Modulation of activation-loop phosphorylation by JAK inhibitors is binding mode dependent

Rita Andraos¹, Zhiyan Qian¹, Débora Bonenfant², Joëlle Rubert¹, Eric Vangrevelinghe³, Clemens Scheufler⁴, Fanny Marque¹, Catherine H. Régnier¹, Alain De Pover¹, Hugues Ryckelynck¹, Neha Bhagwat⁵,⁶, Priya Koppikar⁵, Aviva Goel³, Lorenza Wyder¹#, Gisele Tavares⁴, Fabienne Baffert¹, Carole Pissot-Soldermann³, Paul W. Manley³, Christoph Gaul³, Hans Voshol², Ross L. Levine³, William R. Sellers¹, Francesco Hofmann¹, and Thomas Radimmerski¹

Authors’ Affiliations: ¹Disease Area Oncology, ²Developmental and Molecular Pathways, ³Global Discovery Chemistry, ⁴Center for Proteomic Chemistry, Novartis Institutes for BioMedical Research, Basel, Switzerland. ⁵Human Oncology and Pathogenesis Program and Leukemia Service, Department of Medicine, Memorial Sloan Kettering Cancer Center. ⁶Gerstner Sloan Kettering Graduate School of Biomedical Sciences. #Current address: Actelion Pharmaceuticals Ltd., Allschwil, Switzerland.

Contributed equally.

Running title: Binding mode-dependent modulation of JAK phosphorylation

Keywords: JAK2, STAT5, chronic myeloproliferative neoplasm, polycythemia

Footnotes:

Corresponding Author: T. Radimerski, Disease Area Oncology, Novartis Institutes for BioMedical Research, Klybeckstrasse 141, 4057 Basel, Switzerland. E-mail: thomas.radimerski@novartis.com

Disclosure of Potential Conflicts of Interest: N.B., P.K., A.G. and R.L. declare no competing financial interests. Note that all other authors are or have been full-time employees of Novartis Pharma AG.

Author Manuscript Published OnlineFirst on May 3, 2012; DOI: 10.1158/2159-8290.CD-11-0324
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Abstract

JAK inhibitors are being developed for the treatment of rheumatoid arthritis, psoriasis, myeloproliferative neoplasms and leukemias. Most of these drugs target the ATP-binding pocket and stabilize the active conformation of the JAK kinases. This type-I binding mode can lead to an increase in JAK activation-loop phosphorylation, despite blockade of kinase function. Here we report that stabilizing the inactive state via type-II inhibition acts in the opposite manner, leading to a loss of activation-loop phosphorylation. We used X-ray crystallography to corroborate the binding mode and report for the first time the crystal structure of the JAK2 kinase domain in an inactive conformation. Importantly, JAK inhibitor-induced activation-loop phosphorylation requires receptor interaction, as well as intact kinase and pseudokinase domains. Hence, depending on the respective conformation stabilized by a JAK inhibitor, hyperphosphorylation of the activation-loop may or may not be elicited.

Significance

This study demonstrates that JAK inhibitors can lead to an increase of activation-loop phosphorylation in a manner that is binding-mode dependent. Our results highlight the need for detailed understanding of inhibitor mechanism of action, and that it may be possible to devise strategies that avoid target priming using alternative modes of inhibiting JAK kinase activity for the treatment of JAK-dependent diseases.
Introduction

The Janus kinase (JAK) family of non-receptor tyrosine kinases has crucial roles in cytokine signaling and development. In mammals four JAK family members, JAK1, JAK2, JAK3 and TYK2, have been identified (1-3). The JAKs contain a carboxyl-terminal tyrosine kinase domain and an adjacent pseudokinase domain (1), termed JAK homology (JH)-1 and -2 domains, respectively. It is thought that the JH2 domains lack protein kinase activity, although this has been challenged for the JAK2 JH2 (4), and that they may negatively regulate the JH1 kinase (5). However, regions of the JH2 domains appear to be required for signal transduction (6, 7). Through their amino-terminal domain the JAKs associate with cytokine receptors, which upon cytokine binding undergo a conformational change that allows JAK activation via either auto- or trans-phosphorylation on tyrosine residues in the activation-loop (8). Subsequently, the JAKs phosphorylate cognate receptors and STAT proteins that then translocate into the nucleus to initiate transcription of target genes (9).

JAK3 has emerged as a target for the prevention of transplant rejection and treatment of auto-immune diseases (10), and a JAK3-biased inhibitor is currently undergoing evaluation in patients with rheumatoid arthritis and other immunological diseases. JAK2 is an important oncology target due to STAT activation in many cancers, recurrent translocations in leukemias creating constitutively active JAK2 fusion proteins (11), and most notably activation of JAK2 through somatic mutation in polycythemia vera (12). Interestingly, the latter mutation occurs within the JH2 domain (12), exchanging valine at position 617 to phenylalanine, and is believed to disrupt the repressive function of the pseudokinase. The JAK2 V617F mutation is also frequently
found in the myeloproliferative diseases essential thrombocythemia (ET) and primary myelofibrosis (PMF) (13).

Different mechanistic approaches can be taken to inhibit protein kinases (14). The first JAK inhibitors to be described were the unselective tyrphostins, such as AG-490 (15), which are thought to act in a substrate-competitive manner, either non-competitive or mixed-competitive for ATP (16). More recently ATP-competitive compounds binding to the kinase-active conformation (type-I inhibitors) emerged as potent and selective JAK inhibitors (17, 18). Several type-I JAK inhibitors have been developed and some have demonstrated promising activity in patients with rheumatoid arthritis, psoriasis and myelofibrosis (19). In contrast, type-II inhibitors engage kinases in their inactive conformation (14). Such type-II inhibitors occupy a hydrophobic pocket adjacent to the ATP-binding site, which becomes accessible through a conformational shift of the activation-loop to the DFG-out state. Several type-II inhibitors targeting either a spectrum of kinases or directed towards BCR-ABL have been developed for the treatment of solid tumors or chronic myeloid leukemia, respectively. To date, however, type-II inhibitors targeting JAK2 have been relatively unexplored.

Here, we analyze the effects of JAK inhibitors having different binding modes on JAK activation-loop phosphorylation. Expanding on previous studies (20-22), we find that compounds, which inhibit JAKs with a type-I mechanism can increase JAK activation-loop phosphorylation, despite blockade of kinase function and inhibition of STAT phosphorylation. Importantly, we show that stabilizing JAKs in the inactive state by a type-II inhibitor leads to the loss of activation-loop phosphorylation. The increase
of JAK activation-loop phosphorylation by type-I inhibitors is staurosporine-sensitive, ATP-dependent and requires receptor interaction, as well as intact kinase and pseudokinase domains.

**Results**

*Structurally diverse ATP-competitive JAK inhibitors can increase JAK activation-loop phosphorylation*

Upon profiling JAK inhibitors in JAK2<sup>V617F</sup> dependent SET-2 cells, which have JAK2 amplification and predominantly express mutant JAK2 (23), we noticed an increase in JAK2 activation-loop phosphorylation with JAK inhibitor exposure, despite suppression of STAT5 phosphorylation (Fig. 1A). This phenomenon was seen with different inhibitors, including the pan-JAK inhibitor “JAK Inhibitor 1” (17), the JAK3-biased pyrrolo[2,3-d]pyrimidine CP-690,550 (18) and the JAK2-biased quinoxaline NVP-BSK805 (24). A similar disconnect between the changes in phosphorylation of JAK2 and STAT5 was previously observed for JAK Inhibitor 1 treated HEL cells (20-22), which only express JAK2<sup>V617F</sup> and have JAK2 amplification (23). Similar results were obtained in Ba/F3 cells expressing mutant MPL<sup>W515L</sup> (Supplementary Fig. S1A), which is found in ≤ 10% of JAK2<sup>V617F</sup>-negative ET and PMF cases (25). In B-cell precursor acute lymphoblastic leukemia (ALL) MHH-CALL-4 cells with deregulated CRLF2 expression and JAK2<sup>I682F</sup> mutation (26), the different JAK inhibitors suppressed STAT5 phosphorylation without appreciably altering JAK2 phosphorylation (Supplementary Fig. S1B). In Ba/F3 cells expressing TEL-JAK2, a cytoplasmic fusion protein of the oligomerization domain of TEL with the JAK2 kinase domain (27), NVP-BSK805 partially suppressed activation-loop phosphorylation (Supplementary Fig. S1C). Basal
JAK2 phosphorylation was minimal in SET-2 cells, but incubation with increasing concentrations of NVP-BSK805 increased activation-loop phosphorylation, reaching a plateau at concentrations of ≥ 300 nM, which coincided with suppression of STAT5 phosphorylation (Fig. 1B). As the JAK2 phospho-Tyr1007/Tyr1008 antibody can cross-react with the analogous TYK2 phosphorylation sites, we verified JAK2-specificity by depleting JAK2 in HEL92.1.7 cells, which appear to have largely lost dependency on JAK2V617F for proliferation (28). This approach should avoid potential confounding effects resulting from apoptosis induction after JAK2 depletion in JAK2V617F-dependent cells. Both baseline and JAK2 inhibitor-induced phospho-JAK2 levels were blunted in JAK2-depleted HEL92.1.7 cells (Fig. 1C), supporting specific detection of JAK2 activation-loop phosphorylation. TYK2 depletion did not impact induction of JAK2 phosphorylation upon JAK2 inhibitor treatment (data not shown).

In SET-2 cells treated with JAK inhibitors we failed to immunoprecipitate JAK2 using a carboxyl-terminus directed antibody (Fig. 1D), indicating that the inhibitors engage the kinase either in a conformation or multi-protein complex that masks the epitope. Accordingly, immunoprecipitation of JAK2 from inhibitor treated cells with an antibody recognizing an amino-terminal epitope was feasible and the kinase had increased levels of activation-loop phosphorylation, as compared to JAK2 immunoprecipitated from control cell extracts (Fig. 1D). Similar results were obtained using GM-CSF stimulated TF-1 cells with wild type JAK2 pretreated with NVP-BSK805 (Supplementary Fig. S1D).

Next, we assessed whether JAK inhibitors could also increase activation-loop phosphorylation on other JAK family members. CMK cells express JAK3 bearing an
activating A572V mutation (29) and constitutive STAT5 phosphorylation in these cells is dependent on both JAK3 and JAK1 (24, 29, 30). Treatment of CMK cells with JAK Inhibitor 1, CP-690,550 or NVP-BSK805 induced JAK3 activation-loop phosphorylation (Fig. 1E), with the extent being consistent with their rank order of potency towards JAK3 (18, 24). In TF-1 cells IFN-α stimulation led to weak TYK2 activation-loop phosphorylation together with robust STAT5 activation. Pre-treatment of TF-1 cells with JAK Inhibitor 1, CP-690,550 or NVP-BSK805 suppressed IFN-α-induced STAT5 phosphorylation and markedly increased TYK2 phosphorylation (Fig. 1F). However, pretreatment with JAK Inhibitor 1 prior to IFN-α stimulation did not discernibly augment JAK1 phosphorylation (Supplementary Fig. S1E). JAK Inhibitor 1 treatment did increase JAK1 activation-loop phosphorylation in HEL92.1.7 cells transiently transfected with JAK1 bearing the V658F activating pseudokinase mutation (31) (Supplementary Fig. S1F). These results show that structurally diverse ATP-competitive JAK inhibitors (Supplementary Fig. S2) can induce an increase in JAK activation-loop phosphorylation.

**Differential kinetics of JAK2 and STAT5 phosphorylation recovery after JAK inhibitor washout in cells**

The findings above raise the question of what happens to JAK/STAT signaling following inhibitor dissociation from their activation-loop phosphorylated target. To address this, SET-2 cells were treated with JAK inhibitors possessing different in vitro binding kinetics (Fig. 2A, Supplementary Fig. S3A-C). Following a pulse of JAK Inhibitor 1, CP-690,550 or NVP-BSK805, cells were washed, returned to medium without inhibitor and extracted at different times. All inhibitors suppressed STAT5 phosphorylation and increased JAK2 phosphorylation during drug-treatment (Fig. 2B).
After compound washout, STAT5 phosphorylation recovered between 30 minutes to 4 hours. Interestingly, JAK2 hyperphosphorylation was only lost in CP-690,550 treated samples (Fig. 2B), while in the samples treated with JAK inhibitor 1 or NVP-BSK805, levels of JAK2 phosphorylation remained elevated. These differences were not readily explained by the kinetic parameters of the inhibitors (Fig. 2A). Potentially on-target drug residence time in cells differs from that determined in biochemical assays with JH1 alone, e.g. due to the conformation adopted by JAK2 in cells. Alternatively, drugs with a slow off-rate may not have dissociated completely during the washing step. Incubation of factor-starved TF-1 cells with CP-690,550 did not lead to detectable induction of phospho-JAK2, and upon drug washout there was no appreciable STAT5 phosphorylation in the absence of GM-CSF (Supplementary Fig. S4A). These results indicate that JAK inhibitor-induced activation-loop hyperphosphorylation requires specific activating JAK mutations or, in the case of wild-type JAKs, cytokines engaging receptor complexes.

**Increased JAK2 activation-loop phosphorylation following JAK2 inhibitor treatment in vivo**

Induction of JAK2 activation-loop phosphorylation following kinase inhibition was also observed in vivo. In a mouse model of erythropoietin-induced polycytemia (24), the hormone induced STAT5 phosphorylation in spleen extracts (Fig. 2C), while signals for phospho-JAK2 were below detection limits. Treatment with NVP-BSK805 blunted erythropoietin-induced STAT5 phosphorylation, but increased phospho-JAK2 levels (Fig. 2C). Similarly, in mice transplanted with bone marrow expressing MPL<sub>W515L</sub>, causing significant thrombocytosis and myelofibrosis (25), NVP-BSK805 treatment
increased phospho-JAK2 in spleen extracts, while levels of phosphorylated STAT3 and MAPK were reduced (Fig. 2D).

Type-II JAK inhibition suppresses activation-loop phosphorylation and downstream STAT phosphorylation

The inhibitors assessed stabilize the JAK active conformations through a common binding mode (type-I binding mode), raising the question whether compounds stabilizing the inactive conformation (type-II binding mode) would differ in terms of effects on activation-loop phosphorylation. Compounds with low activity in enzymatic assays with activated (phosphorylated) JAK2 kinase, but good potency in JAK2-dependent cellular assays, as well as structural elements typical of type-II inhibitors were identified by database mining. The dihydroindole NVP-BBT594, a potent type-II inhibitor of wild-type and T315I mutant BCR-ABL (32), blocked proliferation of JAK2V617F mutant cells, but displayed limited activity in the JAK2 enzymatic assay (Supplementary Table S1). Importantly, incubation of SET-2 cells with NVP-BBT594 blunted STAT5 and JAK2 activation-loop phosphorylation (Fig. 3A). NVP-BBT594 also inhibited JAK2 activation-loop and STAT5 phosphorylation in Ba/F3 TEL-JAK2 cells and in MHH-CALL-4 cells (Supplementary Fig. S4B, C). Furthermore, NVP-BBT594 suppressed JAK3 and STAT5 phosphorylation in JAK3A572V mutant CMK cells (Fig. 3B), indicating that it inhibits JAK3, JAK1, or both. Accordingly, NVP-BBT594 suppressed growth of CMK cells with a GI50 of 262 nM in proliferation assays (Supplementary Table S1) and blunted IFN-α-induced JAK1, TYK2, STAT1, STAT3 and STAT5 phosphorylation in TF-1 cells (Supplementary Fig. S4D).
Immunoprecipitation studies provided additional evidence for a distinct JAK2 conformation being stabilized by NVP-BBT594. In contrast to type-I compounds (Fig. 1D), the ability to immunoprecipitate JAK2 from NVP-BBT594 treated cells was largely indistinguishable using antibodies directed to the amino- or carboxyl-terminal epitopes (Fig. 3C). In compound pulse-treatment/compound washout experiments with NVP-BBT594, STAT5 phosphorylation was initially inhibited and gradually returned to baseline (Fig. 3D). Hence, there is a marked difference in the changes of JAK activation-loop phosphorylation imposed by JAK inhibitors, depending on their binding mode.

**NVP-BBT594 binds to JAK2 in the DFG-out conformation**

The type-II binding mode hypothesis for NVP-BBT594 (Fig. 4A) was confirmed by X-ray crystallographic analysis of the inhibitor in complex with the JAK2 kinase domain at 1.35 Å resolution (Fig. 4B). In contrast to previous JAK2 crystal structures obtained with NVP-BSK805 (24) or JAK Inhibitor 1 (33), which are bound to the active conformation of JAK2 (“DFG-in” conformation), NVP-BBT594 binds to JAK2 in the inactive conformation (“DFG-out” conformation), where F995 of the DFG motif is translocated (10 Å) from its position in the kinase active state. In addition to the usual salt-bridge with catalytic K882, the carboxyl side-chain of conserved helix C residue E898 is also engaged in an H-bond interaction with NVP-BBT594. We believe that this is the first report of the JAK2 JH1 structure in an inactive conformation. The coordinates of residues 1000-1012 of the activation-loop are missing because of lack of electron density, as frequently seen in inactive kinase structures. NVP-BBT594 also interacts with the hinge region of JAK2 (Fig. 4B, C), with the pyrimidine occupying the adenine-binding pocket of the ATP binding site and making an H-bond interaction with
the backbone-NH of L932. An H-bond is also observed between the amide-NH and the backbone-CO of L932. In contrast to type-I inhibitors, NVP-BBT594 binds to the pocket made accessible through translocation of the DFG-motif with H-bonds between the urea-CO and the backbone-NH of D994 and side-chain of E898, H-bonds between the protonated N-methylpiperazine and the backbone-CO of both I973 and H974, and with the trifluoromethyl moiety participating in lipophilic interactions.

**JAK type-I versus type-II inhibition differentially affects JAK activation-loop phosphorylation as assessed by mass-spectrometry**

Tyrosine-phosphorylated peptide enrichment followed by mass-spectrometry was used to assess the effects of JAK type-I versus type-II inhibitors at the phospho-tyrosine proteome level. SET-2 cells were treated with DMSO, NVP-BSK805, CP-690,550 or NVP-BBT594. Between 100-200 different phospho-tyrosine peptides were identified (Supplementary Table S2), with a sub-set displaying marked changes upon JAK inhibition. Consistent with Western blotting, JAK2 activation-loop phosphorylation was elevated in NVP-BSK805 and CP-690,550 treated samples, and below baseline in NVP-BBT594 treated samples (Table 1). Similar findings were made for a peptide encompassing the TYK2 activation-loop phosphorylation site Tyr1054. As expected, treatment with the different JAK inhibitors suppressed STAT tyrosine phosphorylation (Table 1). Furthermore, JAK inhibition decreased JAK2 Tyr570 phosphorylation, which is a negative regulatory site (34). Loss of Tyr570 phosphorylation upon JAK2 inhibition, irrespective of inhibitor binding mode, was confirmed in JAK2V617F mutant cells by Western blotting (Supplementary Fig. S5A-F).
Type-I inhibitor-induced increase of JAK activation-loop phosphorylation is staurosporine-sensitive and ATP-dependent

In principle, very different mechanisms could cause increased JAK activation-loop phosphorylation upon kinase inhibition by type-I inhibitors: In the frame of feedback regulation, JAKs might phosphorylate and regulate their own phosphatase in the removal of activation-loop Tyr-phosphorylation (35). However, this hypothesis was inconsistent with RNAi-mediated depletion of tyrosine-phosphatases SHP-1, SHP-2 and PTP1B (data not shown). A second model favoring a JAK kinase conformation no longer recognized by negative regulatory proteins of the SOCS family appears unlikely since SOCS proteins interact with the phosphorylated activation-loop (36). Thirdly, JAK activation-loops exposed by type-I inhibitor binding (33) might be hyperphosphorylated by another tyrosine kinase. To investigate this possibility, SET-2 cells were firstly treated with the pan-kinase inhibitor staurosporine, and then with NVP-BSK805. Staurosporine alone had little effect on basal JAK2 phosphorylation, but suppressed the NVP-BSK805-induced increase (Fig. 5A). Since staurosporine can bind JAK2 in a type-I fashion (37), consistent with phospho-STAT5 suppression (Fig. 5A), SET-2 cells were pretreated (5 or 10 minutes) with NVP-BSK805, followed by treatment with staurosporine, which also suppressed the induction of phospho-JAK2 (data not shown). To investigate which tyrosine kinase activity might account for JAK activation-loop phosphorylation following JAK type-I inhibition, cells were pre-treated with inhibitors of ABL/KIT/PDGFR/DDR (imatinib), SRC-family kinases (dasatinib), FAK (NVP-TAE226) or IGF-1R (NVP-AEW541), but these drugs failed to suppress NVP-BSK805-induced JAK2 phosphorylation (Fig. 5A and data not shown). siRNA-mediated depletion of BTK, LYN or PYK2, which have been implicated in JAK2 signaling (9, 38), did not suppress NVP-BSK805-induced JAK2 phosphorylation (Fig.
5B, 5C and data not shown). JAK2\textsuperscript{V617F} transphosphorylation by another JAK family member appeared unlikely, as increased activation-loop phosphorylation was also seen with pan-JAK type-I inhibitors (Fig. 1A) and was not suppressed by simultaneous depletion of JAK1, JAK3 and TYK2 by RNAi in SET-2 cells (data not shown). Acute ATP-depletion in SET-2 cells, by blocking both glycolysis and oxidative phosphorylation, suppressed the NVP-BSK805-induced JAK2 activation-loop hyperphosphorylation (Fig. 5D, E), further implicating a kinase activity in the effect. These experimental conditions led to an approximately 5-fold reduction of cellular ATP levels, triggering AMPK activation (Fig. 5E).

\textit{Intact FERM, JH2 and JH1 domains are required for type-I inhibitor induced increase of JAK activation-loop phosphorylation}

These results led us to assess whether JAK2 itself might be involved in increasing activation-loop phosphorylation when a type-I inhibitor engages the JH1 domain in the active conformation. Accordingly, we probed the impact of introducing defined point mutations in the FERM, JH2 and JH1 domains, as previous studies indicated they interact \textit{in vivo} (5, 39). First, mutation of the JAK2 pseudokinase was investigated. Modeling of the JH2 domain revealed a structure in accordance with a previous model (40) and possibly compatible with nucleotide binding. Indeed, it was recently reported that the JAK2 pseudokinase can bind ATP and autophosphorylate both Ser523 and Tyr570 (4). Thus, if the JH2 domain assumes an active-like conformation and maintains catalytic activity under particular conditions, mutation of Lys581 in \(\beta\)-strand 3 would be predicted to abrogate catalysis (4). This lysine and the surrounding amino acids are conserved in the pseudokinase domains of JAK1, JAK3 and TYK2. JAK2\textsuperscript{V617F} or JAK2\textsuperscript{K581R/V617F} were transiently transfected into JAK2\textsuperscript{V617F} mutant HEL92.1.7 cells,
followed by NVP-BSK805 treatment. Compared to controls, JAK2\textsuperscript{V617F} transfected cells had higher levels of both basal and JAK2 inhibitor-induced phospho-JAK2.

Importantly, in JAK2\textsuperscript{K581R/V617F} transfected cells phospho-JAK2 levels were comparable to control cells and induction of phospho-JAK2 levels by NVP-BSK805 treatment was suppressed (Fig. 6A). As a negative control we mutated K607, situated adjacent to the predicted helix-\(\alpha\)C in the JH2 domain. In cells transfected with JAK2\textsuperscript{K607R/V617F} NVP-BSK805-induced activation-loop phosphorylation was not suppressed (Supplementary Fig. S6A). Transfection of JH1 domain catalytically dead JAK2\textsuperscript{V617F/K882E} also suppressed NVP-BSK805-induced activation-loop phosphorylation (Supplementary Fig. S6B). These results were corroborated in Ba/F3 cells stably expressing the human erythropoietin receptor by transient transfection with JAK2\textsuperscript{V617F}, JAK2\textsuperscript{K581R/V617F} and JAK2\textsuperscript{V617F/K882E} (Fig. 6B). To assess the potential involvement of the FERM domain, we transiently transfected cells with JAK2\textsuperscript{Y114A/V617F}, a FERM domain mutation that precludes receptor binding (41), which also suppressed NVP-BSK805-induced activation-loop phosphorylation (Fig. 6B).

These results suggest that defined conformations at receptor complexes are required for increased JAK2 activation-loop phosphorylation after type-I inhibitor treatment. In JAK2\textsuperscript{V617F} mutant cells, to enable this phenomenon we believe that the enzyme has to be bound to homo-dimeric type-I cytokine receptor complexes. To assess JAK signaling from oligomeric cytokine receptor complexes, we used JAK3\textsuperscript{A572V} mutant CMK cells (29). As JAK3 signals in conjunction with JAK1, which plays a dominant role in signaling via common gamma-chain containing cytokine receptor complexes (30), we tested the effect of JAK1 depletion on JAK inhibitor-induced JAK3 activation-loop phosphorylation. JAK1 depletion reduced basal levels of phospho-JAK3
and phospho-STAT5 and also strongly suppressed CP-690,550-mediated induction of phospho-JAK3 (Supplementary Fig. S6C). We then investigated interferon signaling via the heterodimeric IFN-α receptor complex. Depletion of JAK1 in TF-1 cells suppressed STAT5 phosphorylation and JAK Inhibitor 1-mediated induction of phospho-TYK2 upon IFN-α stimulation (Supplementary Fig. S6D). As we could not preclude that JAK1 depletion might impact receptor-complex conformations and/or receptor interaction of the respective cooperating JAK family members, we assessed the impact of exogenous JAK1 expression. TF-1 cells were either transiently transfected with empty vector, wild-type JAK1, or a variant with a mutation in the conserved lysine in β-strand 3 (K622R). Enforced expression of JAK1 resulted in constitutive JAK1 phosphorylation but modest inducibility of TYK2 and STAT5 phosphorylation by IFN-α. Nevertheless, JAK Inhibitor 1 pretreatment resulted in elevated levels of phospho-TYK2, although less than that of vector control (Supplementary Fig. S6E). In contrast, phospho-JAK1 was undetectable in cells expressing JAK1K622R, either at baseline following factor starvation, or after IFN-α stimulation, and levels of phospho-TYK2 and phospho-STAT5 were lowest. Finally, in transient transfection experiments using HEL92.1.7 cells, the JAK1K622R protein (Fig. 6C), but not wild-type JAK1 (Supplementary Fig. S6F) or a negative control mutant JAK1K648R (Fig. 6D), suppressed JAK Inhibitor 1-mediated increases in JAK1V658F activation-loop phosphorylation. Thus, the increase of JAK activation-loop phosphorylation by type-I inhibitors requires JAK receptor interaction, as well as intact kinase and pseudokinase domains.

**Discussion**

Several examples of inadvertent kinase activation by small molecule inhibitors have been reported (42, 43), one of the most striking being the cross-activation of RAF
family members by B-RAF kinase inhibitors in B-RAF wild-type cells (44). Often the involved kinases exhibit elevated phosphorylation on regulatory sites, which may enable rapid catalytic reactivation upon dissociation of the inhibitors. Thus, it is important to recognize and understand feedback and priming mechanisms in the process of drug discovery, as it may be possible to devise strategies that avoid target priming.

We find that inhibitors targeting the active conformation of JAK2 can increase activation-loop phosphorylation, corroborating and expanding on previous studies (20, 21). In cells with either deregulated CRLF2 expression together with JAK2I682F mutation or expressing TEL-JAK2, type-I kinase inhibitors either did not enhance or partially suppressed activation-loop phosphorylation. We speculate that JAK2 may already be maximally phosphorylated in MHH-CALL-4 cells, whereas chimeric TEL-JAK2 lacks the regulatory elements of native JAK2. Importantly, complete loss of JAK activation-loop phosphorylation occurred with a compound that stabilized the inactive state. We also report the first co-crystal structure of JAK2 in complex with a type-II inhibitor, which might enable the design of more potent inhibitors. It will be of particular interest to assess if type-II inhibitors differ in terms of MPN disease-modification as compared to type-I inhibitors, which could be explored in animal models of MPN-like disease (12, 25).

Currently, it is unclear how engagement of JAKs in their active conformation by type-I inhibitors results in increased activation-loop phosphorylation. The process appears to be ATP-dependent and staurosporine-sensitive. It is thought that JAK activation involves JAK trans- and/or auto-phosphorylation. Furthermore, there appears to be an interplay between the JH1 and JH2 domains, and the latter may have both
negative and positive impacts on JH1 kinase activation. On one hand, disruption of the JAK2 JH2 domain dramatically increases JH1 activity \textit{in vitro} (5), but on the other hand an intact JH2 domain is crucial for JAK2, JAK3 and TYK2 activation in cells by cytokines and interferons (6, 7, 45). In severe combined immunodeficiency, JAK3 JH2 mutations (\textit{e.g.} C759R) were described that paradoxically result in a hyperphosphorylated, but catalytically inactive kinase (46). Interestingly, the phosphorylation is lost when K855 is mutated in the kinase domain of C759R mutant JAK3 (45). In JAK3, either deletion of the JH2 domain or its inclusion in a construct otherwise only containing JH1 abrogated kinase activity (45). These findings were reconciled in a model in which the FERM, JH2 and JH1 domains interact to form a signaling-competent kinase.

From this perspective, our findings that specific mutations either in the FERM, JH1 or JH2 domain suppress JAK type-I inhibitor-induced increase in JAK2^{V617F} activation-loop phosphorylation are intriguing. This suggests that JAK-receptor complex interactions, ligand binding or activating mutations are required to permit increased activation-loop phosphorylation after type-I inhibitor treatment. Furthermore, the kinase domain itself needs to be intact, which could be interpreted as transphosphorylation of the inhibitor-bound JAK by the other JAK partner at the receptor complex, if its conformation precludes inhibitor binding, but not ATP binding and phosphorylation of activation-loop tyrosines. However, we cannot exclude the possibility that JAK2^{V617F/K882E} predominates in the inactive conformation, providing a potential explanation for reduced type-I inhibitor-induced activation-loop phosphorylation.
JH2 β-strand 3 mutation also suppressed JAK inhibitor-induced JAK activation-loop phosphorylation, indicating that an intact pseudokinase domain is required for the phenomenon to occur. Functional impact of specific JH2 domains is underscored by the recurrence of the V617F mutation in myeloproliferative neoplasms (12, 13) and of R683 mutations in ALL (47), which suggests these hotspots could constitute a gain of function, rather than a loss of function. Interestingly, modeling and mutagenesis approaches have identified F595 in the JH2 helix-αC to be required for constitutive JAK2V617F activation (40). The model postulates that F595 and F617 interact, which may impact orientation of the JH2 helix-αC and thereby may lead to modulation of JH1 activity. Notwithstanding this stacking model, F595 is also required for constitutive activity of JAK2 exon 12 mutants and R683G mutant JAK2 (40).

The conserved lysine of JH2 β-strand 3 might interact with a conserved glutamate in the helix-αC to impose the correct JH2 architecture. Our findings with JAK2K581R and JAK1K622R are consistent with a model in which the JH2 assumes an active-like conformation and perhaps even catalytic activity under particular circumstances. Recent studies suggested that proteins previously assumed to be pseudokinases, such as CASK (48) or ErbB3 (49), might have protein kinase activity in vivo, despite lacking the canonical catalytic residues. The JAK JH2 domains lack the Asp in the HRD motif of the catalytic loop. Whether key residues required for catalysis might be provided under specific conditions by a substrate, structurally compensated for by appropriate residues in adjacent sub-domains or by interacting domains can only be speculated.
Ungureanu et al. reported that the JH2 domain of JAK2 has dual-specificity protein kinase activity *in vitro*, auto-phosphorylating the negative regulatory sites Ser523 and Tyr570. This suggests that JH2 catalytic activity maintains low basal JAK2 JH1 activity, supported by the finding that K581A mutation led to elevated phosphorylation of the JAK2 activation-loop, STAT1 and STAT5, along with loss of JAK2 Ser523 and Tyr570 phosphorylation (4). However, in our studies with JAK2V617F/K581R and JAK1K622R proteins, we did not detect elevated baseline JAK activation-loop (as compared to JAK2V617F and JAK1, respectively) or STAT phosphorylation. Our results suggest that when JH1 is inhibited by a type-I inhibitor exposing the activation-loop, JH2 has a positive role in regulating its phosphorylation. A potential shift in the phosphorylation pattern on regulatory sites upon JAK2 inhibition is also supported by the finding of reduced Tyr570 phosphorylation in our phosphoproteomics analysis. Taken together, our data suggest that alternate modes of inhibiting JAK kinase activity might present a novel therapeutic strategy for the treatment of JAK-dependent diseases.

**Materials and methods**

*Compounds*

NVP-BSK805 (50), NVP-BBT594, staurosporine and imatinib were synthesized internally. JAK Inhibitor 1 was from Calbiochem (San Diego, CA, USA) and CP-690,550 from Cardiff Chemicals (St Mellons, Cardiff, UK). Compounds were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO).

*Cell culture*
SET-2, CMK and TF-1 cells were cultured as described (24). HEL92.1.7 cells (ATCC, Manassas, VA, USA) were cultured in RPMI medium supplemented with 10% of fetal calf serum, 2 mM L-glutamine, 1% sodium pyruvate and 1% (v/v) penicillin/streptomycin. Cell lines were verified in 2006 by isolating genomic DNA and sequencing JAK2 exon 14 (and JAK3 exon 13 in CMK cells), and later by genotyping using Affymetrix Genome-Wide Human SNP Array 6.0. MHH-CALL-4 cells (DSMZ, Braunschweig, Germany: Performs characterization by short tandem repeat DNA typing) were cultured in 24-well plates in medium as described above, but supplemented with 1% HEPES and 1% sodium pyruvate.

Western blotting

Cells were treated with inhibitors and extracted essentially as described (24). Typically, 20 μg of protein lysates were resolved by NuPAGE Novex 4-12% Bis-Tris Midi Gels (Invitrogen, Carlsbad, CA, USA) and transferred to PVDF membranes by semi-dry blotting. The following antibodies were used: Phospho-STAT5 (Y695) (#9359), phospho-JAK1 (Y1022/Y1023) (#3331), JAK1 (#3332), phospho-JAK2 (Y1007/Y1008) (#3776), JAK2 (#3230), phospho-TYK2 (Y1054/Y1055) (#9321), TYK2 (#9312), phospho-STAT1 (Y701) (#9171), phospho-STAT3 (Y705) (#9131), phospho-AMPKα (T172) (#2535), AMPKα (#2532), BTK (#3533) and LYN (#2796) were from Cell Signaling Technology (Beverly, MA, USA). JAK2 (#sc-34480), phospho-JAK3 (Y980) (#sc-16567), TEL (#sc-11382) and STAT5 antibodies (#sc-835) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-JAK2 (Y570) (#09-241) was from Millipore (Billerica, MA, USA) and 4G10 was made in-house. The β-tubulin (#T4026) antibody was from Sigma (St. Louis, MO, USA). Antibodies were typically incubated overnight at 4°C followed by washes and incubation with HRP-
conjugated secondary antibodies. Immunoreactive bands were revealed with ECL reagents.

**JAK immunoprecipitation**

Cells were extracted as described above. Typically, lysates were adjusted to 0.5 mg total protein input in 200 μL of lysis buffer. Antibodies for immunoprecipitation of JAK2 (#sc-34480, amino-terminal epitope) or JAK3 (#sc-513) were from Santa Cruz Biotechnology, and of JAK2 (#3230, carboxyl-terminal epitope) or TYK2 (#9312) from Cell Signaling Technology. Then, 25 μL of UltraLink Immobilized Protein A/G beads (Pierce, Rockford, IL, USA) were added and samples were incubated for 1 hour with rotation at 4°C. After washing, bound fractions were released by heating at 70°C for 10 minutes in 20 μL NuPAGE LDS sample buffer for Western blot analysis as described above.

**X-ray crystallography**

Crystals were grown at 20°C using the hanging drop vapor-diffusion method. Purified JAK2 kinase domain (JAK2(840-1132)::Y1007F, Y1008F) with mutations Y1007F and Y1008F in complex with NVP-BBT594 (molar ratio 1:3) at 9 mg/mL in 20 mM Tris, 250 mM NaCl, 1 mM DTT, pH 8.5 was mixed with an equal volume of a reservoir solution containing 1.8 M sodium malonate pH 6, 0.1 M glycyl-glycine pH 8.2. Plate-like crystals grew within a few days. Prior to flash-cooling the sample in liquid nitrogen it was transferred to a cryoprotectant solution consisting of 2.7 M sodium malonate pH 6, 0.1 M glycyl-glycine pH 8.2. During data collection the crystal was cooled at 100 K. Diffraction data were collected at the Swiss Light Source (beamline X10SA) using a Marresearch CCD detector and an incident monochromatic X-ray beam with 0.9999 Å.
wavelength. Raw diffraction data were processed and scaled with XDS/XSCALE software. The structure was determined by molecular replacement with PHASER as implemented in CCP4 using as search model the coordinates of JAK2 in complex with a pan-JAK inhibitor (PDB code 2B7A, (33)). The program BUSTER was used for structure refinement (Supplementary Table S3) using all diffraction data between 20 and 1.34 Å resolution, excluding 5% of the data for cross-validation. The final model converged at $R_{\text{work}} = 17.6\%$ ($R_{\text{free}} = 18.9\%$). The refined coordinates of the complex structure have been deposited in the RCSB Protein Data Bank under accession code 3UGC.

Mouse models of MPN-like disease

The mouse models of erythropoietin-induced polycythemia (24) and MPL$^{W515L}$ driven myelofibrosis-like disease (25) have been described previously. Animal experiments were performed in strict adherence to the Swiss law for animal welfare and approved by the Swiss Cantonal Veterinary Office of Basel-Stadt, or performed according to an animal protocol that has been approved by the MSKCC Instructional Animal Care and Utilization Committee.

Acknowledgements

The authors wish to thank Hans Drexler for the generous gift of SET-2 cells. The authors thank Sébastien Rieffel, Bernard Mathis, Markus Kroemer, Céline Be, Nina Baur, Francesca Santacroce and Violetta Powajbo for excellent technical assistance. We thank Micheal Eck for fruitful discussions and Ralph Tiedt, Patrick Chêne and David Weinstock for critical reading and comments on the manuscript.
References


23. Quentmeier H, MacLeod RAF, Zaborski M, Drexler HG. JAK2 V617F tyrosine kinase mutation in cell lines derived from myeloproliferative disorders. Leukemia 2006;20:471-6.


Tables

Table 1. Analysis of changes in tyrosine phosphorylation by mass-spectrometry following treatment of SET-2 cells with different JAK inhibitors.
Table 1. Log2 ratios of selected phospho-tyrosine peptides following JAK inhibitor treatment. Changes in phosphopeptide abundance following JAK inhibitor versus DMSO treatment in the different experiments are displayed as log2 ratios calculated from duplicate runs. The table shows a subset of the detected peptides and the respective affected phospho-tyrosine residues are indicated. ND: Not detected.

<table>
<thead>
<tr>
<th>P-Tyr site</th>
<th>BSK805 Exp. 1</th>
<th>BSK805 Exp. 2</th>
<th>CP-690,550 Exp. 1</th>
<th>CP-690,550 Exp. 2</th>
<th>BBT594</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK2 Y570</td>
<td>-1.59</td>
<td>-2.80</td>
<td>-3.62</td>
<td>-2.10</td>
<td>-2.68</td>
</tr>
<tr>
<td>JAK2 Y1007</td>
<td>3.94</td>
<td>6.95</td>
<td>5.34</td>
<td>3.58</td>
<td>-0.88</td>
</tr>
<tr>
<td>JAK2 Y1007, Y1008</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-2.16</td>
</tr>
<tr>
<td>LYN Y266</td>
<td>6.71</td>
<td>-0.76</td>
<td>0.25</td>
<td>-0.31</td>
<td>-2.85</td>
</tr>
<tr>
<td>PTPN11 Y63</td>
<td>2.50</td>
<td>-0.88</td>
<td>0.07</td>
<td>0.30</td>
<td>-1.09</td>
</tr>
<tr>
<td>PTPN18 Y389</td>
<td>-1.64</td>
<td>-3.40</td>
<td>ND</td>
<td>-2.13</td>
<td>-2.78</td>
</tr>
<tr>
<td>PTPN6 Y564</td>
<td>-2.50</td>
<td>-2.65</td>
<td>-0.74</td>
<td>-0.53</td>
<td>-2.37</td>
</tr>
<tr>
<td>PTPRA Y798</td>
<td>-8.64</td>
<td>-6.01</td>
<td>-0.49</td>
<td>0.11</td>
<td>-3.28</td>
</tr>
<tr>
<td>SKAP2 Y11</td>
<td>3.89</td>
<td>-0.27</td>
<td>2.36</td>
<td>1.69</td>
<td>-1.83</td>
</tr>
<tr>
<td>SKAP2 Y197</td>
<td>-1.09</td>
<td>0.28</td>
<td>0.26</td>
<td>0.63</td>
<td>-0.75</td>
</tr>
<tr>
<td>STAT1 Y701</td>
<td>ND</td>
<td>ND</td>
<td>-3.84</td>
<td>ND</td>
<td>-3.96</td>
</tr>
<tr>
<td>STAT3 Y705</td>
<td>-2.32</td>
<td>-3.67</td>
<td>-7.73</td>
<td>-2.80</td>
<td>1.37</td>
</tr>
<tr>
<td>STAT5A Y90</td>
<td>-0.98</td>
<td>0.85</td>
<td>-0.40</td>
<td>0.49</td>
<td>-0.54</td>
</tr>
<tr>
<td>STAT5A Y694</td>
<td>-2.47</td>
<td>0.32</td>
<td>-2.60</td>
<td>-3.25</td>
<td>-2.24</td>
</tr>
<tr>
<td>TYK2 Y292</td>
<td>4.99</td>
<td>-1.32</td>
<td>-5.35</td>
<td>-0.89</td>
<td>1.94</td>
</tr>
<tr>
<td>TYK2 Y1054</td>
<td>1.51</td>
<td>1.02</td>
<td>5.18</td>
<td>3.22</td>
<td>-4.56</td>
</tr>
</tbody>
</table>

SET-2 cells were treated for 30 minutes with the indicated JAK inhibitors and extracted. Changes in phosphopeptide abundance following JAK inhibitor versus DMSO treatment in the different experiments are displayed as log2 ratios calculated from duplicate runs. The table shows a subset of the detected peptides and the respective affected phospho-tyrosine residues are indicated. ND: Not detected.

Figure legends

Figure 1. Increase of JAK activation-loop phosphorylation by JAK inhibitors. A, SET-2 cells were treated for 30 minutes with JAK inhibitors at 1 μM or DMSO and then extracted for Western blot analysis of JAK2 Y1007/Y1008 and STAT5 Y694 phosphorylation. JAK2 and STAT5 served as loading controls. B, SET-2 cells were treated with increasing concentrations of NVP-BSK805 for 30 minutes and then
assessed as described above. C, Non-targeting (Ctrl) or JAK2 targeting siRNA oligos were transfected into HEL92.1.7 cells. After 72 h, cells were treated for 30 minutes with 1 μM NVP-BSK805 or DMSO and then assessed as described above. D, SET-2 cells were treated with 1 μM NVP-BSK805 or DMSO for 30 minutes. JAK2 was immuno-precipitated (IP) using an amino- or carboxyl-terminal antibody, followed by Western blot analysis of P-JAK2 and JAK2. E, CMK cells were treated for 30 minutes with JAK inhibitors at 1 μM or DMSO and then extracted for Western blot analysis of JAK3 Y980 (following JAK3 IP) and STAT5 phosphorylation. F, TF-1 cells were starved in medium without GM-CSF overnight and then either pre-treated with DMSO or JAK inhibitors at 1 μM for 30 minutes. Cells were then stimulated or not with 10 ng/mL IFN-α for 10 minutes, followed by extraction for Western blot analysis of TYK2 Y1054/Y1055 (after TYK2 IP) and STAT5 phosphorylation.

**Figure 2.** JAK inhibitor-induced JAK activation-loop phosphorylation can be transient or sustained and is also seen in vivo. A, JAK inhibitor kinetic parameters determined in biochemical assays with JAK2 JH1. B, SET-2 cells were treated for 2 h with different JAK inhibitors at 1 μM, followed by washing, transferring back into medium and extraction at indicated time-points. Control cells were treated with DMSO. JAK2 Y1007/Y1008 and STAT5 Y694 phosphorylation were detected by Western blotting. JAK2 and STAT5 served as loading controls. C, mice received a subcutaneous injection of 10 U rhEpo and were orally administered 25, 50 or 100 mg/kg NVP-BSK805. Control animals received either a subcutaneous injection of saline or 10 U rhEpo and were orally administered vehicle. After 3 h, spleen samples were processed for detection of P-JAK2 and P-STAT5 levels as described above. D, irradiated mice transplanted with bone marrow transduced with MPLW515L were followed for
development of leukocytosis and thrombocytosis. Mice were then administered either vehicle or NVP-BSK805 at indicated dose-levels. After 24 h, levels of JAK2, STAT3 and MAPK phosphorylation in spleen extracts were assessed by Western blotting. Actin served as loading control.

Figure 3. Type-II mode of JAK inhibition suppresses both JAK activation-loop and substrate phosphorylation. A, SET-2 cells were treated for 30 minutes with 1 μM NVP-BBT594 or DMSO and then extracted for Western blot analysis of JAK2 Y1007/Y1008 phosphorylation and STAT5 Y694 phosphorylation. JAK2 and STAT5 were probed for as loading controls. B, SET-2 or CMK cells were treated with increasing concentrations of NVP-BBT594 for 1 h and then extracted for detection of P-JAK2, P-JAK3 (Y980) and P-STAT5 by Western blotting. C, SET-2 cells were treated with 1 μM NVP-BBT594 or DMSO for 30 minutes. JAK2 was immuno-precipitated using an amino- or carboxyl-terminal antibody, followed by Western blot analysis of levels of P-JAK2 and JAK2 protein that was IPd. D, SET-2 cells were treated for 2 h with 1 μM NVP-BBT594, followed by washing, transferring back into medium and extraction at the indicated time points. Control cells were treated with DMSO. P-JAK2 and P-STAT5 levels were assessed by Western blotting.

Figure 4. JAK2 JH1 in complex with NVP-BBT594 at 1.34 Å resolution. A, chemical structure of NVP-BBT594. B, overall ribbon representation of the JAK2 kinase domain with the bound inhibitor NVP-BBT594 illustrated as stick model. Inhibitor binding occurs to the DFG-out conformation of the kinase domain. Residues 1000-1012 from the activation-loop did not show electron density and are omitted from the final model. C, stereo view of NVP-BBT594 bound to JAK2. Polar contacts...
between the protein, the inhibitor molecule and solvent are indicated with dotted green lines.

**Figure 5. JAK2 type-I inhibitor-induced increase of JAK2 activation-loop phosphorylation is staurosporine sensitive and suppressed by reduction of ATP levels.**  
**A,** SET-2 cells were pretreated for 30 minutes with DMSO control (Ctrl), 10 μM staurosporine or imatinib, followed by treatment for 1 h with 1 μM NVP-BSK805 or DMSO. JAK2 Y1007/Y1008 and STAT5 Y694 phosphorylation were assessed by Western blotting.  
**B,** non-targeting or BTK targeting siRNA oligos were transfected into SET-2 cells. After 72 h, cells were treated for 1 h with 1 μM NVP-BSK805 or DMSO and P-JAK2 and P-STAT5 levels were assessed as above. JAK2, STAT5 and BTK served as loading controls and to verify siRNA-mediated target knockdown.  
**C,** non-targeting or LYN targeting siRNA oligos were transfected into SET-2 cells, followed by treatment and analysis as described above.  
**D,** SET-2 cells were treated for 30 minutes with 10 mM 2-deoxy-D-glucose and 20 μM oligomycin A or drug vehicle. Cells were then treated for 20 minutes with 1 μM NVP-BSK805 or DMSO. P-JAK2 and P-STAT5 levels were assessed as above.  
**E,** Extracts from the experiment shown in **D** were also probed (left panels) for phosphorylated AMPKα. AMPKα and β-tubulin served as loading controls. The histogram depicts relative ATP levels (means of four independent experiments ± SD) in SET-2 cells treated as described in **D.**

**Figure 6. JAK type-I inhibitor-induced increase of JAK activation-loop phosphorylation requires intact JH1 and JH2 domains.**  
**A,** HEL92.1.7 cells were transiently transfected with the indicated JAK2 constructs. Control cells were transfected with empty vector (Ctrl). After 24 h, cells were treated with 1 μM NVP-
BSK805 or DMSO for 30 minutes, followed by extraction for Western blot detection of P-JAK2 (Y1007/Y1008) and P-STAT5 (Y694) levels. B, Ba/F3 EpoR cells were transiently transfected with the indicated JAK2 constructs or empty vector (Ctrl). After 24 h cells were treated as described above. C, HEL92.1.7 cells were transiently transfected with the indicated JAK1 constructs or empty vectors (Ctrl). After 24 h, cells were either treated with 1 μM JAK Inhibitor 1 or DMSO for 1 hour, followed by extraction for Western blot detection of JAK1 Y1022/Y1023 phosphorylation, and of P-JAK2 and P-STAT5 as above. Total levels of JAK1, JAK2 and STAT5 served as loading controls. D, HEL92.1.7 cells were transiently transfected and treated as in C to assess the impact of the JAK1^K648R control mutant on JAK Inhibitor 1-induced activation-loop phosphorylation of co-transfected JAK1^V658F.
<table>
<thead>
<tr>
<th></th>
<th>JAK Inh. 1</th>
<th>CP-690,550</th>
<th>BSK805</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (M)($\times 10^8$)</td>
<td>0.84 ± 0.03</td>
<td>1.51 ± 0.26</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>$k_{on}$ (M$^{-1}$·s$^{-1}$)($\times 10^6$)</td>
<td>7.3 ± 1.4</td>
<td>3.5 ± 0.5</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>$k_{off}$ (s$^{-1}$)($\times 10^4$)</td>
<td>61.1 ± 13.0</td>
<td>54.0 ± 3.0</td>
<td>7.3 ± 1.7</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>2</td>
<td>2</td>
<td>16</td>
</tr>
</tbody>
</table>
### Figure 3

#### A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>P-JAK2</td>
</tr>
<tr>
<td>BBT594</td>
<td>P-JAK2</td>
</tr>
<tr>
<td></td>
<td>JAK2</td>
</tr>
<tr>
<td></td>
<td>P-STAT5</td>
</tr>
<tr>
<td>SET-2</td>
<td>STAT5</td>
</tr>
</tbody>
</table>

#### B

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>P-JAK2</td>
</tr>
<tr>
<td>100 nM</td>
<td>P-JAK2</td>
</tr>
<tr>
<td>300 nM</td>
<td>P-JAK2</td>
</tr>
<tr>
<td>500 nM</td>
<td>P-JAK2</td>
</tr>
<tr>
<td>1000 nM</td>
<td>P-JAK2</td>
</tr>
<tr>
<td>5000 nM</td>
<td>P-JAK2</td>
</tr>
<tr>
<td></td>
<td>JAK2</td>
</tr>
<tr>
<td></td>
<td>P-STAT5</td>
</tr>
<tr>
<td></td>
<td>STAT5</td>
</tr>
<tr>
<td>SET-2</td>
<td>STAT5</td>
</tr>
<tr>
<td>CMK</td>
<td>P-JAK3</td>
</tr>
<tr>
<td></td>
<td>JAK3</td>
</tr>
<tr>
<td></td>
<td>P-STAT5</td>
</tr>
<tr>
<td></td>
<td>STAT5</td>
</tr>
</tbody>
</table>

#### C

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>P-JAK2</td>
</tr>
<tr>
<td>BBT594</td>
<td>P-JAK2</td>
</tr>
<tr>
<td></td>
<td>JAK2</td>
</tr>
<tr>
<td></td>
<td>P-STAT5</td>
</tr>
<tr>
<td>SET-2</td>
<td>STAT5</td>
</tr>
</tbody>
</table>

#### D

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>P-JAK2</td>
</tr>
<tr>
<td>BBT594</td>
<td>P-JAK2</td>
</tr>
<tr>
<td></td>
<td>JAK2</td>
</tr>
<tr>
<td></td>
<td>P-STAT5</td>
</tr>
<tr>
<td>SET-2</td>
<td>STAT5</td>
</tr>
</tbody>
</table>

- **IP:** JAK2 C-term.
- **IP:** JAK2 N-term.
Andraos et al. Figure 4
Andraos et al. Figure 5
Andraos et al. Figure 6
Modulation of activation-loop phosphorylation by JAK inhibitors is binding mode dependent

Rita Andraos, Zhiyan Qian, Débora Bonenfant, et al.

Cancer Discovery  Published OnlineFirst May 3, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-11-0324

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2012/04/12/2159-8290.CD-11-0324.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.