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Inhibitors of JAKs/STATs and the kinases: a possible new cluster of drugs

Cheng Luo and Pauliina Laaja

JAK(s)/STAT(s) relay cytokine signals through tyrosine site-specific phosphorylation of the proteins involved in cellular responses for the activation and proliferation of bone marrow-derived cells. In recent years, the constitutive or elevated expression of JAK/STAT has been found in cancer cells and oncogene transfected cells, and has been shown to be involved in the immune rejection of allografts and the inflammatory processes of autoimmune diseases. This review discusses the strategies for screening and rational design of selective, potent JAK/STAT and kinase inhibitors that are either ATP-competitive or non-ATP competitive, naturally derived or synthetic, as well as other unique inhibitors and analogues for different therapeutic indications.

Cheng Luo
Oklahoma Medical Research Foundation
Oklahoma City
OK 73104, USA
e-mail: Cheng-Luo@mail.omrf.ouhsc.edu
Pauliina Laaja
Turku Center for Biotechnology
University of Turku and
Åbo Academy University
PO Box 123
20520 Turku, Finland

▼ Janus kinases (JAKs), which have an apparent molecular weight of ~130 kDa, are associated with the intracellular domains of receptors and are bound to endoplasmic membranes by myristoylated N-terminal lipids. They are crucial signal transducers for a variety of cytokines, growth factors and interferons [1–3]. JAKS comprise seven distinct structural domains that are numbered from the C-terminus. JAK homology (JH) 1 is the kinase domain. The more proximal kinase-like domain is JH2, which functions as a negative regulator. There are five other N-terminal regions (JH3–JH7) that predominantly participate in the binding of the receptor. JAK was identified initially in the process of erythropoietin (EPO) -mediated haematopoiesis, which promotes the conversion of bone marrow cells to red blood cells. EPO, a glycoprotein hormone that is produced in the kidney, has long been used for treating anaemia in end-stage renal disease, which suggested that JAK might participate in the activation and proliferation of marrow-derived cells. From this observation, it was hypothesized that inhibition of JAK kinases might prove to be a possible therapy for leukaemia. JAKs relay the signals initiated by extracellular stimuli via corresponding receptors that comprise two heterosubunits: α and β. The heterodimerization of these receptor subunits induces the signalling cascade, which consists of autophosphorylation of the receptor, followed by phosphorylation of JAK. Either the receptor and/or JAK can alternatively recruit the STAT (signal transduction and activation of transcription) protein via recognition of the SH2 domain near the phosphorylated sites [4]. STAT, an unbound, cytosolic, soluble protein (77–80 kDa) that dimerizes upon phosphorylation, subsequently translocates to the nucleus where it binds to the enhancer regions of DNA for transcription of cytokine-responsive genes. JAKs/STATs represent a large family; together they integrate the signal transduction of cytokines in haematopoietic cells, lymphocytes and other bone-derived mammalian cells.

Src homology domains

The Src homology (SH) domain has phosphorylation sites in kinases and docking sites in STATs. The tyrosine kinases typically include SH1, SH2, SH3 and SH4 domains. SH1 is the kinase domain and is ~350 amino acid (aa) residues in length. The SH2 domain is 80–120 aa in length and functions in binding phosphotyrosine (p-Y) residues. The SH3 domain is involved in interactions with proline-rich regions and is ~60 aa in length. The SH4 domain is a small region (15–17 aa) that is located near the N-terminus and contains the signal for fatty acylation [5]. Src kinases, for example, lyn kinase and Bruton's tyrosine kinase (BTK), have a unique domain (UD) and a domain sequence of SH4-UD-SH3-SH2-kinase. The domain sequence of protein tyrosine phosphatases is typically SH2-SH2-kinase. In JAK proteins, SH2 is located between the JH2 and FERM (4.1, ezrin, radixin, moesin) domains, whereas in STAT, SH2 is located between the

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transcriptional activation domain (TAD) and the DNA binding linker in the C-terminus [2]. Non-myristoylated Src molecules do not bind to membranes, but some Src kinases carrying this modification can be found in the cytosol. Myristoylation probably does not guarantee association of the protein with the membrane, which could indicate the function of Src – for example, the recruitment of Lck kinase in assembled glycolipid-enriched membrane (GEM) domains or lipid rafts and its subsequent translocation into the immune synapse in T-cell receptor (TCR) signalling [6]. Palmitoylation is a reversible process, but myristoylation is irreversible. Depalmitoylation and repalmitoylation are thought to be mechanisms for modifying the localization of the Src family kinases in response to different stimuli [7].

Table 1. Effector molecules and the STATs involved in signalling of JAKs

Effector molecule	Signalling component	JAK that relays the signal
IL-2, -7, -9, -15	STAT3, STAT 5a and STAT5b	JAK1 and JAK3
IL-3	STAT5	JAK2
IL-4	STAT5a, STAT5b and STAT6	JAK1 and JAK3
IL-6, -11, LIF	STAT1 and STAT3	JAK1 and JAK2
IL-10	STAT3	JAK1 and TYK2
IL-12	STAT4	JAK2 and TYK2
IL-13	STAT3 and STAT6	JAK1, JAK2 and TYK2
IL-21	STAT1, STAT3 and STAT5	JAK1 and JAK3
IL-22	STAT1, STAT3 and STAT5	JAK1 and TYK2
Angiotensin II	STAT1 and STAT2	JAK2 and TYK2
Growth hormone	STAT3 and STAT5	JAK2
EGF	STAT1, STAT3 and STAT5	JAK1 and JAK2
EPO	STAT5a and STAT5b	JAK2
IFN-α, -β	STAT1–STAT6	JAK1 and TYK2
IFN-γ	STAT1 and STAT2	JAK1 and JAK2
Leptin	STAT3	JAK2

Adapted from Refs [2,3,5]. Abbreviations: EGF, epidermal growth factor; EPO, erythropoietin; IFN, interferon; IL, interleukin; JAK, Janus kinase; LIF, leukaemia inhibitory factor; STAT, signal transduction and activation of transcription; TYK, tyrosine kinase.

JAK/STAT coordination

The JAK family includes JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2), and subsequent signalling components, STAT1–STAT5, STAT5a, STAT5b and STAT6. They are structurally and functionally similar to each other, but have a different gene map locus, which is illustrated by the location of the human *JAK*s. *JAK1* is located in 1p31.3, whereas *JAK2* is in 9p24. Conversely, *JAK3* (19p13.1) and *TYK2* (19p13.2) are located close to one another. STATs are also distributed on different chromosomes and in different gene map loci. In contrast with the large number of cytokines and their superfamily of receptors in mammalian cells, JAKs/STATs coordinate to different receptor complexes. Interferon (IFN)-γ signalling is relayed by JAK1 and JAK2, and IFN-α or IFN-β by JAK1 and TYK2 (Table 1). The combination of JAKs and STATs represent efficiency and economy in cell regulation. STATs typically contain the following domains: the N-terminus, coiled-coil, SH2, linker, DNA binding, and TAD in the C-terminus [5]. JAK3 has been found to be more restrictive in its coordination with ligands, receptors and STATs (Table 1). JAK3 is primarily expressed in haematopoietic cells and is an important pharmaceutical target (Table 2).

Negative regulation of JAK/STAT

Suppressor of cytokine signalling (SOCS) proteins are widely present in cytokine-stimulated cells. Cytokine regulation can occur in three ways, massive, transitive and coordinative. SOCS-1 is a commonly detectable negative regulator of JAK. It has been shown that the knockout of SOCS-1 is lethal in mice because myeloproliferative disorders are driven by excessive IFN-γ signalling. Interleukin (IL)-6 upregulates the expression of SOCS-1, which results in the inhibition of JAK/STAT activity and, therefore, downregulates IFN-γ expression in lymphocytes. [8]. SOCS efficiently inhibit proliferation signals induced by a variety

Table 2. IC_{50} (μM) of ATP-competitive kinase inhibitors

Inhibitor	Kinase or receptor				
	JAK1	JAK2	JAK3	EGFR	Lck
PP1	>50	na	na	>0.25	0.005
ZM39923.HCl	4.40	na	7.10	5.60	na
ZM449829	4.70	na	6.80	5.00	na
AG490 (Tyrphostin B42)	na	0.10	4.30	0.10	na
AG1295 (Tyrphostin)	na	na	na	0.30–0.50	na
WHI-P131	na	na	9.10	na	na
Glivec™ (Imatinib)	na	>100 [40]	na	na	na

Adapted from Ref [49]. Abbreviations: EGFR, epidermal growth factor receptor; JAK, Janus kinase; na, not available; PP, pyrazolo-pyrimidine.

of oncogenes or other abnormal signals [9]. The second inhibitor of the JAK/STAT pathway is Bcl-6, which normally functions as a transcriptional repressor by binding to STAT6. Bcl-6 knockout mice develop an inflammatory disease characterized by increased levels of IgE, which is analogous to human atopic skin disease. The third negative regulation point is the pseudo-kinase, JH2 domain, of the JAK protein. Recently, negative regulation of JH2 was demonstrated in an *Escherichia coli* expression system in which the kinase neoprotein had a higher level of autophosphorylation when it contained JH1 alone, compared with the construct that expressed JH1–JH2 together. The kinase assay used in the *E. coli* expression system avoided possible interference from other similar kinases that are always present in mammalian cells [10]. JAK/STATs are also negatively regulated by protein tyrosine phosphatases (PTPs), for example, the SH2-domain-containing PTP1 (SHP1, SHP2) and CD45, as well as the nuclear protein inhibitor of activated STAT (PIAS), through distinct mechanisms [11].

Inhibition mechanisms of JAKs/STATs

Physiological and pathological roles of JAKs/STATs

Understanding the accurate inhibition mechanism in physiological and pathological conditions has helped to identify high-quality prospective targets for precise inhibition. *Jak1*–/– mice are runted at birth and die perinatally or neonatally, which indicates multiple, obligatory roles for JAK1 in biological responses [12]. *Jak2*–/– mice do not respond to IFN-γ, but develop a form of acute myeloid leukaemia. *Jak3* knockout mice present a severe combined immune deficiency (SCID) phenotype [13]. Mutations that lead to SCID have been found in several domains of JAK3, but the majority of spontaneous mutations that result in truncation or rearrangements of the JAK3 protein occur in the pseudokinase domain. In these mutants, JAK3 kinase activity remains normal but the function is eventually impaired because the enzyme cannot effectively bind to its substrate(s) [14]. *Tyk2*–/– mice are viable and fertile, but TYK2 is involved in IL-13R–JAK/STAT signalling in goblet cell hyperplasia of the airways. Furthermore, *Tyk2*–/– mice have enhanced T-helper (Th) cell 2-mediated antibody production and allergic inflammation, which would suggest that TYK2 plays a role in the downregulation of allergic inflammation. TYK2 could also balance Th1 and Th2 through a bilateral role in the regulation of allergic inflammation [15]. Interestingly, as yet no TYK2 inhibitor has been reported, which could indicate that TYK2 has a suppressive role with respect to the abnormal expression of other genes. Involvement of aberrant JAK activation in human cancers is typically linked to a chromosomal abnormality because the translocation of the short arm of chromosome 9, which contains the kinase domain of JAK2, into the short arm of chromosome 12 forms a fusion protein called TEL–JAK2. This recombinant protein possesses constitutive kinase activity in β-progenitors and is associated with T-cell childhood acute lymphoblastic leukaemia (ALL). JAK1 in cells transformed by either v-Abl or Epstein–Barr virus might participate in the malignant processes. JAK2, TEL-JAK2 fusion protein and STAT3 have been frequently shown to be constitutively activated in human cancers [16–18], including breast, prostate and ovarian cancer, as well as leukaemia and other haematopoietic malignancies. Constitutive STAT activation is even more strongly associated with carcinogenesis. For example, STAT1, STAT3 and STAT5 have been detected in almost all types of cancers [19]. The T-cell transformation from IL-2 dependent to independent has been shown to be correlated with the activation of a group of JAK/STAT kinases [20]. The dominant negative form of STAT3, broadly equivalent to the defect phenotype, was able to prevent Src-induced transformation of NIH3T3 cells [21]. In addition to carcinogenesis, JAKs/STATs are strongly implicated in the pathology of asthma as regulators of the high proliferation and differentiation rates of B-lymphocytes. Imbalanced T-helper cells produce abnormal cytokine profiles that significantly contribute to IgE secretion by B-cells. They eventually provoke mast cell degranulation with the subsequent release of a variety of mediators that trigger early and late inflammatory asthmatic responses [22].

JAK/STAT and lymphocyte differentiation

Receptor-mediated activation of JAK/STAT leads to T-cell differentiation in the thymus, or at sites of inflammation. For example, IL-2 classically converts naive T-cells (Th0) into subsets Th1 and Th2. In a similar fashion, IL-4 and IL-6 effect preferential conversion to Th2, whereas IL-12 effects preferential conversion to Th1. In addition, IL-6 can convert monocytes into macrophages. The imbalance of cytokines frequently initiates and establishes inflammatory processes in many types of immune or autoimmune disease, for example, type 1 diabetes, allergy, inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis and immune rejection of transplantation. The over population of Th1 (versus Th2) in pancreatic islets is normally regarded as a precursor to insulitis as a result of the breakdown of mucosal tolerance, which eventually leads to pancreatic β-cell destruction by T-cell-mediated cytosol porin draining, or apoptosis [23]. A potent pyrazolo-pyrimidine (PP) inhibitor, PP1 (Figure 1), increases IL-4 production, but reduces IL-2 and interferon production in cultures of splenocytes from ovalbumin-specific T-cell receptor (TCR)-transgenic BALB/c mice. PP1 favours Th2 subset differentiation, even

though it strongly blocks the activation of lymphocytes [24], thus reducing the risk of insulitis in type 1 diabetes [25]. PP1 is thought to act by direct inhibition of the kinases Lck, Fyn or Hck, which are involved in TCR signalling and subsequently T-cell activation. PP1 inhibits JAK1 kinase *in vitro*, but it is a much more potent inhibitor of the cytosolic soluble tyrosine kinase Lck (Table 2), which phosphorylates intracellular domains of multiple components in co-stimulation of T-cell activation with antigen-presenting cells (APC) [26]. Thus PP1, its analogues and molecules with similar activity are potential therapeutic agents in autoimmune diseases.

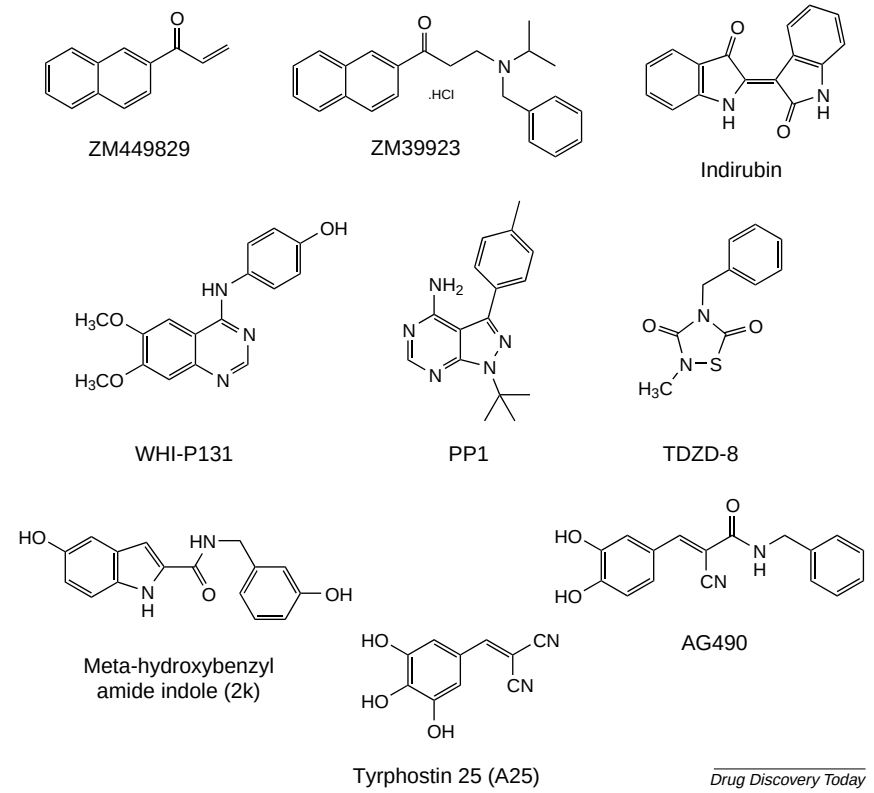

Figure 1. Small molecules that inhibit JAK and other kinases in *in vivo* and *in vitro* assays.

JAK/STAT and immune rejection of allografts

T-cells respond to allografts by trafficking to specific locations including, the graft itself, secondary lymphoid organs and other lymphoid tissues. Recent studies *in vitro* have shown that proper trafficking is important in developing tolerance to allografts. Lymphocyte trafficking is controlled by a complex interaction of a variety of receptors and ligands including adhesion molecules and chemokines and their receptors. Acute allograft rejection is driven by cytokines (e.g. IL-2) that activate and expand alloreactive T-cells. Alloantigen tolerance was demonstrated by a complete signalling blockade as a result of administration of anti-IL-2Rα monoclonal antibody [27]. However, IL-10 is a highly immunosuppressive ligand that influences T-cell, B-cell and APC function. In general, IL-10 downregulates immune response in a manner similar to IL-6 via JAK/STAT and a variety of SOCS molecules, which commence important downstream effects on cellular quiescence and alleviate immune rejection. Recently, a variant of the undecylprodigiosin family of antibiotics, PNU156804, was found to prolong allograft survival synergistically with the inhibitor cyclosporine A, and additively with rapamycin, by blocking allograft rejection through the targeting of JAK3 [28]. Furthermore, FK778 alone extends allograft survival by inhibiting JAK3 [29]. Although it is clear that cytokines and JAKs are involved in immune rejection, the range of inhibition, specific targeting sites, and accurate roles of individual JAKs needs to be studied further.

Inhibitors of JAK/STAT and tyrosine kinases

Precise inhibition of abnormally expressed or activated kinases, or antagonism of receptors might represent strategies for the development of chemotherapies. Through random or selectively sorted screening, several recognized candidates of inhibitors of JAKs/STATs are under further investigation (Figure 1). High-throughput screening (HTS) methods using ELISA and virtual screening (VS) methods have been developed to identify inhibitor candidates. Although advantages and disadvantages have been identified for ELISA and VS, these two techniques complement each other in the early stages of drug discovery [30].

Identification of random small molecule inhibitors of kinases by high throughput screening

The purification and crystallization of membrane proteins, or membrane-associated proteins, is still an issue for modern drug discovery. Small molecule receptor antagonists could potentially change the affinity of other ligands and receptors via protein–protein and protein–nucleic acid interactions [31]. At present, several inhibitors of JAK/STAT kinase have been identified by different HTS methods. For example, the screening of a 50 000 compound library led to the identification of a few positives for kinase inhibition,

including AS701173, which was further characterized as a potent non-ATP competitive inhibitor (Table 3). At present, several recognized, non-selective inhibitors of JAK kinase have been identified by HTS. AG490 (tyrphostin B42) was identified from kinase assays and was found to inhibit ErbB1 and ErbB2 autophosphorylation with an IC_{50} of 0.5 and 12.0 μM, respectively. In addition, AG490, and its analogue A25, inhibit JAK2 and JAK3 (Table 2), which blocks the downstream counterpart substrates including STAT1, STAT3, STAT5a and STAT5b [32]. Initially, AG490 was regarded as a specific JAK2 kinase inhibitor because JAK2 was found abundantly expressed and constitutively phosphorylated in acute lymphoblastic leukaemia cells [33]. However, AG490 actually suppresses IL-2-induced T-cell proliferation by inhibiting JAK3 in a dose-dependent manner in D10 and CTLL-2 T-cell lines [34]. Dimethoxyquinazoline derivatives, WHI-P154 and WHI-P131, inhibit JAK3 (Table 2), but not JAK1 or JAK2. Interestingly, WHI-P131 induces apoptosis in JAK3 expressing human leukaemia cell lines NALM-6. In cell-based assays, STI571 selectively inhibits chronic myeloid leukaemia (CML)-related kinases. Recently, STI571 was shown to inhibit the receptor for the platelet-derived growth factor (PDGF-R) and c-kit, a receptor tyrosine kinase, which consequently blocks stem cell factor-mediated cellular signalling, including downstream effects of ligand-stimulated receptor autophosphorylation, inositol phosphate formation, and mitogen-activated protein (MAP) kinase activation [35]. STI571 is currently in clinical trials as an adjuvant therapeutic agent for the treatment of non-small cell lung cancers because it inhibits lung cancer cell growth, probably by inhibiting PDGF-R-α phosphorylation [36]. Similarly, AG490, PP1 and their analogues inhibit the JAK-STAT pathway involved in PDGF-stimulated proliferation of human airway smooth muscle cells, an important pathway in the airway remodelling observed in some asthmatic patients [37].

Rational design of small molecule inhibitors of kinases by virtual screening

High-resolution 3D protein structure determination by X-ray crystallography is frequently the starting point for the design of selective and reversible inhibitors using docking software [38]. In most cases, it is preferable for docking to be reversible and for the inhibitor to have a high affinity of binding to the enzyme or receptor. Internal coordinated mechanics (ICM) is one of the approaches used in VS that can perform atom and charge assignment, and recognition of rotatable bonds in each unique structure. These methods enable parts of ligands to be automatically constrained to a pre-defined position during docking. Furthermore, these approaches generate multiple conformations of the receptor or the enzyme that is free to dock with small molecules. The simulation can display potential binding pockets on the protein surface and other interaction properties. Docking scripts under a grid framework modify flexible side chains to encompass induced fits. A classic example of the utility of molecular modelling in the development of compounds is that of the kinase inhibitor Glivec™ [also known as Gleevec™; STI571; Imatinib (Novartis; http://www.novartis.com)]. Inadvertent activation of the Abelson tyrosine kinase (Abl) causes CML. In nearly all cases of CML, the reciprocal translocation between chromosome 9 and 22 results in the fusion of Abl to the breakpoint cluster region (BCR). Glivec™ acts by ATP-competitive inhibition of the BCR–Abl tyrosine kinase and its chemical structure was designed with reference to the crystal structure of the kinase domain of c-Abl [39] and docking studies [40]. It effectively stopped CML *in vivo*, and gave excellent effects in clinical trials and, in May 2001, Glivec™ became the first drug on the market to inhibit protein kinases, a landmark in modern precision drug development. Another attractive lymphocyte-specific target for designing novel T-cell immunosuppressants is Lck, a decisive kinase in TCR-APC-mediated T-cell activation. The crystal structure of the SH2-SH3 domains of Lck was determined in monomeric and multimeric forms. Furthermore, the structure of the kinase domain was resolved in its activated state at 1.7 Å resolution [41]. The structure revealed a phosphoryl group at Tyr394 that generates a competent active loop, but phosphorylation at Tyr505 results in inactivation. Comparisons with other kinase structures have indicated that tyrosine phosphorylation and ligand binding could typically elicit two distinct hinge-like movements between the kinase subdomains. WHI-P131 and WHI-P154 were designed with the aid of a 3D homology model of the JAK3 kinase domain [33]. It is generally believed that the structure–activity relationships (SAR) and the 3D homology model can be used for drug design. Many challenges still remain, for example, the 3D structures of JAKs or the individual domains (including the kinase domain) are currently unresolved, and it will remain a substantial challenge to perform computer-aided drug design in the absence of a high-resolution structure.

Pharmacophore perception drug design from pseudo-substrate-based peptide inhibitors

In addition to common rational drug design methods, pseudo-substrate-based peptide inhibitors have been used for pharmacophore mapping studies. These studies will eventually impact on inhibitor design. Such rational design is possible because many kinase sequences are known and the ATP binding domains are relatively conserved. Peptidomimetic strategies are difficult, but the approach

can be used to obtain information for small molecular drug design, which is exemplified by the use of conformationally and topographically constrained combinatorial chemistry libraries generated by the 'split-mix synthesis' method [42]. Although the ATP binding domain has been used for drug design in the past, the SH2 domain plays a crucial role in organizing coherent signal transduction complexes that are essential for the appropriate intracellular response to extracellular stimuli. Blocking or inactivating SH2 domain-dependent signalling has been a useful strategy in developing therapeutic agents. Because of their similarity to the substrate of the binding domain of the kinase, tripeptides and tetrapeptides are the optimum size to mimic the interactions formed between the substrate and the surface of the SH2 domains, including the pY pocket. From peptide inhibitor studies on the SH2 domain of Src and Lck, it has been shown that these domains exhibit a marked preference for the sequence pYEEIE. Short peptides incorporating this sequence exhibit a reasonably high affinity for Src family SH2 domains. Interestingly, rosmarinic acid (RosA), which can be extracted from *Prunella vulgaris*, strongly inhibits the Lck SH2-pYEEIE interaction, which has been shown using ELISA, indicating that RosA is an inhibitor of the Lck SH2 domain. A threefold increase in the inhibitory effect was observed when a negatively charged amino acid was appended to RosA, for example, Asp or Glu. These analogues, which are specific for SH2 domains of Src family protein tyrosine kinases, are unique novel non-phosphopeptide SH2 inhibitors [43]. RosA inhibited TCR-induced-Ca^{2+} mobilization and IL-2 promoter activation, but not phorbol 12-myristate 13-acetate (PMA) induced IL-2 promoter activation, which indicates that its point of inhibition is at the membrane proximal site of TCR signalling. Therefore, RosA inhibits TCR-induced splenocyte proliferation by targeting the SH2 domain of Lck.

Modes of action of potential therapeutic kinase inhibitors

All kinases share certain structural similarities, for example, the presence of three disulfide bonds and similar ATP-binding clefts. The design of inhibitors for kinases is further complicated by their conformational flexibility, or plasticity, and interactions with other ligands and receptors [44]. Here we focus on two significant aspects related to ATP-binding inhibition.

Non-ATP competitive inhibition of kinases

In non-ATP competitive inhibition, the inhibitor binds to a loop or pocket outside the ATP-binding cleft of the kinase. It has been estimated that there are 2000 kinases and between 300 and 500 phosphatases in the human genome that use ATP as the second substrate when the concentration of ATP is high. Non-ATP competitive inhibition creates the possibility for high selectivity and potency, because these inhibitors compete with only nM concentrations of protein substrates, rather than μM or mM levels of ATP in the case of ATP-competitive inhibition. The small heterocyclic thiadiazolidinones (TDZD; Figure 1) are the first non-ATP competitive inhibitors for glycogen synthase kinase (GSK)-3β. They are relatively potent inhibitors with *in vitro* IC_{50} values in the μM range, independent of ATP concentration [45]. GSK-3β is involved in glycogen metabolism, but is also now known to regulate a diverse array of cell functions. The structure of GSK-3β contains a loop binding site for a pre-phosphorylated substrate, which is referred to as a priming phosphorylation site. This creates opportunities for the development of non-ATP competitive inhibitors that would selectively inhibit some functions of GSK-3β, but not others. TDZD has been suggested for therapeutic use in neurodegenerative diseases, type II diabetes, bipolar disorder, stroke, cancer and chronic inflammatory disease [46]. Although PP1 binds to the ATP pocket in Lck, as revealed by its co-crystal structure, PP1 is characteristic of non-ATP and ATP-competitive kinase inhibitors. However, reducing the ATP concentration in the assay did not improve the affinity between PP1 and Src kinases. Examination of the homology in the kinase domains of Src, Hck and Lck revealed significant differences outside the ATP binding pocket, whereas they are identical within the ATP binding domain. This indicates that an inhibitor must bind outside of the ATP-binding cleft to enable selective inhibition. Because activated Src is the hallmark of numerous cancers, understanding the mechanism of PP1 inhibition of activated Src should facilitate the discovery of potent and selective Src kinase inhibitors [47]. There have been several ATP-competitive inhibitors for MEK-1 (mitogen-activated protein kinase kinase-1), but recently Serono (http://www.serono.com/index.jsp) found that the inhibitory activity of AS701173 was independent of ATP concentration at the ranges used in the MEK-1 assay [48]. Similarly, PP1 inhibits Lck ATP-competitively, but inhibits $pp60^{c\text{-}src}$ non-ATP competitively. Screening for non-ATP competitive kinase inhibitors is particularly challenging because the mechanism by which these molecules bind is unclear, which makes rational design difficult. Interestingly, some naturally derived compounds that have recently been shown to be non-ATP competitive inhibitors are being used for analogue development (Table 3).

ATP-competitive inhibition of kinases

In contrast with sequence variation in non-ATP binding sites, the active sites are highly conserved in kinases. With

Table 3. IC_{50} (μM) of non-ATP competitive kinase inhibitors

Inhibitor	Target	IC_{50} (μM)	Refs
TDZD-8	GSK-3β	2.00	[46]
PP1	$pp60^{c\text{-}src}$	0.05	[47]
AS701173	MEK-1	0.03	[48]
Tyrphostin A47 (AG213)	EGFR-k	2.40	[55]
Piceatannol (ST-638)	PTK	50.00	[56]
	EGFR-k	2.10	[56]
Iminochromene 9TA	$pp60^{c\text{-}src}$	0.12	[56]
Meta-hydroxybenzyl amide indole (2k)	$pp60^{c\text{-}src}$	38.00	[57]

Abbreviations: AG213, 3,4-dihydroxy-α-cyanothiocinnamamide; EGFR-k, epidermal growth factor receptor-kinase; GSK, glycogen synthase kinase; MEK, mitogen-activated protein kinase kinase; PP, pyrazolo-pyrimidine; PTK, protein tyrosine kinase; ST-638, α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide.

respect to the substrate binding site, tyrosine kinases have a deeper loop and fewer consensus regions than serine/threonine kinases. Thus far, the majority of kinase inhibitors are based on ATP-competitive inhibition interfering with substrate binding. They are relatively non-selective ATP mimicking molecules or ubiquitous kinase inhibitors. One of the known ATP-competitive JAK3 inhibitors, ZM449829 (Figure 1), functionally inhibits T-cell proliferation, even though it also inhibits other tyrosine kinases to some extent [49]. Interestingly, ZM39923 (Figure 1), breaks down to form the JAK3 inhibitor ZM449829 and the compounds exhibit similar IC_{50} values (Table 2). At present, there is no structural information available for the ATP-binding site of JAK kinase. The ATP-binding sites of some kinases, including insulin receptor kinase (IRK) [50] and BTK [51], have been resolved. These structures show that the crucial components are associated with the active state conformation and include the closure of two lobes and the position of a c-helix relative to the N-terminal lobe. The two lobes in the BTK structure adopt a closed conformation in the ATP-binding domain. The distance of the c-helix from the active site is larger in BTK than in the IRK ternary complex structure, whereas the distance between Glu445 and Lys430 is 10.2 Å in BTK, but in IRK the corresponding distance is 3.0 Å [52].

Perspectives: rewards and remaining challenges

JAK/STAT and the tyrosine kinases play important roles in relaying the signals of cytokines. Cytokines are produced in abnormal amounts in cancer cells and lymphocytes under inflammatory conditions. Inhibition of JAK/STAT has advanced the basic and clinical studies of tyrosine kinase inhibitors as anti-cancer, anti-inflammation and anti-allograft rejection agents. At present, several tyrosine kinase inhibitors, but no JAK kinase inhibitors, have entered clinical trials. Some promising inhibitors, such as the JAK3 inhibitor FK778 (a leflunomide analogue) are in a highly advanced stage of preclinical development for the prevention of acute heart and kidney allograft rejection [28]. CP-690,550, another orally active, low molecular weight inhibitor of JAK3, has tripled survival time following kidney transplant in animal models, and is being developed by Pfizer (http://www.pfizer.com/main.html) to prevent the rejection of transplanted organs. Today, alternative strategies for 3D structural determination could also provide the means of translating the information from genome sequencing into knowledge that can aid the discovery of drugs based on common cross correlations [53]. This is an extremely complex system, but it can be more reliable than the simple theory of structure similarity versus biological activity in terms of drug screening strategy. There is almost no correlation of structure and function between inhibitors and targets. These similar compounds do not necessarily interact with the targeted macromolecule in similar ways [54]. However, it can be expected that the current environment of rapidly advancing structural information will greatly contribute to the theoretical and experimental 3D elucidation of target protein structure, which will hopefully result in greater ease of drug design.

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