Review Article

Therapeutic inhibition of the Janus kinases

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Since the success of imatinib in the treatment of chronic myeloid leukemia, tyrosine kinase inhibitors have been shown to be both efficacious and well tolerated despite absolute specificity for a single kinase. Consequently, multiple tyrosine kinase inhibitors have been approved and many more are in development. The JAK family of tyrosine kinases consists of 4 cytoplasmic proteins, which are obligatorily required for Type I/II cytokine receptors to signal and activate intracellular signaling pathways. They are critical for the function of over 60 cytokines and are therefore attractive targets for the generation of new immunomodulatory and oncology drugs.

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Introduction

Reversible phosphorylation is now recognized as one the most fundamental signaling in eukaryotic cells. This covalent modification is a major means for transmitting information from outside the cell and between sub-cellular components within the cell. Phosphorylated enzymes typically show altered activity and the presence of phosphorylated groups allows the association of key multiprotein complexes, which in turn trigger further signaling events. The importance of protein phosphorylation is supported by evidence that mutations and dysregulation of protein kinases play causal roles in human disease. This is especially true in cancer, in which mutant protein kinases or their upstream activators function as oncogenes.

From the point of view of an immunologist, protein phosphorylation is also the major mechanism by which key immune receptors signal. Examples include but are not limited to the T cell receptor, B cell receptor, NK, and Fc receptors. In addition, immune cells communicate an array of secreted polypeptides designated cytokines and many of these are couple to receptors that induce protein phosphorylation. Cytokines play critical roles in the development of hematopoietic and immune cells and in immunoregulation[1]. In this regard, they are key for both innate and adaptive immunity[2]. The receptors for some cytokines, like stem cell factor and platelet derived growth factor (PDGF) are receptor tyrosine kinases (RTKs), whereas the receptors for transforming growth factor family cytokines are receptor serine-threonine kinases. In contrast to these cytokine receptors, Type I and II cytokine receptors are devoid of intrinsic kinase activity, instead the signal through receptor-associated Janus kinases (JAKs, Fig.1).
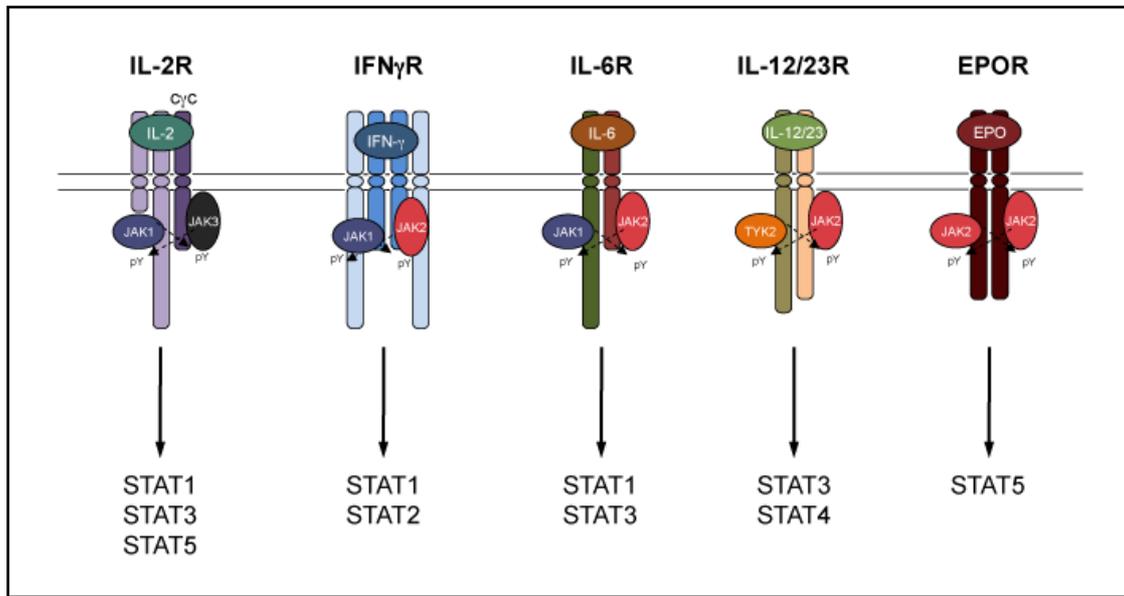
Fig.1 JAK family members associate with Type I/II cytokine receptor subunits

The non-redundant functions of various kinases in different immune cell protein kinases are well exemplified by different knockout mice and humans with mutations. (Fig.1). The JAK family consists of four members, the importance of which has been documented by knockout mice and by patient mutations[3]. Two members, JAK1 and JAK2, are essential for normal development, whereas patients that lack JAK3 and TYK2 have been described. Mutation of *JAK3* results in a severe combined immunodeficiency, characterized by an almost complete absence of T cells and natural killer cells with defective B cells[4-7].

Based on these findings and many others, targeting protein kinases has come to be recognized as a useful strategy in the development of novel immunosuppressant drugs. As will be discussed, the targeting of protein kinases is one of the most active areas of pharmaceutical drug development, much of the impetus coming from oncology. More recently, kinase inhibitors have been designed to address immune cell activation in order to obtain new immunosuppressive agents in the treatment of transplantation and autoimmune disease.

Structure and function of protein kinases

There are 518 kinases in the human genome divided into eight major families. Protein kinases or phosphotransferases, catalyze the transfer of γ phosphate of a purine nucleotide triphosphate, (i.e. ATP and GTP) to the hydroxyl groups of their protein substrates. Protein kinases can be classified by the amino acid substrate preference: serine/threonine kinases, tyrosine kinases and dual kinases (meaning that both serine/threonine and tyrosine residues can be phosphorylated). The protein tyrosine kinase (PTK) family has 90 members, one third of which are receptor tyrosine kinases (RTK) and the remainder are cytoplasmic proteins that typically function in close proximity to, and downstream of receptor / ligand complexes. Many signaling pathways are initiated by tyrosine kinases that lead to the subsequent activation of downstream, more numerous serine / threonine kinases. The existence of numerous kinases and phosphatases, which can target phosphotyrosine, phosphoserine or both, means that protein phosphorylation can be rapidly induced and reversed.

Almost all protein kinases have catalytic domains that belong to a single eukaryotic protein kinase (ePK) superfamily. The common evolutionary ancestry of the kinase domain (also known as the catalytic domain), which consists of 250-300 amino acid residues, manifests as a highly conserved three-dimensional structure. This consists of two lobes (N-lobe and C-lobe) that surround the nucleotide binding site[8]. The smaller N-lobe consists of a cluster of β-pleated sheets with a single α-helix. The larger C-lobe is made up of α-helices. Within the C-lobe lies the substrate-binding site, typically a groove on the surface. A hinge region connects the two lobes. The hinge, together with two loops emerging

from each lobe form the ATP binding pocket: the primary target for most kinase inhibitors.

Given the high degree of structural conservation, a priori one might assume that developing a therapeutically useful kinase inhibitor would be unlikely: many kinases serve critical cellular functions, any lack of specificity could theoretically produce a drug whose efficacy could be crippled by adverse effects. Fortunately though, this is not the case.

Imatinib and the first generation protein tyrosine kinases inhibitors

The success of the first FDA approved kyrosine kinase inhibitor did much to establish the efficacy of this group of drugs. Imatinib was designed as an inhibitor of Abl tyrosine kinase. BCR-Abl represents a fusion protein that is the result of a chromosomal translocation (Philadelphia chromosome) observed in patients suffering from chronic myeloid leukemia (CML)[9]. The Abl kinase is constitutively active within the fusion protein and has been implicated in initiating numerous signaling pathways that mediate cell survival and proliferation and the pathognomonic presence of BCR-Abl in CML led its being one of the most intensively studied protein tyrosine kinases. Since its introduction, imatinib has revolutionized the treatment of CML. Key to its success has been both the drugs ability to arrest the progression of the CML, and its modest side effect profile especially compared with the chemotherapies that it replaced[10]. The ability of patients to tolerate this drug for years of therapy lies in part with its kinase specificity, how is this possible?

The ATP binding region is made up of six polar amino acid residues that are invariant across whole families of kinases; similarly there are a number of lipophilic residues that are highly conserved. Nonetheless, it has been possible to identify inhibitors with a reasonable degree of specificity; although different kinases are structurally similar in an active ATP-bound confirmation, the inactive confirmation of these enzymes is more unique and can be used to generate selective inhibitors[11]. Within the critical ATP binding region there is an amino acid whose amide carbonyl binds to N-6 of adenine in the active confirmation. The side chain of this amino acid extends into the reaction pocket in the inactive state and for this reason is referred to as "the gate keeper residue" [12]. As the side chain is not involved in direct ATP binding it varies across kinases, and variation of this gatekeeper residue is exploited by a number of inhibitors that are able to bind the inactive confirmation of specific kinases. In the case of Abl kinase the gatekeeper residue is a threonine residue, which interacts with a methyl group of the phenyl ring of imatinib[11]. Across the collective kinase superfamily almost any amino acid can appear as the gate-keeper, although in practice it is typically a bulky non-polar residue (methionine, tyrosine, phenylalanine, lysine)[12].

Nonetheless, none of the existing kinase inhibitors are absolutely specific for a single kinase. Imatinib is a less specific inhibitor of Abl kinase than was initially assumed[13, 14]. As it turns out, this is has been beneficial; imatinib has been found to be efficacious for malignancies other than CML, which do not have abnormal Abl kinase activity. It has been used to treat gastrointestinal stromal tumor, and hypereosinophilic syndrome through its ability to inhibit Kit[15] and PDGFR-FIPIL1[16] kinases respectively. Thus the partial inhibition of multiple kinases can be both well tolerated and contribute to the efficacy of many inhibitors.

The long-term use of imatinib and other kinase inhibitors in cancer is associated with the acquisition of drug resistance. Interestingly one of the commonest sites of mutation is the otherwise conserved "gatekeeper residue"[17]. For this reason, "multikinase" inhibitors that are less selective than imatinib, like dasatinib and sunitinib, are therapeutical useful agents and remarkably, have acceptable levels of toxicity[18-20].

Targeting Cytokine Signaling by inhibiting Janus kinases: Tofacitinib, Ruxolitinib, and related compounds

The success of inhibitors of both tumor necrosis alpha (TNFα) and IL-6 in the treatment of rheumatoid arthritis and other autoimmune diseases suggests that targeting intracellular pathways downstream of cytokine receptors may also be useful[7, 21, 22]. The profound phenotype associated with absence of different JAKs led to the suggestion that inhibiting these kinases might be a strategy for the development of a new class of immunosuppressive drugs[23].

There are now many JAK inhibitors in clinical trials and under development, most of which inhibit multiple JAK family members. A number of these compounds have been found to be efficacious in preclinical models of autoimmune disease and allograft rejection[24-27]. Tofacitinib, formerly designated CP-690,550, was one of the first JAK inhibitors to enter the clinic. Tofacitinib inhibits JAK3 and JAK1 and to a lesser extent JAK2, but has little effect on TYK2[22, 28, 29]. Consequently, tofacitinib potently inhibits cytokines that bind

to the common γ chain, which associates with JAK3 (Fig.1). These include the cytokine IL-4, which is important for T helper (Th)2 development and allergic disease, IL-7 and IL-15, which are important for T cell homeostasis and memory, IL-21, which is an important product of follicular helper T cells that drives of B cell maturation and IL-9, which promotes mucus production. Given the importance of Th2 cells in the development of atopic disease[2], it is not surprising that tofacitinib is efficacious in models of these diseases[30]. It seems likely that tofacitinib would be useful in the treatment of allergy and asthma, assuming that that it safe (see below).

Inhibition of JAK1 by tofacitinib blocks IFN-γ, IL-6 and to a lesser extent IL-12 and IL-23, which collectively are required for differentiation of both Th1 and Th17 cells[22]. Both Th1 and Th17 cells are implicated in the pathology of rheumatoid arthritis[31]. In addition to T helper cells, innate immune mechanisms are also key drivers of pathology in arthritis.

Through its ability to block JAK1 and JAK2, tofacitinib has the capacity to block the action of interferons, IL-6, and other cytokines secreted by cells of the innate immune system[22, 32]. In a sepsis model, tofacitinib blocks the production of TNFα and IL-1, cytokines that are not themselves associated with JAKs[22]. For all these reasons, it is perhaps not surprising that tofacitinib was efficacious in preclinical arthritis models[33]. It is noteworthy that in both animal models and early clinical studies that the beneficial effect of tofacitinib is rapid, suggesting that this initial benefit may be derived from the action of this drug on the innate immune system[22, 34]. Phase II and phase III clinical trials investigating the role of tofacitinib in rheumatoid arthritis have demonstrated efficacy[34, 35]. Importantly, these studies show that tofacitinib is effective even in patients that have failed to respond to biological agents[36]. The ability of tofacitinib to suppress innate immune responses may help explain in the rapid responses seen in patients starting tofacitinib. In addition to rheumatoid arthritis, Tofacitinib is under clinical investigation for inflammatory bowel disease, psoriasis and prevention of transplant rejection.

Despite inhibiting multiple JAK's, tofacitinib has been well tolerated. As expected, there is an increase in infections; however, opportunistic infections are uncommon[32, 37]. In a study in patients receiving multiple immunosuppressive agents for renal transplantation, BK virus nephropathy was noted in patients that received tofacitinib, but this was thought to be due to over-immunosuppression[38]. Use of tofacitinib is not associated with significant declines in T-lymphocyte num-

bers. NK cell numbers do decline in patients on tofacitinib and the effect of long-term-therapy on lymphocyte cell numbers has yet to be fully evaluated[39, 40]. Dose dependent neutropenia and anaemia has been recorded in some of the trials and presumably this is due to the inhibition of JAK2[25]. The medium term use of tofacitinib is associated with a 15 ～ 30% increase in serum cholesterol although the ratio of total to HDL cholesterol is not altered[35]. The mechanism is unclear but is seen in a number of biological immuno-supressives including TNFα inhibitors and the anti IL-6 receptor antibody, tocilizumab[41, 42]. In all cases the increases can be reversed by the use of HMG-CoA inhibitors.

As gene targeting of *Jak2* in mice was embryonically lethal, it was initially assumed that inhibition of JAK2 would be undesirable. However, the discovery that gain-of-function mutations of *JAK2* underlie polycythemia vera and myelofibrosis led to the idea that pharmacologically targeting JAK2 could be useful. Ruxolitinib is the first of a list of JAK2 inhibitors for the treatment of myeloproliferative disease. Phase II and phase III clinical trials on patients with myelofibrosis have demonstrated that ruxolitinib was significantly superior to best available therapy in reducing symptoms of myelofibrosis[43] although anemia remains a problem and can be excerbated by ruxolitinib. Ruxolitinib inhibits both JAK1 and JAK2 and can cause anemia and thrombocytopenia in patients that do not have myeloproliferative disease although these can be mitigated by close control of dosing[44]. Patients with myelofibrosis have a significant and cumulative risk of developing acute leukemia in a manner similar to patients with CML. The ability of imatinib to prevent the development of acute leukemia in patients with CML was critical for its success. It remains to be seen if ruxolitinib is able to arrest the development of acute leukemia in patients with myelofibrosis.

As cγc cytokines employ both JAK1 and JAK3 for signaling, it might be expected that ruxolitinib and tofacitinib might block some of the same cytokines. It is therefore of interest to note that in a phase II study in rheumatoid arthritis, ruxolinitib had efficacy that was not dissimilar from tofacitinib[44]. Other JAK1/JAK2 inhibitors are also in development and clinical trials and it is notable that existing tyrosine kinase inhibitors appear to have activity against JAKs[45-48].

Conclusions

The scientific advances in the 1990's has led to the discovery of many novel intracellular signalling pathways that

link receptor and cytokine signalling with alteration of gene expression and cellular activation necessary to trigger an immune cell response. Many of these pathways are inter-linked to make up a complex array of networks made up of enzymes, adaptor proteins and transcription factors, all of which are potential targets for drug discovery in the quest to make a therapy that will be able to treat a specific auto-immune disease without an unacceptable degree of immuno-supression. Now more than a decade since the identification of many new targets, the first generation of drugs designed to interfere with specific immune cell signals are being brought to the clinic. The success of the anti cancer BCR-Abl inhibitor, imatinib has placed the protein kinases center stage as targets of future drug discovery. As many of the key steps in the activation of an immune cell are often shared with those that allow a cancer cell to proliferate, many of these agents are being tested as anti-cancer drugs rather than as immuno-suppressives. Despite this, agents originally intended for the treatment of cancer have been successfully used in the field of immunology and this may continue to be true for future modifiers of cell signalling. Conversely JAK inhibitors could potentially be used in the treatment of leukemia. Either way, we are likely to see a large number of novel immunosuppressants appear both serendipitously and intentionally as new protein kinase inhibitors are licensed for a wide range of debilitating illnesses.

Given their ability to block innate and adaptive responses, it seems likely that Jak inhibitors will be useful in a wide variety of setting for which other drugs that have more toxicities are presently being used. The extent to which Jak inhibitors supplant the use of these other drugs remains to be seen. Nonetheless, one wonders if Jaks can be used in place of steroids in disorders ranging from asthma to vasculitis; clearly this will require appropriate clinical trials. Topical Jak inhibitors may also be useful in inflammatory dermatologic and pulmonary disease; and indeed, preclinical data suggest that this is likely to be the case[49].

Disclosure of conflict of interests

Sources of funding

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