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STAT signaling in the pathogenesis and treatment of myeloid malignancies

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STAT transcription factors play a critical role in mediating the effects of cytokines on myeloid cells. As STAT target genes control key processes such as survival, proliferation and self-renewal, it is not surprising that constitutive activation of STATs, particularly STAT3 and STAT5, are common events in many myeloid tumors. STATs are activated both by mutant tyrosine kinases as well as other pathogenic events, and continued activation of STATs is common in the setting of resistance to kinase inhibitors. Thus, the targeting of STATs, alone or in combination with other drugs, will likely have increasing importance for cancer therapy.

Introduction

In the two decades since the STAT signaling pathway was first described, enormous strides have been made in understanding the critical role that this pathway plays in diverse hematopoietic cancers. STATs are typically oncogenic through the constitutive activation of tyrosine kinases, and through the years, a number of mutant kinases have been characterized that activate STAT signaling. Because of the critical role of tyrosine kinases in many cancers, much effort has gone into the search for inhibitors of these kinases that may be effective for cancer therapy. However, because of the critical role that STATs play in mediating the effect of kinases, they may also be directly targeted and may be effective anti-cancer agents. As we come to better understand STAT signaling in cancer, our ability to directly target the STAT pathway as a means of cancer therapy will be enhanced, thus contributing to a more personalized approach of treating patients.

STAT Signaling

Signal transducer and activator of transcription (STAT) proteins are a family of transcription factors that regulate critical cellular processes, such as proliferation, differentiation and apoptosis.1 When a growth factor or cytokine binds to its receptor, it either activates its intrinsic tyrosine kinase activity or causes the receptor chains to aggregate, bringing associated tyrosine kinases, usually JAKs, into juxtaposition.2 This activates their kinase activity, which mediates the subsequent tyrosine phosphorylation of the JAKs themselves as well as the cytokine receptor chains. The highly tyrosine phosphorylated receptor-kinase complex then serves as a docking site for proteins, such as STATs, which possess src-homology-2 (SH2) domains that allow binding to specific tyrosine-phosphorylated amino acid sequences.3 The STATs recruited in this way become phosphorylated on unique tyrosine residues necessary for activation,4 then dissociate from the receptor-kinase complex and dimerize via reciprocal phosphotyrosine-SH2 interactions.5 The STAT dimers translocate to the nucleus where they bind to a nine base pair sequence in the regulatory regions of target genes, thereby modulating their expression.6

STATs may also function as monomers and as non-phosphorylated dimers; however, in most circumstances it is the tyrosine phosphorylated dimer that is the critical mediator of signal transduction of the pathway. In some instances, the activity of the STAT transcription factor can be further modulated by the phosphorylation of the STAT protein on a serine residue.7 The activation of STATs is normally both rapid and transient and is subject to tight regulation. Such regulation includes not only kinase activation, but also inhibitory proteins that mediate the inactivation of STATs and prevent further signaling. These inhibitory regulators include phosphatases, suppressors of cytokine signaling (SOCS), protein inhibitors of activated STATs (PIAS) and nuclear ubiquitin E3 ligases.

STAT-mediated gene expression is involved in many normal physiological processes, such as proliferation, survival and differentiation. In hematopoietic cells in particular, cytokines whose effects are transduced by STATs play a central role in regulating the production of red blood cells, platelets and the full spectrum of white blood cells. Thus, it is not surprising that inappropriate activation of STATs plays a critical role in the formation and maintenance of the full spectrum of hematopoietic cancers, particularly those of the myeloid lineage. This can occur by a variety of mechanisms, including the autocrine or paracrine production of growth factors, activation of kinases by mutations, or loss of negative regulators (Fig. 1).

Acute myeloid leukemia. Deregulated STAT signaling is associated with increased cellular proliferation, disturbed differentiation and arrested apoptosis, which are the hallmarks of leukemogenesis. Constitutive activation of two of the family
members, STAT3 and STAT5, either alone or together, has been demonstrated in leukemic cell lines and blasts in a substantial proportion of patients with acute myeloid leukemia (AML).\textsuperscript{8-12} Constitutive activation of STAT3 and the presence of a truncated isoform, STAT3B, were correlated with a poor clinical outcome.\textsuperscript{12} Moreover, expression of the STAT3B isoform was more prevalent in relapse as compared with diagnosis.\textsuperscript{13} Recently, it was suggested that induced phosphorylation of signaling intermediates was more informative for understanding the biology of leukemic cells than the basal phosphorylation state. Using single cell flow cytometry, it was shown that potentiated STAT3 and STAT5 phosphorylation post growth factor stimulation was associated with a negative outcome for patients receiving standard AML chemotherapy.\textsuperscript{14,15}

Several mechanisms have been implicated for the constitutive activation of STATs in leukemias, including autocrine/paracrine stimulation by cytokines\textsuperscript{16} and the effects of kinases activated through mutations. Some of these mutations include chromosomal translocations generating fusion proteins with constitutive tyrosine kinase activity, such as BCR/ABL, which is a kinase fusion protein in chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL) that leads to the constitutive activation of STAT5. Mutations in FMS related tyrosine kinase 3 (FLT3), either involving internal tandem duplications (ITD) or point mutations in the activating loop of the tyrosine kinase domain, are observed in approximately 30% of AML patients and are associated with poorer prognosis.\textsuperscript{17} FLT3 ITD mutations cause the constitutive activation of FLT3, leading to aberrant activation of multiple downstream pathways, including STAT5.\textsuperscript{18-20} The activation of STAT5 by FLT3 ITD is independent of Src and JAK kinases.\textsuperscript{21} Further supporting the pathogenic role of this mutation, FLT3-ITD expression confers factor independent growth in murine IL-3-dependent cell lines and causes a fatal myeloproliferative disorder in murine bone marrow transplantation models and in FLT3-ITD knock-in mice.\textsuperscript{22,23}

Several small molecule FLT3-tyrosine kinase inhibitors (TKI) have been developed and examined in AML patients as single agents or in combination with chemotherapy. The induction of cytotoxicity by FLT3 inhibitors is closely correlated with deactivation of STAT5, while resistance to FLT3 inhibition is associated with persistent activation of STAT5.\textsuperscript{24} It is hypothesized that upregulation of FLT3 ligand and the silencing of SOCS expression by methylation of its genetic regulatory elements combine to enhance STAT signaling activity. These data support the use of combination therapy of FLT3 inhibitors with agents targeting the STAT pathway as treatment for AML patients with FLT3 mutations.\textsuperscript{25}

**Chronic myelogenous leukemia.** Chronic myelogenous leukemia (CML) is characterized by the presence of the Philadelphia...
chromosome, the reciprocal translocation of chromosomes 9 and 22 that generate the fusion protein BCR/ABL. This protein functions as a tyrosine kinase and can transform hematopoietic cells.\textsuperscript{26} STAT5 is constitutively activated by both the 190 kD and 210 kD isoforms of BCR/ABL.\textsuperscript{27-30} STAT5 activation is correlated with functional effects on cell cycle progression and resistance to apoptosis through increased expression of cyclin D1 and Bcl-xl, respectively\textsuperscript{31,32} and is essential for leukemic cell survival.\textsuperscript{33,34} Murine STAT5A-null bone marrow cells were inefficient in generating and maintaining a CML-like disease, suggesting an important role of STAT5 in the pathogenesis of CML.\textsuperscript{35} STAT5 activation may play a critical role in drug resistance in CML through the induction of P-glycoprotein and the modulation of telomerase activity,\textsuperscript{36} and high expression of STAT5 accounts for TKI resistance.\textsuperscript{37,38}

Currently, BCR/ABL kinase inhibition by imatinib, and the related kinase inhibitors nilotinib and dasatinib, is considered standard therapy for CML.\textsuperscript{38,39} Imatinib leads to complete inhibition of STAT5 activation, and this is likely a key part of the effectiveness of this therapeutic approach. However, resistance to imatinib develops in a subset of patients, generally through mutations in BCR/ABL that impair binding of the inhibitors to the ATP-binding site.

Several approaches have been taken to identify targets other than BCR/ABL for treating CML resistant to kinase inhibitors.\textsuperscript{40,41} Given the central role of STAT5 in mediating the pathogenic effects of BCR/ABL, this is an appealing target, as discussed in more detail below.\textsuperscript{42,43}

Another suggested mechanism for imatinib resistance is activation of STAT3 within the bone marrow microenvironment. This novel mechanism suggests the utility of using STAT3 inhibitors to increase the efficacy of BCR-ABL inhibitors.\textsuperscript{44}

**Myeloproliferative neoplasms.** Myeloproliferative neoplasms (MPN) are a group of clonal disorders that arise from the transformation of hematopoietic stem cells. For many years the molecular pathogenesis of these diseases remained unknown. It was reported that a subset of the patients with polycythemia vera (PV) displayed constitutive STAT3 activation in their peripheral granulocytes.\textsuperscript{45} By applying a panel of inhibitors, it was also shown that spontaneous erythropoietin-independent differentiation in PV is due to a constitutive activation of signaling pathways including JAK2-STAT5, PI3K and Src.\textsuperscript{46} STAT5 nuclear translocation and activation was detected in megakaryocytes and in circulating CD34\textsuperscript{+} cells from the majority of patients with idiopathic myelofibrosis and the spontaneous growth of these cells was abolished by STAT5 or JAK2 inhibition.\textsuperscript{47}

In 2005, several groups reported a single acquired point mutation in JAK2 in the majority of patients with Philadelphia chromosome (Ph)-negative MPN.\textsuperscript{48-51} This JAK2 mutation is a valine to phenylalanine substitution at position 617 (JAK2 V617F) in the kinase-dead JH2 domain. It is believed that this mutation disrupts the auto-inhibitory effect of the JH2 domain on the JH1 domain, which leads to both constitutive activation and hypersensitivity to the effect of cytokines.\textsuperscript{52} This mutation is believed to play a critical role in the pathogenesis of these disorders; mice transplanted with bone marrow cells transduced by a retrovirus expressing JAK2 V617F rapidly develop erythrocytosis, progressing to a myelofibrotic state within a few months.\textsuperscript{52-54} Reflecting the central role of STAT5 in the pathogenesis of these diseases, there is constitutive activation of STAT5 in JAK2 V617F-expressing Ba/F3 cells and a significant increase in phosphorylated STAT5 in the bone marrow and spleens of JAK2 V617F animals.\textsuperscript{55,56}

The ability of JAK2 V617F to induce cytokine-independent activation of the JAK2 and STAT5 pathways and transformation to cytokine independence requires the coexpression of homodimeric Type I cytokine receptors, such as the erythropoietin receptor (EpoR), thrombopoietin receptor (TpoR) or granulocyte colony-stimulating-factor receptor (GCSFR). EpoR mutations that impair erythropoietin-mediated JAK2 or STAT5 activation also impair transformation mediated by the JAK2 V617F kinase, indicating that JAK2 V617F requires a cytokine receptor scaffold for its transforming and signaling activities.\textsuperscript{55,56} Introduction of a constitutively active form of STAT5 and the overexpression of the STAT5 target gene Bcl-xl into human erythroid progenitors induces an erythropoietin-independent terminal differentiation and endogenous erythroid colony (EEC) formation, which is a hallmark of PV. STAT5 and Bcl-xl knock-down in human erythroid progenitors inhibits colony-forming unit-erythroid (CFU-E) formation in the presence of erythropoietin. These results suggest that JAK2 V617F may induce EEC via the STAT5-dependent Bcl-xl expression.\textsuperscript{57}

The mutational frequency of JAK2 V617F is estimated at over 95% in PV, 50% in essential thrombocytosis (ET) and primary myelofibrosis (PMF), 20% in certain other MPNs including refractory anemia with ringed sideroblasts and thrombocytosis (RARS-T) and less than 5% in AML or myelodysplastic syndrome.\textsuperscript{58} Thus, JAK2 V617F plays a critical role in a significant subset of MPNs, suggesting that targeting this kinase may be a useful treatment strategy.

In addition to STAT5, STAT3 is also activated in MPNs. Immunostaining of bone marrow biopsies show three specific patterns of phosphorylated STAT3 and STAT5 that differed significantly from the normal pattern. Specifically, there is uniformly increased STAT3 and STAT5 phosphorylation in PV; increased STAT3 phosphorylation and reduced STAT5 phosphorylation in ET; and uniformly reduced STAT3 and STAT5 phosphorylation in PMF. Interestingly, in all evaluated MPNs, the STAT5 and STAT3 phosphorylation pattern is not influenced by the presence of the JAK2 V617F mutation.\textsuperscript{59} By contrast, in another study examining phospho-STAT5 immuno-histochemistry of bone marrow biopsies of chronic MPN, all patients with the JAK2 V617F mutation showed abnormal nuclear STAT5 phosphorylation. In the JAK2 wild-type group, STAT5 phosphorylation was observed in about a third of the patients.\textsuperscript{60,61}

In addition to JAK2 V617F, other JAK2 mutations have been described in MPN. Mutations in exon 12 of JAK2 are consistently associated with increased levels of tyrosine phosphorylated JAK2 and STAT5. When transduced into Ba/F3 cells, all four JAK2 exon 12 mutations caused growth factor hypersensitivity and activation of biochemical pathways associated
Thus, inhibition of a common downstream mediator of the effects of these activated kinases holds out the promise for increased efficacy even in the setting of additional kinase mutations, the ability to block the effects of other activated kinases and the potential to synergize with kinase inhibitors and other therapies. The large number of tyrosine kinases that can be activated in hematological cancers converges on a small number of transcription factors, which then regulate the transcription of genes driving the tumor phenotype. Therefore, an appealing strategy is to directly target key transcription factors, such as STAT3 and STAT5, which may have broad applicability for cancer therapy (Fig. 4).

Figure 2. In myeloid leukemias and myeloproliferative neoplasms, a variety of mutations can lead to the activation of tyrosine kinases that can phosphorylate STATs, particularly STAT3 and STAT5. These STATs then drive the transcriptional activation of genes regulating survival, proliferation, self-renewal and other phenotypes characteristic of these diseases.

Targeting the STAT Pathway for the Treatment of Hematologic Malignancies

Since STATs are activated in numerous blood cancers and are essential to the pathogenesis of these tumors, targeting STATs is an attractive approach for therapeutic intervention. The activation of STATs can occur through the constitutive activity of tyrosine kinases, such as BCR/ABL, FLT3 and JAK2, as well as activation by autocrine and paracrine factors, loss of negative regulators and other mechanisms. Inhibiting tyrosine kinases is an appealing strategy for treating these diseases, in that it addresses the driving mutation in the malignant cell and can shut down several downstream pathways simultaneously (Fig. 3). In fact, the development of imatinib and other BCR/ABL kinase inhibitors represents a triumph of the molecular therapy of cancer. However, there are several limitations to this strategy. First, resistance often emerges to kinase inhibitors. This can occur through further mutations of the kinase, blocking the ability of the drug to bind to the target. In addition, activation of other kinases may occur to circumvent the dependence on the inhibited kinase. Thus, inhibition of a common downstream mediator of the effects of these activated kinases holds out the promise for increased efficacy even in the setting of additional kinase mutations, the ability to block the effects of other activated kinases and the potential to synergize with kinase inhibitors and other therapies. The large number of tyrosine kinases that can be activated in hematological cancers converges on a small number of transcription factors, which then regulate the transcription of the genes driving the tumor phenotype. Therefore, an appealing strategy is to directly target key transcription factors, such as STAT3 and STAT5, which may have broad applicability for cancer therapy (Fig. 4).
Strategies to developing STAT inhibitors: cell-based screens. The multiple steps through which an unphosphorylated STAT molecule in the cytoplasm proceed to activate gene transcription in the nucleus affords a number of opportunities for targeted inhibition. One strategy to identify inhibitors of the various steps in the STAT signaling pathway is to establish a cell-based assay in which the transcriptional activity of STATs can be monitored using a reporter, such as luciferase. Coupled with a counter screen to exclude non-specific effects, this approach allows the ability to rapidly screen thousands of compounds to identify specific STAT inhibitors. The open-ended nature of this screen allows for the identification of STAT inhibitors at any step in the signaling pathway, although it can be challenging to deconvolute how a hit derived from this assay specifically blocks STAT function. One compound that has emerged from this approach is pimozide, which inhibits both STAT3 and STAT5 in hematopoietic tumors including CML, AML and MPNs. Pimozide inhibits STAT3 and STAT5 phosphorylation, but several lines of evidence strongly suggest that it does not inhibit kinases such as BCR/ABL, FLT3 and JAK2. As expected by virtue of its targeting a downstream mediator, pimozide is effective in models of CML driven by BCR/ABL mutations, such as T315I, that render it resistant to currently available kinase inhibitors.

Pimozide has also displayed anti-leukemic effects in in vivo models. In a mouse model of AML driven by a FLT3 ITD mutation, pimozide results in a notable reduction in tumor burden (Nelson and Frank, manuscript under revision). Pimozide, which is FDA approved for neurological disorders, is known to have a good safety profile in humans. Reflecting this, pimozide is effective in models of CML driven by BCR/ABL mutations, such as T315I, that render it resistant to currently available kinase inhibitors.

Other STAT inhibitors that have been identified by this approach include nifuroxazide, which appears to act through kinase inhibition, and pyrimethamine, whose mechanism of action is still being elucidated. Thus, cell-based screens represent one useful strategy for developing STAT inhibitors with potential for clinical development.

The role of phosphatases in STAT inhibition. STAT activation represents a balance between phosphorylation by tyrosine kinases and deactivation largely by phosphatases. In some cancers, negative regulators of STATs exhibit low activity through decreased expression, often through promoter methylation. Therefore, one method of reducing STAT signaling is by
enhancing the activity of these negative regulators. There are several reports of drugs whose effects may be dependent on these negative regulators. For example, sunitinib and sorafenib are kinase inhibitors that are approved for use in solid tumors, though they may have benefit in hematopoietic tumors as well. However, several recent reports have also suggested a role for phosphatases in the action of these drugs. By inhibiting the activity of phosphatases using sodium vanadate or by siRNA-mediated knock-down, the ability of sorafenib or sunitinib to decrease STAT3 tyrosine phosphorylation was reduced. In addition, the reduction of phosphatase activity inhibited the ability of sunitinib to kill tumor cells in vitro. Therefore, it is possible that the activation of phosphatases is a critical component of the clinical effects of sorafenib, sunitinib and other kinase inhibitors. It is not surprising that phosphatases play an important albeit indirect role in dephosphorylating activated STATs after kinase inhibition. However, these studies on sorafenib and sunitinib suggest a more active role of these drugs on phosphatases, and thus, the dephosphorylation of the STATs may occur through both the inhibition of the kinase and activation of the phosphatase. Thus, the inhibition of STAT phosphorylation by the activation of phosphatases may gain increased importance in the near future.

Inhibition of DNA binding: decoy oligonucleotides. Transcription factors bind to DNA to regulate expression of their target genes, and thus one method of inhibiting their activity is to prevent them from binding to their target sequence. One means of doing so is by competing the protein away from binding to the regulatory regions of genes. Synthetic double stranded oligonucleotides containing a STAT binding sequence can act as decoys, such that activated STATs bind to this sequence, rather than to the regulatory regions of their endogenous target genes. The K562 CML cell line depends on the constitutive activation of STAT5, making it a logical model to test this approach. Introducing a decoy oligonucleotide into these cells reduces STAT5 transcriptional activity leading to the reduction in expression of critical STAT5 target genes and the induction of apoptosis. Importantly, this decoy oligonucleotide has no effect on the myeloid HL-60 cell line, which has no constitutively activated STATs, demonstrating that the effect of the decoy oligonucleotide depends on the presence of activated STAT5. Therefore, preventing STAT transcriptional activity through the use of a decoy oligonucleotide may be an effective way of reducing STAT activity in tumor cells.

Inhibition of DNA binding: small molecules. Since double-stranded oligonucleotides may have pharmacological properties limiting their applicability in vivo, alternate approaches to inhibit STAT-DNA binding are also of potential importance. To identify small molecules that directly inhibit STAT3 DNA binding activity, a library of compounds was screened using an in vitro binding assay. A platinum (IV) complex, IS3 295, was identified...
by its ability to inhibit STAT3 DNA binding. Though other platinum-based compounds such as cisplatin bind to DNA, their binding is non-specific. In contrast, IS3 295 inhibits STAT1 and STAT3 homo- and heterodimers from binding DNA, while having no effect on the ability of STAT5 homodimers or the unrelated E2F1 protein to bind to DNA. In contrast, cisplatin has no effect on the DNA binding activity of any of these proteins, demonstrating the specificity of IS3 295 to STAT1 and STAT3. Significantly, IS3 295 did not disrupt the binding of STATs that were already bound to DNA, suggesting that they could only bind to free STAT proteins. This led to the suggestion that IS3 295 directly interacts with the DNA binding domain of STATs, thereby preventing them from binding to DNA. IS3 295 inhibited STAT-mediated gene transcription and it led to apoptosis in cells containing constitutively activated STAT3.

Flavopiridol is a drug that has well-known antineoplastic activity due to its ability to inhibit cyclin-dependent kinases, but intriguing data suggest it might also disrupt STAT3-DNA binding. Using a variety of cell-free assays, it has been shown that flavopiridol inhibits the DNA binding activity of STAT3, while not affecting DNA binding of other proteins. In addition, flavopiridol decreases the transcription of Mcl-1, a STAT3 target gene important in apoptosis regulation. Though flavopiridol affects RNA polymerase II phosphorylation, it does not cause a global reduction in gene transcription, suggesting that flavopiridol has some selectivity to STAT3. Therefore, the data on flavopiridol and IS3 295 suggest that it is possible to inhibit the interaction of STAT3 with DNA and kill tumor cells, and thus targeted DNA binding inhibitors may have significant therapeutic potential.

**Dimerization inhibitors.** The activity of STATs is critically dependent on their SH2 domains. These are required for recruitment of STATs to activated receptor-kinase complexes where they become phosphorylated, and for each monomer to bind to the phosphorylated tyrosine of its binding partner, allowing active dimers to form. Though there may be biological effects of STAT monomers, tyrosine phosphorylated STAT dimers are likely the predominant active molecule for transcriptional regulation. Therefore, small molecules that specifically block SH2 domains would likely be useful STAT inhibitors. Using a structure-based virtual screen as well as the interrogation of chemical libraries, several such dimerization inhibitors have been identified. The first dimerization inhibitor discovered was STA-21. This compound disrupts dimer formation, has no effect on STAT3 phosphorylation and lacks any effect on STAT1 or STAT5. Treatment of cells with STA-21 reduced the expression of STAT3 target genes and induced apoptosis in cancer cell lines containing activated STAT3. Significantly, this compound was used in a small clinical trial. Topically applied STA-21 was successfully used to treat the skin lesions of psoriasis, a disease characterized by constitutive STAT3 activity. It is unclear what would be the bioavailability of this compound should it be given systemically for cancer patients.

Other similar approaches have led to both peptide-based and non-peptide small molecules that target the STAT3 SH2 domain. For example, C188-9, which selectively blocks STAT3 but not STAT1 phosphorylation, induces apoptosis in AML cell lines. Taken together, these studies suggest that STAT dimerization inhibitors may be important approaches to the treatment of cancer.

**Concluding Remarks**

Advances in our understanding of myeloid diseases has revealed that STAT transcription factors play a key role in activating genes driving the inappropriate proliferation, survival and self-renewal characteristic of these diseases. In addition to providing insights into their pathogenesis, these findings have also opened up new possibilities for targeted therapies of these diseases. The increasing use of tyrosine kinase inhibitors in clinical practice and in clinical trials has been a major advance in cancer therapy, and many of these drugs exert some or all of their effects through inhibition of STATs. However, directly targeting STATs is also likely to have a clinical benefit that may complement or exceed that of kinase inhibitors. Even in CML, where the targeting of BCR/ABL has led to great success, issues such as the emergence of drug resistance or the inability to eradicate the leukemic stem cell may be overcome with direct STAT5 inhibitors. In MPNs and AML, kinase inhibitors targeting JAK2 or FLT3 have been significantly less effective, perhaps due to co-activation of other pathways. While STATs and other transcription factors had traditionally been viewed as difficult targets to modulate pharmacologically, it is clear that with the use of a variety of strategies significant progress is being made. Thus, STAT inhibitors, alone or combined with kinase inhibitors and other therapies, may lead to enhanced clinical benefit.

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