deep concave area on the C-terminal lobe side (highlighted by red arrows), which plays a critical role in enhancing potency during inhibitor design. From left to right: structures of JAK1:tofacitinib, JAK2:ruxolitinib and JAK3:delgocitinib complexes (PDB IDs 3EYG, 6VGL and 7C3N, respectively).

tofacitinib. In the JAK2 complex structures with ruxolitinib and baricitinib, the pyrazole ring extends from the head into the hydrophobic pocket, aligning almost within the same plane as the pyrrolopyrimidine head, leaving a deep concave area at the C-terminal lobe side, which is occupied by the cyan group folding back from the tail (Fig. 4D)[90]. The crystal structure of delgocitinib (JTE-052) in complex with JAK3 reveals a similar mechanism for filling the hydrophobic pocket as seen in ruxolitinib. Notably, the methyl group extending from the four-membered ring inserts into a profound concave region (Fig. 4D)[96]. Kris Meier and colleagues used pyrrolopyrimidine as the inhibitor head and explored various structures of the inhibitor body, including complicated unconventional multiple ring structure. Despite their bulkier size, some of these compounds demonstrated potent inhibitory effects. This observation may indicate the flexibility and size tolerance of the JAK hydrophobic pocket in inhibitor body design [97].

The inhibitor's tail can complement the body in effectively occupying the hydrophobic pocket or anchoring to a site beneath the P-loop [80,90]. This is crucial for enhancing potency, selectivity, and modulating the pharmaceutical properties of the inhibitor. As previously mentioned, while the body of abrocitinib may seem less optimal for accommodation in the hydrophobic pocket, its potency can be enhanced by the tail. Our recent study also demonstrated the effectiveness of enhancing potency and selectivity through tail modifications of the inhibitors [98]. Additionally, tail modifications sometimes play a role in fine-tuning hydrophobic characteristics, which are pivotal factors influencing the compound's logP. However, the design of tail segments in JAK inhibitors tends to exhibit less predictable patterns. In pharmaceutical industry, a common strategy involves creating a head-body core compound that can readily be derivatized with various functional groups, such as different alkyl, acyl compounds, and aromatic moieties [94,99]. Subsequent systematic testing is employed to identify the optimal lead compound for further development.

The design of the side-chain involves its extension from the head toward the solvent, with a key principle being the inclusion of charged or polar groups on the side facing the solvent [88,95]. In certain instances, the side-chains need to be tailored to attach to specific residues in particular JAKs, aiming to enhance selectivity – a topic we will discuss in the inhibitor selectivity section below. The length and composition of the side-chain tend to be less prescriptive, often varying case by case, particularly when the head structure is altered [81,88].

6. JAK inhibitor selectivity

Due to the shared similarity in the active sites of all four JAKs, many JAK inhibitors exhibit poor selectivity, thereby potentially blocking a broad range of downstream signals and raising significant safety concerns when utilizing JAK inhibitors as drugs [100]. For instance, the JAK-STAT pathway is crucial for the hematopoietic system, where cytokines like EPO and TPO utilize JAK2 to activate STATs. In the context of autoimmune disease treatment, non-selective inhibitors might inadvertently inhibit JAK2, leading to side effects such as leucopenia, thrombocytopenia, and anemia. Simultaneously, this JAK-STAT pathway holds critical importance for the immune system. The treatment for MPNs may inadvertently suppress immune signals, leading to side effects like immune suppression and non-serious infections [100–102]. Therefore, narrowing down JAK inhibition specificity is crucial in terms of enhancing the safety profile when using JAK inhibitors as drugs. As a result, the development of selective JAK inhibitors is critical [103].

Within the JAK family, JAK1 plays the most widespread role in the cytokine signaling pathways. It governs the signaling of type II interferons by pairing with JAK2, the signaling of γ_c cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) by pairing with JAK3, and the signaling of type I, type III interferons, and IL-10 family cytokines by partnering with TYK2(Fig. 1A)[62,63]. Due to the fact that blocking one JAK of a JAK pair in the JAK-STAT pathway is capable of inhibiting downstream signaling, selectively inhibiting JAK1 can attenuate a wide array of immunosignals, without significantly affecting hematopoietic signals

primarily mediated by the JAK2-JAK2 pair. Sequence alignment and structural comparisons reveal a few differences between JAK1 and other JAKs, including variations at F958 and E966, corresponding to tyrosine and aspartic acid in other JAKs (Fig. 3D). F958, situated at the hinge region where the inhibitor's head attaches via hydrogen bonding to the main chain, poses challenges for attaching the side-chain. Therefore, differences in the F958 position might only exert a marginal impact on inhibitor binding, making it less suitable for clear-cut selective inhibitor design. By contrast, E966 emerges as the most viable candidate for designing selective JAK1 inhibitors (Figs. 5A and 5B). Mark Zak and colleagues designed a series of C-2 hydroxyethyl imidazopyrrolopyridines with high selectivity over JAK2. They started from imidazopyrrolopyridines, with the methyl group 3.4 Å from the carboxyl of JAK1's E966, but 5.1 Å from the corresponding carboxyl of JAK2's D939 (Fig. 5C). These compounds exhibited some selectivity due to their attachment to E966 [104]. The subsequent modification of the methyl group to hydroxyethyl resulted in inhibitors with enhanced selectivity and potency (Fig. 5A). The crystal structure further elucidated that the hydroxyethyl group forms a hydrogen bond with the carboxyl group of the side-chain of E966 in JAK1, greatly contributing to binding (Fig. 5D). In contrast, there is no direct contact between these inhibitors and D939's carboxyl in JAK2. Instead, a water molecule bridged the inhibitor and D939, resulting in a weaker binding affinity (Fig. 5E) [105]. A recent example involves our design of C-5 pyrazole-substituted pyrrolopyridine compounds that could potentially form a hydrogen bond between pyrazole and the side-chain of E966. Yet, the side-chain from corresponding aspartic acid residue in other JAKs fall short of reaching the pyrazole group (Fig. 5B)[95].

JAK2 plays a unique role in that it not only collaborates with other members in the JAK family, with the exception of JAK3, to regulate signaling across a wide range of cytokine receptors, but it is also unique in its ability to dimerize with itself [62,63]. JAK2 is an important target

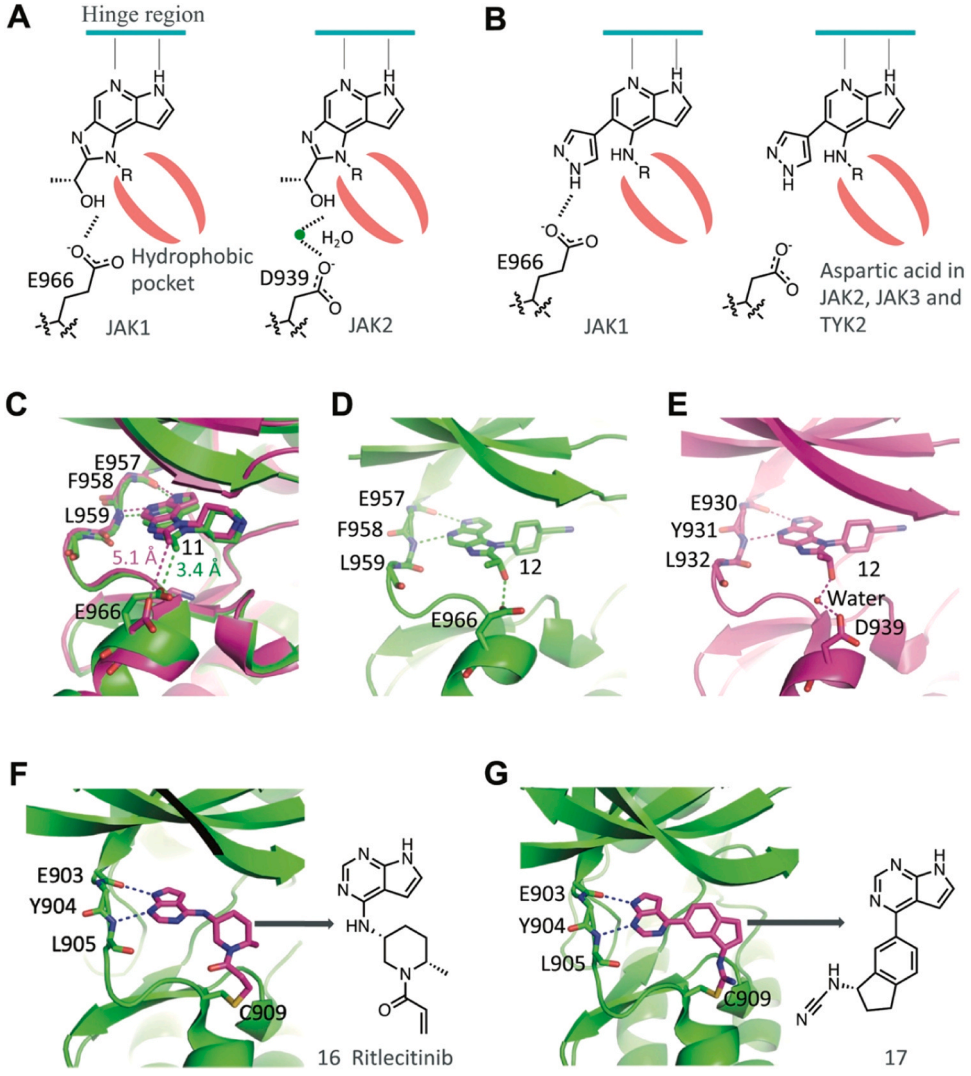

Fig. 5. Design of selective inhibitors for JAK1 and JAK3. (A) Design of a JAK1-selective inhibitor by introducing a hydroxyethyl group to form hydrogen bond with the side-chain of E966, but not the corresponding residues in other JAKs, such as D939 in JAK2. **(B)** Design of a JAK1-selective inhibitor by distinguishing E966 from aspartic acid at the corresponding position in other JAKs, by introducing a pyrazole group on pyrrolopyridines. **(C)** A comparison of an imidazopyrrolopyridine-based JAK inhibitor (compound 11), which shows JAK1 selectivity over JAK2, in complex with both JAK1 (green, PDB ID: 4EHZ) and JAK2 (magenta, PDB ID: 4F09). **(D)** Structure of a hydroxyethyl side-chain-based JAK1-selective inhibitor (compound 12) in complex with JAK1 (PDB ID: 4IVB), with enhanced potency through the direct formation of a hydrogen bond between the hydroxyethyl group and the side-chain of E966. **(E)** Structure of compound 12 in complex with JAK2 (PDB ID: 4IVA), where the hydroxyethyl group indirectly interacts with the side-chain of D939 via a water molecule. **(F)** Structure of compound 16 (PF-06651600), an acryloyl-based JAK3-selective inhibitor in complex with JAK3 (PDB ID: 5TOZ), with major selectivity achieved through the formation of a covalent bond between the inhibitor and C909 in JAK3. **(G)** Structure of a cyanamide-based JAK3 inhibitor (compound 17) in complex with JAK3 (PDB ID: 6DUD), with a covalent bond formed between the cyanamide and the side-chain of C909 in JAK3.

for hematopoietic diseases such as MPNs [106]. The endeavor to design a JAK2-selective inhibitor is intricate due to the absence of distinct residues at the active site that are exclusively unique to JAK2. The sequence identities between the JAK2 TK domain and TK domains of other JAKs lie below 60% though, introducing subtle structural variations between JAK2 and other JAKs. Isolating these distinctions for the purpose of developing JAK2-selective inhibitors, however, remains difficult. The lack of ligand-free structures or uniform ligands in complex with all four JAKs, apart from the exception of tofacitinib, hampers the comparison of structural differences between JAK2 and the others, crucial for informing selective inhibitor design [79,80]. For instance, while the structures of JAK2 in complex with the JAK2 inhibitors ruxolitinib and baricitinib (which exhibit similar potency against JAK1) have been elucidated, a lack of corresponding structures with other JAKs impedes the guidance for enhancing JAK2 potency [90]. Fedratinib, a potent selective JAK2 inhibitor FDA-approved for treating MPNs after ruxolitinib, exhibits an IC_{50} value of 3 nM for wild-type JAK2 and the V617F mutant, which is 30, 100, and 300-fold more potent than JAK1, JAK2 and TYK2, respectively [107]. However, it encounters a similar challenge due to the absence of co-crystal structures with JAK1, JAK3 and TYK2, which might offer important information for the selectivity mechanism.

Compared to its counterparts in the JAK family, JAK3 exclusively partners with JAK1 to regulate a relatively narrow range of cytokine signaling pathways, primarily γ_c cytokines [62,63]. In theory, a selective JAK3 inhibitor could offer a more favorable safety profile than inhibitors targeting JAK1 or JAK2. Structural comparisons reveal that the JAK3 active site shares similarities with other JAKs, albeit with subtle differences. One variation involves the residue at position A966, which is glycine in other JAKs, which could be taken advantage of during the design of a JAK3-selective inhibitor. In practice, a more intriguing aspect is a distinct divergence observed at the cysteine residue at position 909, where the corresponding residue in other JAKs is a serine. Cysteine residues are frequently targeted for the construction of covalent inhibitors [108]. Atli Thorarensen and colleagues introduced an acryloyl group to a series of pyrrolopyrimidine derivatives, to create irreversible JAK3-selective covalent inhibitors. Among these compounds, ritlecitinib (PF-06651600) exhibited remarkable potency against JAK3 with >50-fold selectivity over other JAKs (IC_{50} 33 nM for JAK3; 1640 nM for JAK1; and >10,000 nM for other JAKs)[109]. Ritlecitinib, the first JAK3-selective inhibitor with suitable pharmacokinetic and pharmacodynamic properties, was approved by the FDA in June 2023 for treating severe alopecia areata [110]. The crystal structure of ritlecitinib complexed with JAK3 confirmed that the inhibitor's head binds to the hinge region, while the inhibitor's tail forms a covalent bond with the C909 in JAK3 (Fig. 5F)[111]. Various acryloyl-based JAK3 inhibitors have been developed to date, and other chemical groups, such as cyanamide, can also be employed to target the cysteine residue for the development of JAK3-selective inhibitors [112]. Agustin Casimiro-Garcia and colleagues designed a series of cyanamide-based JAK3 inhibitors. The crystal structure confirms the cyanamide forms covalent bond with Cys909 in JAK3 (Fig. 5G)[113]. While the cysteine residue is an effective target for JAK3 inhibitor development, it is worth noting that among over 500 human kinases, more than ten, including EGFR, Bruton's tyrosine kinase, and B-cell lymphocyte kinase, possess a cysteine near the active site. This raises concerns about potential off-target effects of JAK3 inhibitors that interact with cysteines from other kinases [108,114]. During the development of ritlecitinib, numerous compounds were screened, and some exhibited inhibitory effects on other kinases containing cysteine residues near the active site [109]. Nonetheless, systematic testing of various compounds can mitigate off-target effects, ensuring the selection of the most suitable candidate for further development.

Within the JAK-STAT pathway, TYK2 plays a crucial role by associating with receptors for IL-12, IL-23, type I IFNs, and IL-10 family cytokines (Fig. 1. A). These interactions are critical for the function of T helper 1 (Th1), Th17, B cells, and myeloid cells, which are involved in autoimmune and chronic inflammatory diseases [115–117]. An intriguing TYK2 variant, P1104A, renders it inactive and provides protection against various autoimmune diseases [118]. Remarkably, individuals homozygous for this inactive variant do not experience an increased susceptibility to malignancies, bacterial, mycobacterial, fungal, or viral infections, suggesting potential tolerability for TYK2 inhibition. Consequently, developing a selective TYK2 inhibitor could offer a superior safety profile in autoimmune disease treatment [116, 119]. Comparing the structures of TYK2 and other JAKs reveals some differences at the active site (Fig. 3D). For instance, residues I960 and V981, adjacent to the hinge region, differ from valine and leucine in other JAKs. Additionally, the residue R901 at the edge of the active site corresponds to arginine, asparagine, and serine in JAK1, JAK2, and JAK3, respectively. These distinct amino acid variations present an opportunity for the rational design of selective TYK2 inhibitors. In the case of pyrrolopyrimidine head JAK inhibitors such as tofacitinib, baricitinib, and ruxolitinib, the pyrrole ring clashes with the larger side-chain of I960 in TYK2, resulting in approximately 30-fold, 20-fold, and 5-fold decreased potency against TYK2 compared to JAK1, respectively [119]. Designing a head that binds to the hinge region while avoiding this steric clash could potentially enhance TYK2 inhibition and selectivity. For instance, in contrast to pyrrolopyrimidine head inhibitors that form hydrogen bonds with the carbonyl of E979 and the amino group of V981, monocyclic amine compounds capable of forming hydrogen bonds with the amino group and carbonyl of V981 effectively avoid potential steric clashes with I960. Andrew Fensome and colleagues reported the discovery of PF-06700841, a dual inhibitor of TYK2 and JAK1 based on a monocyclic amine structure. Its remarkable potency against TYK2 can be attributed, in part, to the absence of steric clashes with I960 (Figs. 6A and 6C)[120]. This inhibitor is currently in clinical development and has demonstrated effectiveness and good tolerance in patients with moderate-to-severe plaque psoriasis [121]. Brian Gerstenberger and colleagues explored head cores to minimize steric clashes (Fig. 6B) [119]. They tested a series of bicyclic head cores that share structural similarities but differ in atom types at specific positions. Despite the slight variations in the head core, these compounds exhibited varying binding affinities due to differences in electrostatic potential against TYK2. One specific compound (24; Fig. 6B), displayed noteworthy potency against TYK2, primarily due to the C-7 H position of the head ring potentially serving as a hydrogen bond donor, enabling it to form hydrogen bonds with the carbonyl of V981. The optimization of this head ring led to the discovery of PF-06826647, which exhibited excellent potency, selectivity, and favorable ADME properties (Figs. 6A and 6D)[119]. This compound is currently in clinical development [122]. Another example involves the development of compound 20 (Fig. 6A), which not only forms hydrogen bonds with the hinge region but also establishes hydrogen bonds with the side-chain of residue R901 (Figs. 6A and 6E). This residue's counterparts in JAK2 and JAK3 are asparagine and serine, respectively. This strategic interaction could potentially enhance potency and selectivity [123].

7. Allosteric inhibitors targeting the PK domain

While small molecules that bind to and inhibit the JAK TK domain have demonstrated success in treating hematological disorders and autoimmune diseases, safety concerns remain due to the challenge of achieving absolute selectivity for one inhibitor over the other JAKs, as well as other kinases in the human kinome [52,75,124]. Over the past decade, alternative approaches have emerged to explore allosteric inhibitors that bind to the PK domain of JAKs [52,125]. The primary function of the PK domain is to act as a switch, maintaining TK activity in a suppressed state through an autoinhibitory mechanism. The autoinhibition is relieved when cytokines bind, allowing for downstream signaling [73,74]. The importance of the PK domain has been demonstrated by the fact that mutations in specific residues within the PK

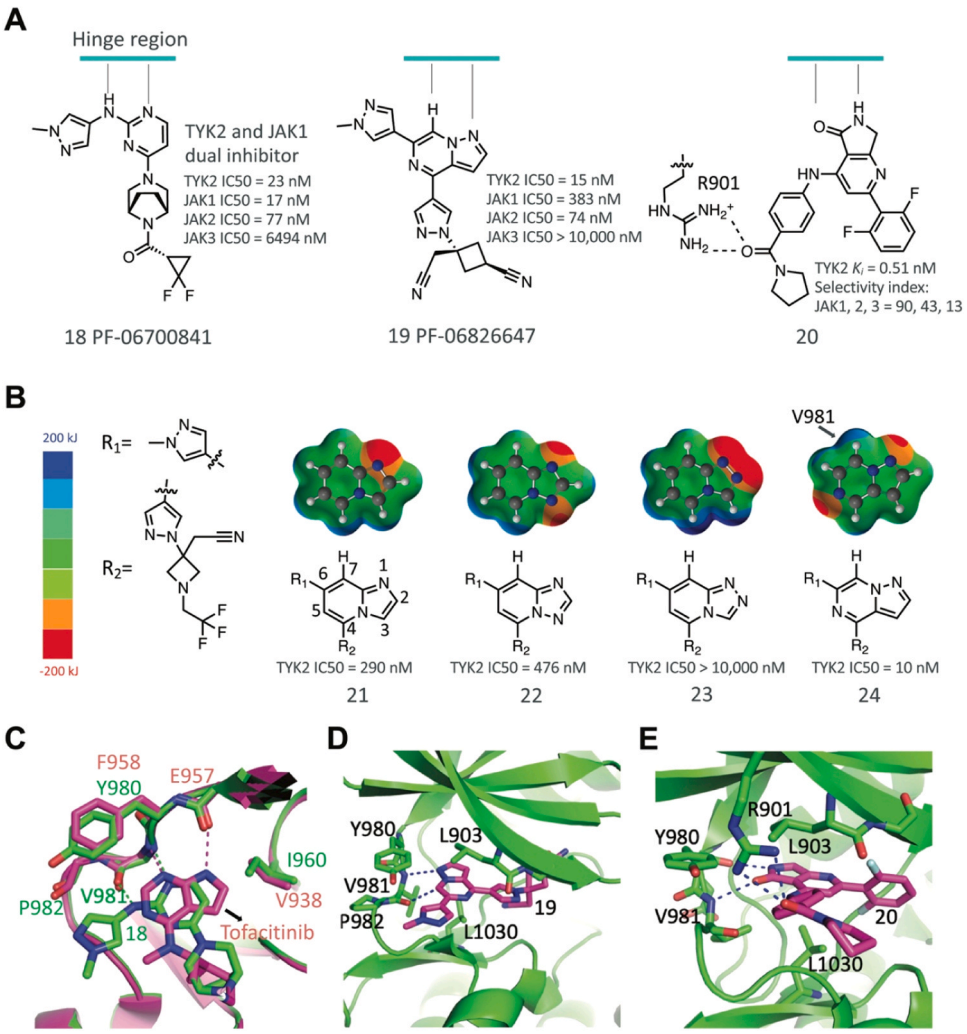

Fig. 6. TYK2 selectivity. (A) Three representative TYK2 inhibitors. **(B)** Optimization of head cores resulting in the discovery of compound 19 [119]. **(C)** Superposition of compound 18 in complex with TYK2 (green, PDB ID: 6DBM) and tofacitinib in complex with JAK1 (cyan, PDB ID: 3EYG). Both inhibitors form hydrogen bonds with the hinge region of JAKs, although in distinct relative positions. In TYK2, I960 (valine in other JAKs) may lead to steric clashes with pyrrolopyrimidine head inhibitors, but monocyclic amines like compound 18 can circumvent this clash, thereby enhancing TYK2 selectivity. **(D)** Interaction of compound 19 with TYK2 (PDB ID: 6×8G). **(E)** Interaction of compound 20 with TYK2 (PDB ID: 7UYU), where the side-chain of R901 forms hydrogen bonds with the inhibitor's carbonyl group.

domain can lead to either gain or loss of function within the JAK-STAT pathway, often resulting in disease conditions [21,25,72]. Although the molecular mechanisms underlying many of these gain or loss-of-function mutations remain unclear, this does not diminish the hypothesis that small molecules binding to and perturbing the conformation of the PK domain can influence its regulatory role in downstream signaling, serving as either activators or inhibitors of the JAK-STAT pathway [73,74].

The concept proof of the alteration of the JAK-STAT pathway signal through the PK domain by using small molecules was pioneered by John Tokarski and colleagues at Bristol-Myers Squibb. They employed a chemogenomics approach to screen a compound library comprising 7908 compounds to identify inhibitors against IL-23 stimulated cells. Compound 25 (Fig. 7A) exhibited potent inhibitory effects with an IC_{50} of approximately 300 nM. It was found to bind to the PK rather than their TK domain. This discovery marked the first demonstration that downstream signal transduction could be inhibited through pharmacological modulation at the PK domain level [126]. A crystal structure was solved of an analog of this compound (compound 26), which also binds to the PK domain and acts as an inhibitor of the JAK-STAT pathway, albeit with reduced potency. This structural information provides a basis for understanding how inhibitors bind to the PK domain (Fig. 7B)[126].

Early attempts to optimize a few hit compounds were unsuccessful in achieving an ideal lead compound for drug development. These efforts encountered various challenges, including off-target effects (inhibiting PDE4 or IKKβ), limited metabolic stability, and inadequate cell permeability [126,127] However, a turning point came with the identification of compound 27, which led to the discovery of deucravacitinib (BMS-986165), the first FDA-approved, highly selective, orally bioavailable allosteric TYK2 inhibitor for treating autoimmune diseases [125]. Unlike early hit compounds, compound 27 (Fig. 7A) exhibited no activity against PDE4 and IKKβ, along with good metabolic stability, albeit with some inhibition of the TK domain of JAKs and other kinases [128]. The optimization of compound 27 began with efforts to enhance selectivity, achieved by attaching an alkyl group to the amide. It was found that a methyl derivatization can significantly improve selectivity against JAKs' TK domain and other kinases, while only slightly reducing potency. However, this methyl group had a propensity to detach in cells, leading to decreased selectivity. This challenge was swiftly resolved by replacing the conventional methyl group with a trideuterium methyl [128]. Other optimizations included eliminating hERG activity, and improving permeability, potency and metabolic stability [76,128]. The

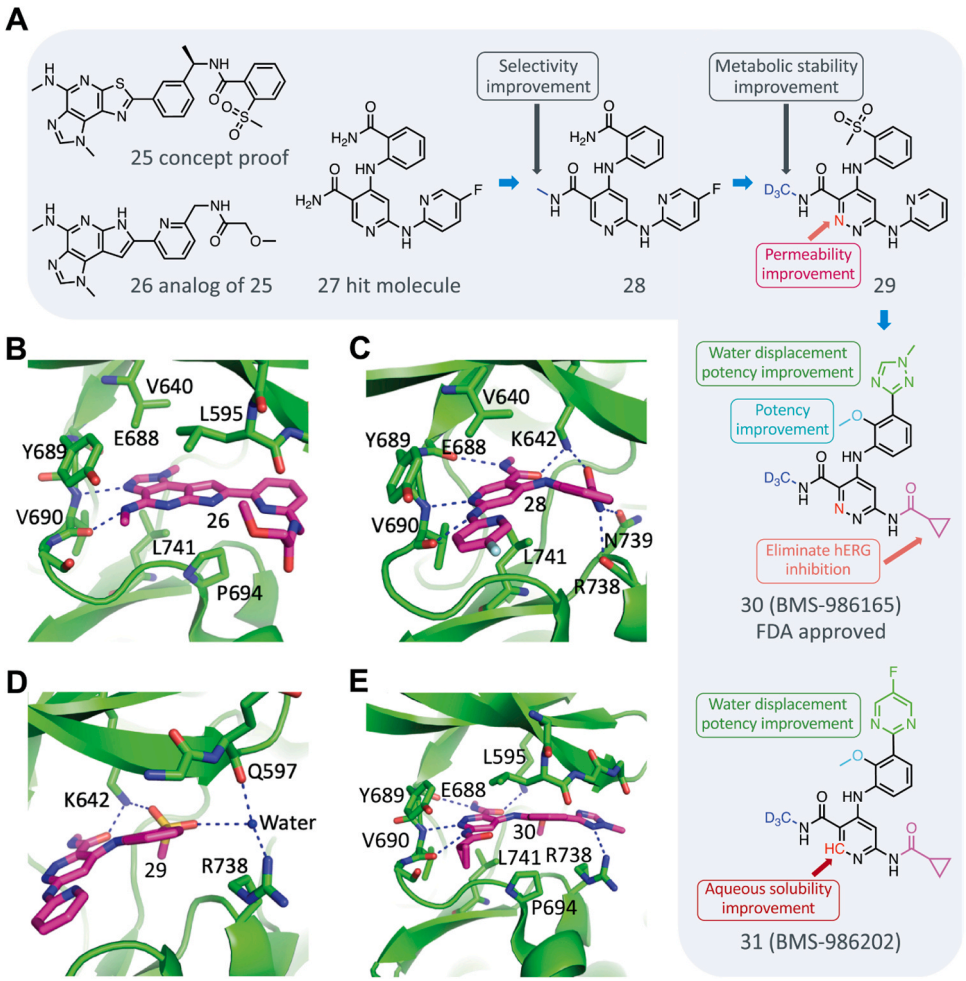

Fig. 7. Discovery of allosteric inhibitors targeting the JAK PK domain and PROTACs targeting STATs. (A) The evolution of highly potent and selective TYK2 allosteric inhibitors, from concept validation to clinical development. **(B)** Interaction of compound 26 (an analog of 25) with the TYK2 PK domain (PDB ID: 4WOV). The hinge region serves as a critical binding site for inhibitor. **(C)** Compound 28 in complex with the TYK2 PK domain (PDB ID: 6NZE). **(D)** Compound 29 in complex with the TYK2 PK domain (PDB ID: 6NZR). A water molecule bridges the carbonyl of Q597 and the side-chain of R738. **(E)** Compound 30 in complex with the TYK2 PK domain (PDB ID: 6NZP). This design establishes direct hydrogen bonding between the inhibitor and the side-chain of R738.

crystal structure of compound 29 in complex with TYK2 PK domain revealed that a water molecule bridged the amino group of R738 and the carbonyl group of Q597 [76]. Further optimization involved attaching a methyl triazole to the C-3 position of the benzene ring, thereby establishing a direct contact with the amino group of R738 to enhance the potency, leading to the discovery of deucravacitinib (Fig. 7E)[76]. A notable advantage of deucravacitinib is its inhibition of TYK2 by binding to its JH2 domain, allowing for maximal efficacy while maintaining a high level of selectivity over the kinome, especially in relation to other JAKs [76]. Clinical trials demonstrated its excellent efficacy and safety profile [125,129]. Continued exploration of allosteric inhibitors targeting the PK domain of TYK2 by researchers at Bristol-Myers Squibb led to the discovery of BMS-986202, which shares a similar structure and binding mechanism with deucravacitinib and is also an excellent TYK2 allosteric inhibitor [130]. The success of deucravacitinib has sparked a surge of interest in the exploration of allosteric inhibitors targeting the PK domains of JAKs, with several JAK allosteric inhibitors now disclosed, including allosteric inhibitor targeting JAK1 PK domain [131–133].

8. STATs binders

Within the JAK-STAT pathway, STATs are recruited and phosphorylated by activated receptor-JAK complexes. Subsequently, phosphorylated tyrosine residues on STATs engage binding to the SH2 domain of their respective partners in trans. This binding event induces a conformational change in the STAT dimer, facilitating the adoption of a configuration suitable for recognizing specific DNA sequences [62,73,74,134]. To date, several crystal structures of STATs have been solved, including those of STAT-DNA complexes and STATs in complex with intracellular segments from receptors. These structures provide the structural basis for STAT recruitment and special dimeric conformation essential for DNA recognition [70,71,135]. Notably, it has been observed that several residues within the SH2 domain form extensive hydrogen bonds with phosphorylated tyrosine residues, highlighting the critical role of phosphorylation in JAK-STAT signaling (Fig. 8A). It has been proposed that molecules that competitively occupy the phosphorylated tyrosine-binding site on the SH2 region of STATs can either inhibit the recruitment and phosphorylation of STATs or prevent them from adopting the conformation required for their function as transcription factors, resulting in the attenuation of downstream signaling. Such molecules show promise as potential therapeutic agents for rectifying aberrant JAK-STAT signaling implicated in various disease conditions [77]. Several research initiatives have been launched to explore these molecules, including those targeting STAT3, STAT5, and STAT6 [77, 136–141]. A prominent characteristic of these molecules is that they are peptidomimetics containing a phosphate group on the aromatic moiety designed to bind to the phosphorylated tyrosine-binding site on

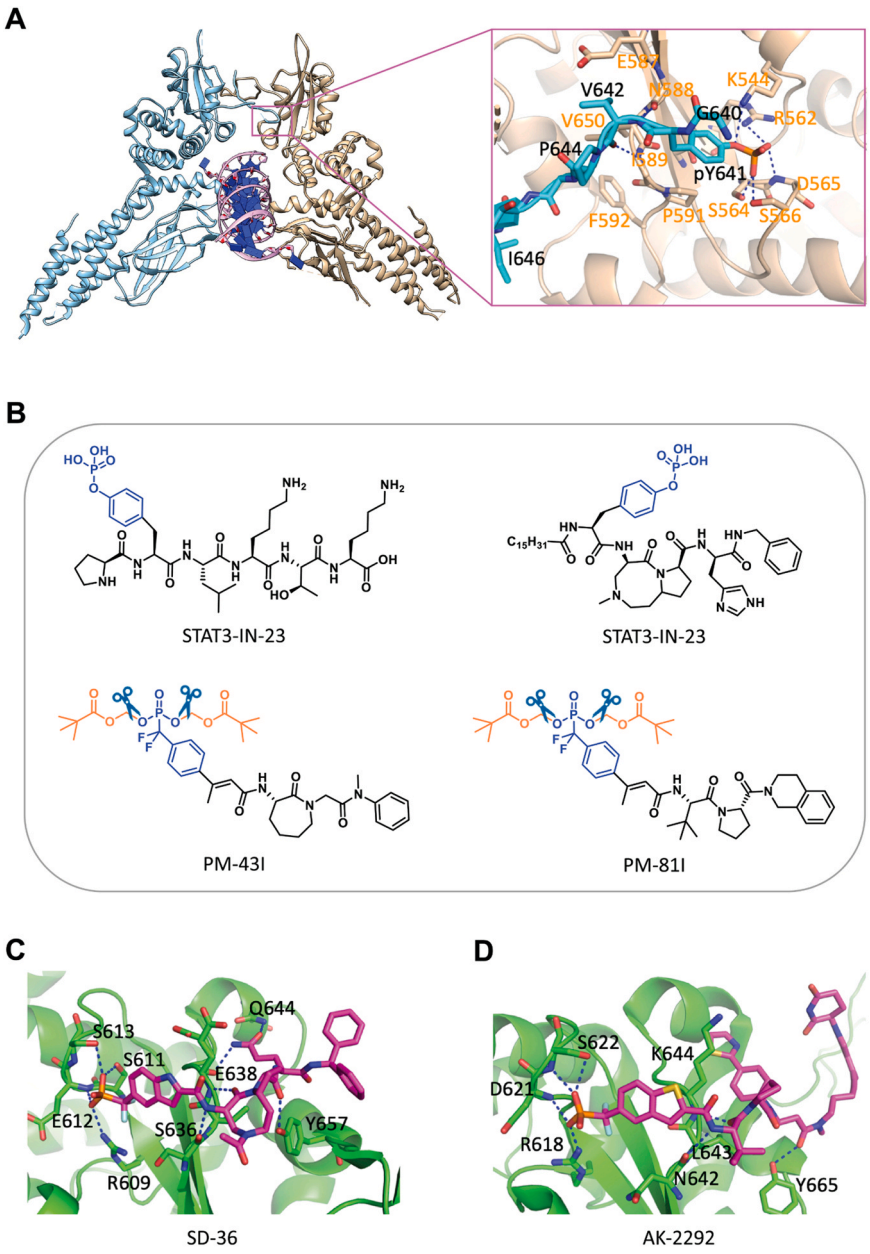

Fig. 8. Discovery of inhibitors targeting the STATs. (A) The structural basis for the binding of phosphopeptides to the SH2 domain of STAT proteins: a mechanism crucial for STAT recruitment, DNA recognition, and STAT inhibition. The interaction between the phosphorylated peptide, derived from one partner of STAT, and the SH2 domain involves extensive hydrogen bonding with its phosphorylated tyrosine residues (exemplified by STAT6, PDB ID: 4Y5W). **(B)** Typical STAT inhibitors. In certain cases, phosphorylated tyrosine is modified with hydrophobic groups to enhance its permeability, these groups can be cleaved cells. **(C and D)** PROTACs targeting STATs and their interaction with STATs. The STATs binder, linker, and E3 ubiquitin ligase ligand are colored in red, blue, and green, respectively. Phosphate groups from aromatic groups in both molecules form multiple hydrogen bonds with STATs, mimicking the binding of phosphorylated tyrosine. **(C)** SD-36 in complex with STAT3 (only the STATs binder portion is visible, PDB ID: 6NJS); **(D)** AK-2292 in complex with STAT5 (PDB ID: 7TVA).

the SH2 domain (Fig. 8B). In some cases, these molecules have been derivatized with alkyl esters to neutralize charges, enhancing their cellular permeability (Fig. 8B). These derivatives function as prodrugs, with the alkyl esters expected to be cleaved off once the molecule enters the cells, leaving behind the active component capable of binding to the SH2 domain. Some of these compounds have demonstrated the ability to block both phosphorylation and transcriptional activity of STATs in cells and animal models, making them promising candidates for further development in cancer or autoimmune disease treatment [77, 136–142]. For instance, one compound, PM-43I, developed by John McMurray's group, potently inhibits both STAT5 and STAT6, effectively addressing allergic airway disease in mice [140]. It demonstrates the ability to reverse preexisting allergic airway disease in mice with a median effective dose (ED_{50}) of 0.25 μg/kg, efficiently cleared through the kidneys, and exhibits no long-term toxicity, rendering it suitable for further clinical development against asthma. Another compound, CJ-1383, developed by Shaomeng Wang's group, binds to STAT3 with an inhibition constant (K_i) of 0.95 μM and effectively inhibits cell growth with IC_{50} values of 3–11 μM in two breast cancer cell lines with high levels of phospho-STAT3 [141]. It is effective in the inhibition of STAT3 activity and induction of apoptosis in the MDA-MB-468 cancer cell line in a time- and dose- dependent manner.

9. PROTACs

Targeted protein degradation (TPD) is an emerging therapeutic

approach with the potential to address disease-causing proteins that have traditionally posed significant challenges for conventional small molecule targeting. A major group of molecules facilitating the modulation of such proteins through TPD is called proteolysis-targeting chimeras (PROTACs), which are heterobifunctional small molecules comprising two ligands interconnected by a linker. One ligand selectively binds to a protein of interest (POI), while the other recruits an E3 ubiquitin ligase. The simultaneous engagement of both the POI and ubiquitin ligase by the PROTAC initiates ubiquitylation of the POI, leading to its subsequent degradation via the ubiquitin–proteasome system. Following degradation, the PROTAC is recycled and prepared to target another POI molecule [143–145]. Over the past two decades, since the inception of the first small-molecule PROTAC in the scientific literature, this technology has transitioned from academia to the pharmaceutical industry. Numerous biotechnology and pharmaceutical companies have unveiled programs focused on preclinical and early clinical development, underscoring the growing momentum and potential of TPD in drug discovery and development [143]. Notably, recent advances have seen the exploration of PROTACs targeting proteins within the JAK-STAT pathway, including the TK domain and PK domain of JAKs, as well as STATs.

Charles Mullighan's group has reported the development of PROTACs to target JAK2 as a potential treatment approach for cytokine receptor-like factor 2 (CRLF2) rearranged ALL [146]. This PROTAC employs analogues of ruxolitinib and baricitinib as ligands that interact with JAK2, along with a linker and an E3 ligase binding moiety derived from thalidomide or its derivatives. These compounds have demonstrated potent antileukemic efficacy. One compound, SJ988497, has demonstrated an astounding >70,000-fold increase in activity in CRLF2r MHH-CALL-4 cells when compared to its parent compounds (ruxolitinib and pomalidomide). Another compound, SJ10542 (a JAK2/3 degrader), displayed high selectivity over GSPT1 and other members of the JAK family, coupled with potency in patient-derived ALL cells harboring both JAK2 fusions and CRLF2 rearrangement [147]. Recently, Jun-ya Kato and colleagues reported the development of a series of TYK2 degraders by conjugating analogs of allosteric TYK2 inhibitor BMS-986165 with a VHL ligand as the E3 ligase binding moiety, connected via alkyl linkers of varying lengths. Through optimization, the PROTAC exhibited potent TYK2 degradation efficacy without affecting the protein levels of other JAKs [148].

The efforts to investigate PROTACs that target STATs have been led by Shaomeng Wang's team [78]. Their earlier work on developing STAT binders served as a valuable reference for their subsequent PROTAC development endeavors [141]. The PROTAC molecules they have developed consist of a STATs binder, a linker, and a cereblon ligand for E3 ubiquitin ligase binding [149–152]. The crystal structures of PROTAC-STAT complexes have been elucidated, shedding light on the binding mechanism of these PROTACs, which bears similarity to the binding segment observed in phosphorylated STAT dimers. These structures reveal extensive hydrogen bonding at the phosphorylated tyrosine binding site, likely making a substantial contribution to the binding. Additionally, other interactions are critical not only for enhancing binding but also for ensuring STAT specificity [149,151]. One notable PROTAC, SD-36 (Fig. 8C), potently induces the degradation of STAT3 protein both in vitro and in vivo. It exhibits high selectivity over other members of the STAT family, resulting in a robust suppression of its transcriptional network in leukemia and lymphoma cells. SD-36 effectively inhibits the growth of a subset of acute myeloid leukemia and anaplastic large-cell lymphoma cell lines, by inducing cell-cycle arrest and apoptosis. In multiple xenograft mouse models, SD-36 achieves complete and long-lasting tumor regression at well-tolerated dose schedules, establishing it as a promising strategy for cancer therapy [78, 149,150]. Another impressive PROTAC, AK-2292 (Fig. 8D), is a potent and selective small-molecule degrader of both STAT5A and STAT5B isoforms. With an outstanding selectivity, AK-2292 inhibits STAT5 activity in cells by inducing the degradation of STAT5 proteins, as opposed to over 6000 non-STAT proteins and all other STAT proteins. AK-2292 effectively induces STAT5 depletion in normal mouse tissues and human CML xenograft tissues. Furthermore, it achieves tumor regression in two CML xenograft mouse models at well-tolerated dose schedules, solidifying its position as a promising lead compound for further development [151,152]. In a separate effort, Jinmei Jin and colleagues introduced a toosendanin-based PROTAC named TSM-1, targeting STAT3. While the exact binding mode of toosendanin to STAT3 remains unknown, microscale thermophoresis assays revealed a K_D value of 296 nM. TSM-1 exhibits exceptional selectivity, potency, and robust antitumor effects in STAT3-dependent head and neck squamous cell carcinoma and colorectal cancer, particularly in clinically relevant patient-derived xenografts and patient-derived organoids [153].

10. Conclusions and outlook

Small molecule JAK inhibitors have proven successful in managing hematological and autoimmune diseases, with the approval of drugs like tofacitinib and ruxolitinib sparking a wave of JAK inhibitor development. Nevertheless, the perpetual pursuit of enhancing therapeutic efficacy and target specificity remains a central theme in JAK inhibitor development. The increasing availability of JAK-inhibitor complex structures sheds light on inhibitor binding mechanisms and expedites the development of highly selective JAK inhibitors. Given that safety remains an enduring goal, the continuous development of more potent and selective JAK inhibitors stands as a perennially prominent topic in the pharmaceutical industry. The success of deucravacitinib, an allosteric inhibitor targeting the PK domain, has introduced a powerful and alternative approach for inhibiting the JAK-STAT pathway. Furthermore, other strategies such as STATs binders hold promise in further exploring JAK-STAT pathway inhibition. Emerging therapeutic modalities like PROTAC offer distinct advantages, including selectivity and inhibitory efficiency, with the potential to address disease-causing proteins that have historically been challenging to target using conventional small molecules. These advancements demonstrate significant potential in the development of drugs targeting the JAK-STAT pathway.

CRediT authorship contribution statement

Jeffrey Babon: Writing – review & editing. **Pengbing Mi:** Writing – original draft, Conceptualization. **You Lv:** Writing – original draft, Conceptualization. **Bostjan Kobe:** Writing – review & editing, Writing – original draft, Conceptualization. **Faming Wang:** Writing – review & editing, Writing – original draft, Project administration, Conceptualization. **Jin Zhang:** Writing – review & editing. **Jiajia Lang:** Writing – review & editing. **Longxing Cao:** Writing – review & editing. **Jianxun Qi:** Writing – original draft. **Guohuang Fan:** Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests was involved in this work.

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Author contributions

Y.L., P.M., J.J.B., F.W., and B.K. conceived the manuscript. Y.L. and P.M. wrote the initial version of the manuscript. Y.L., P.M., J.L., J.Z. and F.W. collected the references and created the figures. P.M., J.J.B., G.F., J. Q., L.C., F.W. and B.K. reviewed, commented and edited the manuscript.

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