CHARACTERIZATION OF NOVEL JAK2 TYROSINE KINASE SMALL MOLECULE INHIBITORS AND SITES OF JAK2 PHOSPHO-REGULATION

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2009

This dissertation is dedicated to my family for the support, love, advice and encouragement they have given me.

ACKNOWLEDGMENTS

First and foremost, I would like to express my sincere gratitude to my adviser, Dr. Peter P. Sayeski, whose excellent guidance has been instrumental in my success as a graduate student. He has been available to answer all my questions, provide constructive criticism and has encouraged me to perform my best. Apart from being a mentor, Peter has been a wise friend whom I can rely on for advice.

I would also like to thank the members of my supervisory committee: Drs. Jeff Harrison, Brad Fletcher and Satya Narayan. They have provided valuable scientific advice that has augmented my graduate work. In addition, I am grateful to Dr. David Ostrov, Dr. György Keseru, and Andrew Magis for their assistance with *in silico* modeling applications. Without their help, our Jak2 inhibitor characterization would not have been possible.

I appreciate the members of the Sayeski laboratory, past and present for their support: Tiffany Wallace, Michael Godeny, Issam McDoom, Robert Blair, Annet Kirabo, Anurima Majumder, Kavitha Gnanasambandan, and Nick Figueroa. They have provided an accommodating and encouraging environment. At times, they have also provided comic relief.

In addition, I am grateful for meeting genuine and wonderful friends in Gainesville. I greatly value the enjoyable time I shared with them throughout my graduate career.

Finally, I would like to thank my mother, father and brothers for their constant love and support. I appreciate the fact that you encouraged me to pursue my interests and always believed that I can achieve my goals.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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May 2009

Chair: Peter P. Sayeski
Major: Medical Sciences-Physiology and Pharmacology

Jak2 is a non-receptor tyrosine kinase that acts as a critical link in coupling ligand binding at the cell surface with gene transcription in the nucleus. Since its discovery 17 years ago, Jak2 has emerged as an important protein in human health. In recent years, much attention has been devoted to understanding the role of the Jak2-V617F mutation and other gain-of -function mutations of Jak2 in myeloproliferative disorders and hematological malignancies. The constitutively active Jak2-V617F mutation has been found in a large percentage of myeloproliferative disorder patients and plays a critical role in the development of these disorders. Control of aberrant Jak2 kinase activity via Jak2-specific inhibitors would therefore serve as a useful research tool and/or therapeutic agent. In this dissertation, we characterized two novel Jak2 inhibitors, termed Z3 and G6. We found that these compounds specifically inhibited Jak2 kinase function with no effect on Tyk2 or c-Src. In addition, Z3 and G6 selectively suppressed Jak2-V617F-mediated human erythroleukemia cell growth *in vitro*. Finally, these compounds reduced the *ex vivo* growth of hematopoietic progenitor cells derived from myeloproliferative disorder patients carrying Jak2 mutations. Z3 and G6 could potentially serve as useful research tools or therapeutic agents against diseases related to elevated Jak2 tyrosine kinase activity.

Since Jak2 has been linked to a number of disease states, it is also important to understand how the structure of Jak2 impacts its function. Here, we investigated structural elements in the FERM domain that influence Jak2 function. We found that tyrosines 372 and 373 are novel sites of Jak2 phosphorylation in the FERM domain of Jak2. We demonstrated that loss of tyrosine 372 phosphorylation inhibited ligand-independent Jak2 autophosphorylation. In contrast, loss of tyrosine 373 phosphorylation resulted in more modest reductions in Jak2 autophosphorylation levels. With specific focus on the more significant tyrosine 372, we also found that loss of tyrosine 372 phoshorylation hindered the ability of Jak2 to activate STAT1 and reduced Jak2-dependent gene transcription. In addition, we found that loss of tyrosine 372 phosphorylation impaired interferon-gamma and epidermal growth factor-dependent Jak2 activation. Our results suggest that tyrosine 372 is important for Jak2 function.

CHAPTER 1
INTRODUCTION

History of Jak2 Tyrosine Kinase

Over seventeen years ago, Wilks and colleagues cloned the Jak2 cDNA sequence and upon analysis found that the gene encoded a protein of roughly 130 kDa in mass (Harpur et al., 1992). The predicted amino acid sequence also revealed that Jak2 possessed a kinase domain immediately adjacent to a pseudokinase domain and had seven conserved Jak homology domains. Interestingly, the tandem manner by which the kinase and psuedokinase domains were linked was suggestive of Janus, the Roman God of two opposing faces, which the Jaks were named after.

Initially, Jak2 was characterized as a mediator of cytokine and growth factor signaling (Argetsinger et al., 1993; Silvennoinen et al., 1993; Witthuhn et al., 1993; Ihle et al., 1994). Later on, it was discovered that Jak2 could transduce its signal through G-protein coupled receptors, such as the AT_1 receptor, and be activated by reactive oxygen species thus revealing that Jak2 is a more versatile signaling mediator than previously thought (Marrero et al., 1995; Simon et al., 1998). Another important finding was that Jak2 is expressed in virtually every cell type and is necessary for animal survival as disruption of the Jak2 allele in mice resulted in embryonic lethality (Parganas et al., 1998; Neubauer et al., 1998). A crucial event in the history of Jak2 tyrosine kinase was when a mutation in the psuedokinase domain of this protein, Jak2-V617F, was linked to high percentage of myeloproliferative patients and shown to be the cause of this disease (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Zhao et al., 2005). Given the above facts, it is not surprising that Jak2 tyrosine kinase has emerged as an important molecule in mammalian development, physiology, and disease.

Jak2 Structure

Jak2 Protein Domains

Although the three-dimensional structure of the entire Jak2 protein has not been obtained, a portion of the Jak2 kinase domain has been resolved which gives us structural insight regarding the catalytic portion of JAK2 and the mechanism by which it is regulated (Lucet et al., 2006). According to the Jak2 crystal structure, a loop structure situated between amino acids 1056 and 1078, termed the Jak2 kinase insertion loop, is a feature that is unique to the Jak family (Lucet et al., 2006). This loop is relatively mobile and solvent accessible. In particular, the serine residue located at position 1056 on this loop is solvent exposed, suggesting that it could play a phosphorylation-dependent regulatory role in Jak2 function. In addition, the Jak2 insertion loop is stabilized by the interaction of antiparallel β-sheets with the base and the tip of the activation loop. Moreover, a number of lysine residues stabilize the conformation of phosphorylated tyrosine 1007, which is highly solvent exposed (Lucet et al., 2007). The exposed nature of phosphorylated tyrosine 1007 permits the binding of negative regulatory proteins such as SOCS-1 to this site (Giordanetto and Kroemer, 2003).

Certain studies have contributed to the generation of a theoretical three-dimensional structure of the entire Jak2 protein through analysis of primary nucleotide and amino acid sequence as well as through application of homology modeling (Lindauer et al., 2001; Giordanetto and Kroemer, 2002). Jak2, like other Jak family members, contains seven highly conserved Jak homology (JH) domains termed JH1 through JH7 (Figure 1-1). The carboxyl terminus of Jak2 consists of the JH1 and JH2 domains. The JH1 region encodes the kinase domain, which contains the adenosine triphosphate-binding region and the activation loop. This domain is a requisite for Jak2 tyrosine kinase function (Duhe and Farrar, 1995). Directly N-terminal to the JH1 domain is the JH2 or pseudokinase domain. Although the JH2 psuedokinase

domain of Jak2 shares conserved motifs with other Jak kinases, this region is catalytically inactive (Frank et al., 1995). It has been suggested that in the absence of ligand, the pseudokinase domain has a role in suppressing the function of the kinase domain by physically interacting with the JH1 domain. (Lindauer et al., 2001 ; Chen et al., 2000 ; Saharinen et al., 2003).

The amino terminal region of Jak2 consists of domains JH3 through JH7. Although the Jak family members are thought to lack a canonical SH2 domain, it was noted that in Tyk2, a portion of the JH4 domain along with the entire JH3 domain weakly resemble an SH2 domain (Bernards, 1991). Following cloning of the murine Jak2 cDNA, it was similarly observed that the sequence GLYVLRWS was weakly homologous to the core sequence element of SH2 domains (FLVRES) (Harpur et al., 1992). However, studies have reported conflicting finding as to whether a fully functional SH2 domain exists within Jak2 (Kampa and Burnside, 2000; Giordanetto and Kroemer, 2002).

Directly N-terminal to the putative SH2 domain of Jak2 resides the FERM domain, which spans from the middle of the JH4 domain through the JH7 domain. The FERM domain is involved in mediating stable interactions with other cellular proteins (Yonemura et al., 1998). In addition, the amino terminal end of Jak2, particularly, the JH6 and JH7 domains, has been shown to be crucial for Jak2-cell surface receptor interactions (Frank et al., 1994; Tanner et al., 1995).

In brief, Jak2 is structurally composed of seven distinct Jak homology domains. Each of these domains has a vital role in Jak2 function. The JH1 domain is essential for Jak2 kinase activation, while the JH2 domain is important for Jak2 autoregulation. Finally, JH3-JH7 domains are involved in interactions with substrates and membrane-bound receptors.

Amino Acid Residues Critical for Jak2 Function

Within the seven Jak2 homology domains, several amino acid residues have been identified as being important for regulating Jak2 function. These amino acids consist of non-tyrosine residues and phosphotyrosines. In the kinase domain, Trp 1020 and Glu 1024 are important for Jak2 catalytic activity as mutation of these amino acid residues together or individually render Jak2 catalytically inactive (Zhuang et al., 1994; Vonderlinden et al., 2002). Furthermore, E1024 and R1113 form a hydrogen bond that is important for Jak2-mediated angiotensin II signaling (Sandberg et al., 2004a). Likewise, the interaction between W1038 and E1046 has been demonstrated to be critical for preventing an inhibitory Jak2 phenotype *in vivo* (Frenzel et al., 2006).

In contrast, there are certain non-phosphotyrosine amino acids within the pseudokinase autoinhibitory domain of Jak2 that when mutated result in constitutive Jak2 activation. The most well known is the Jak2-V617F mutation, which is found in a high proportion of myeloproliferative disorder patients. Most likely, the V617 residue is important for the autoinhibitory interaction between the JH2 and JH1 domain. Current molecular models of the psuedokinase domain suggest that this region interacts with the activation loop of the kinase domain (Lindauer et al., 2001; Giordanetto and Kroemer et al., 2002). Moreover, structure function studies have shown that amino acids located in the vicinity of V617 are critical for maintaining the inhibitory property of the pseudokinase domain (Saharinen et al., 2003).

Jak2 tyrosine autophosphorylation is a significant process as it dictates the relay of signals from the cell surface to the nucleus. Murine Jak2 possesses 49 tyrosine residues and 10 of these are known to be phosphorylated with many more tyrosine residues awaiting characterization. These characterized Jak2 tyrosine residues include: Y119, Y201, Y221, Y570, Y813, Y913, Y966, Y972, Y1007 and Y1008. The 11^{th} and 12^{th} phospotyrosine sites recently characterized in

our lab are tyrosine 372 and 373 and they will be discussed later in detail. Many of these sites play important roles in Jak2 tyrosine kinase regulation. In the kinase domain, tyrosines 1007 and 1008 within the Jak2 activation loop were the first sites shown to be phosphorylated (Feng et al., 1997). While tyrosine 1007 is required for maximal Jak2 tyrosine kinase activity, the adjacent tyrosine at position 1008 seems to be dispensable for Jak2 tyrosine kinase function. The proposed mechanism by which phosphorylation of tyrosine 1007 results in maximal Jak2 activation is that the activation loop is moved away from the ATP binding site of the kinase domain upon phosphorylation, thus unblocking this region so that Jak2 can transfer the γ phosphate from ATP onto its substrates (Feng J et al., 1997). In addition, it has been demonstrated that phosphorylated tyrosine 1007 serves as a binding site for members of the Supperssors of Cytokine Signaling (SOCS) regulatory protein family in order to terminate Jak2 dependent signaling (Ungureanu et al., 2002). A recently characterized tyrosine residue in the kinase domain of Jak2 is tyrosine 972. In 2008, our laboratory demonstrated that tyrosine 972 is a novel Jak2 autophosphorylation site and found that this residue is important in Jak2 tyrosine kinase regulation (McDoom et al., 2008). Specifically, we found that phosphorylation of tyrosine 972 is critical for maximal Jak2 kinase function, in the context of a ligand-independent system. In addition, this site was shown to be essential for angiotensin II-dependent Jak2 Y1007/Y1008 phosphorylation. In contrast, Funakoshi-Tago and colleagues have found that phosphorylation of tyrosine 913, also situated in the kinase domain, negatively regulates Jak2 function by abrogating erythropoietin-dependent Jak2 activation (Funakoshi-Tago et al., 2008). Finally, phosphorylation of tyrosine 966 has been shown to promote the binding of p70 to this residue (Carpino et al., 2002). However, p70 does not have any known effect on any cytokines that employ Jak2. Therefore, the role of tyrosine 966 on Jak2-dependent function, if any, has yet

to be defined. Collectively, phosphorylation of tyrosine residues in the kinase domain serves to upregulate or downregulate Jak2 tyrosine kinase activity.

Certain tyrosine residues have also been characterized in the JH2 domain that have opposing roles in of Jak2 function. For instance, tyrosine 570 has been shown to be phosphorylated in response to growth hormone resulting in reduced Jak2 tyrosine kinase activity (Argetsinger et al., 2004). In contrast, when tyrosine 813 is phosphorylated, it serves as a binding site for the SH2B-β adaptor protein which is a potent activator of Jak2 (Kurzer et al., 2004). This in turn, enhances Jak2 tyrosine kinase activity. Although the JH2 domain is generally known to serve as a Jak2 autoinhibitory region, it is interesting that not all tyrosine residues within this domain contribute to the suppression of Jak2 kinase activity. Therefore, it appears that the role of the pseudokinase domain in Jak2 regulatory function is quite complex, but may become more clear once the crystal structure of the JH2 domain is resolved.

In addition, tyrosine residues within the Jak2 FERM domain are important for the association of Jak2 to a particular receptor. For example, our laboratory has shown that tyrosine 201 is phosphorylated and serves as a binding site for SHP-2 to recruit Jak2 to the AT_1 receptor signaling complex. The consequence of this interaction is to promote downstream Jak2-dependent signaling (Godeny et al., 2007). On the contrary, Y119 has a negative function with regard to the association of Jak2 to a particular receptor. Specifically, phosphorylation of Jak2 at tyrosine 119 results in the dissociation of Jak2 from the erythropoietin receptor (Funakoshi-Tago et al., 2006).

In summary, a number of Jak2 amino acids have been characterized that play diverse roles in regulating Jak2 function. These amino acids consist of tyrosine as well as non-tyrosine residues and they are situated on various Jak homology domains. Phosphorylation of these

amino acid residues can result in either propagation or inhibition of Jak2-dependent signaling. In addition, certain amino acid residues have been shown to be phosphorylated yet their role, if any, in the Jak2 signal transduction pathway is undefined. Although the structural elements of Jak2 play vital roles in regulating Jak2 function, a number of external factors also influence Jak2 so that the cell can bring forth the proper Jak/STAT response. In the subsequent section, we will discuss the external factors that affect the Jak2 signaling pathway.

Jak2 Signal Transduction Pathway

The significance of Jak2 in cell signaling was apparent when investigators showed that Jak2 is a critical mediator of cytokine-dependent signaling (Argetsinger et al., 1993; Silvennoinen et al., 1993; Watling et al., 1993; Witthuhn et al., 1993; Narazaki et al., 1994; Rui et al., 1994). Subsequent work demonstrated a correlation between activation of Jak2 in the cytoplasm and amplified gene transcription in the nucleus. The Jak2 signaling paradigm was further elucidated when studies identified a new class of cytokine-responsive transcription factors, named the Signal Transducers and Activators of Transcription (STAT) proteins which mediate gene transcription (Schindler et al., 1992; Shuai et al., 1992). Thus, within two years, it was defined that the primary cellular role of Jak2 is to relay signals from the cell surface to the nucleus.

Aside from STATs, many different proteins participate in the Jak2 signaling pathway to permit a unique and specific signal to occur in the cell. These proteins consist of different cell surface receptors including cytokine, growth factor and G-protein coupled receptors (GPCRs). Moreover, adaptor/regulatory proteins are also critical components of Jak2-dependent signaling.

Importantly, although Jak2 is expressed in numerous tissues and is activated by different types of cell surface receptors, specificity is maintained in this signaling system by the particular receptor and its associated STAT protein. In detail, the specificity of STAT activation by a

certain receptor is established by the particular SH2 domain situated within each STAT molecule and the specific phosphotyrosine-containing motif that is encoded on a given receptor (Heim et al., 1995; Stahl et al., 1995).

Activation of Jak2 via Cytokine Receptors

Jak2 activation through cytokine receptors is the most well understood Jak2 signaling model. An overview of the Jak/STAT signaling pathway is illustrated in Figure 1-2. In this paradigm, signaling is initiated by the binding of ligand to its cell surface receptor, resulting in receptor dimerization. Upon dimerization, the receptors undergo conformational changes that are thought to position the constitutively receptor-bound Jak2 molecules in close proximity to one another in order to facilitate Jak2 autophosphorylation (Brooks et al., 2007). This ligand-dependent tyrosine phosphorylation occurs principally on Tyr 1007 (Feng et al., 1997). An activated Jak2 then phosphorylates specific tyrosine residues on the cytoplasmic tails of the receptors, creating docking sites for the SH2 domain-containing STAT proteins. Once bound to the receptors, the STATs are themselves phosphorylated by Jak2 on tyrosine residues. The tyrosine-phosphorylated STATs then form active homodimers and heterodimers which translocate to the nucleus, where they bind to STAT recognition sequences and modulate gene transcription. Therefore, Jak2 is responsible for transducing a signal from the cell surface to the nucleus through a tyrosine phosphorylation signaling mechanism.

Activation of Jak2 via GPCR

In addition to cytokine receptors, Jak2 is activated by G protein-coupled receptors, such as the AT_1 receptor. GPCR are composed of a single polypeptide chain that spans the plasma membrane seven times and possess both intracellular and extracellular components (Fong, 1996). There are important differences in the mechanism by which GPCR and cytokine receptors facilitate Jak2 activation, but the outcome is the same in that activation of Jak2 leads to STAT

activation and subsequent modulation of gene transcription. The general differences in activation of Jak2 via the cytokine receptor versus the GPCR are illustrated in Figure 1-2. In the GPCR model, the Jak2 molecules are not constitutively bound to the GPCR in the absence of ligand. However, upon ligand binding, Jak2 autophosphorylation is triggered and Jak2 is recruited from the cytosol to the GPCR (Ali et al., 1997; Sayeski et al., 2001). In contrast, in the cytokine receptor model, Jak2 is constitutively bound to the cytokine receptor. Another key difference is that while STATs directly associate with the cytoplasmic tail of the cytokine receptor, it is thought that Jak2 serves as a molecular bridge linking STATs to the GPCR (Ali et al., 2000).

Jak2 Regulatory Proteins

As previously mentioned, in order for Jak2 to reach an initial state of activation, Tyr 1007 within the Jak2 activation loop must be phosphorylated in response to ligand stimulation. Furthermore, the catalytic state of Jak2 can be driven to even greater levels of activation through the association of Jak2 with activator proteins such as SH2B-β. When it is necessary for the cell to terminate Jak2-dependent signaling, there exist several Jak2 inhibitory proteins that exhibit their effect at different points of the signal transduction cascade to suppress Jak2 function. These Jak2 regulatory proteins will be discussed in detail below.

SH2B family of proteins

The SH2B family members include SH2B1, SH2B2 and SH2B3. These proteins all share a common domain structure that includes an SH2 domain, a PH domain domain, several proline-rich regions and a dimerization domain (Maures et al., 2007). The SH2B1 or the simply stated SH2B transcript undergoes alternative splicing, yielding four protein products, SH2Bα, β, and σ (Maures et al., 2007). The SH2B-β isoform has been shown to bind Jak2 at phosphorylated

Y813 via its SH2 domain and enhance growth hormone mediated Jak2 activation (Kurzer et al., 2004). Two mechanism have been suggested to explain how SH2B-β augments Jak2 activity. One mechanism is that dimerization of SH2B-β leads to dimerization of the associated Jak2 proteins thereby resulting in enhanced Jak2 tyrosine kinase activity (Nishi et al., 2005). An alternative model proposes that SH2B-β increases the number of active Jak2 molecules by sustaining Jak2 in an active state (Kurzer et al., 2006). Nevertheless, both of the suggested mechanisms agree that the capacity of SH2B-β to increase Jak2 activity is directly due to SH2B-β function and not a consequence of recruitment of a Jak2 activator nor competition with a Jak2 inhibitor.

SOCS family of proteins

The suppressor of cytokine signaling (SOCS) family of proteins is a class of negative feedback regulators of cytokine receptor signaling. All SOCS family members share a central SH2 domain, a conserved C-terminal motif called the SOCS box and a variable N-terminal region (Ilangumaran and Rottapel, 2003). The SOCS proteins were first characterized as inhibitors of cytokine-dependent Jak2 signaling (Starr et al., 1997). Specifically, SOCS1 has been shown to be a critical regulator of IFN-gamma as over-expression of SOCS1 inhibits Jak2 and blocks IFN-gamma signaling (Song and Shuai, 1998; Sakamoto et al., 2000). SOCS expression is rapidly induced through transmission of the Jak/STAT signal. Subsequently, SOCS protein binds to Jak2 at phophorylated tyrosine 1007 on the activation loop resulting in ubiquitin-mediated degradation of Jak2 (Starr et al., 1997; Ungureanu et al., 2002; Ilangumaran S and Rottapel, 2003).

Overall, the role of SOCS in physiology is important as it regulates the magnitude and duration of Jak2-dependent signaling to prevent excessive signaling and abnormal cellular

activation that could result in oncogenic transformation. In fact, aberrant methylation of SOCS genes have been associated with down-regulation of SOCS proteins in solid tumors, leukemias and hematopoietic cells exhibiting constitutive Jak2 activity (Lee et al., 2006; Niwa et al., 2005; Jost et al., 2007).

Jak2 and Pathophysiology

Role of Jak2 in Cardiovascular Disease

Jak2 plays an important role in cardiovascular disease as activation of this protein has been implicated in the molecular signaling events that lead to certain cardiovascular diseases such as cardiac hypertrophy, ischemia reperfusion and atherosclerosis. For example, Jak2 becomes activated in response cardiac hypertrophy-inducing stimuli. Specifically, it was demonstrated that acute pressure overload in rats increases Jak2 tyrosine phosphorylation levels by triggering autocrine/paracrine secretion of angiotensin II (Pan et al., 1997). Investigators also showed that treatment of cardiomyocytes with the Jak2 inhibitor, AG490, reduced mechanical stretch-induced Jak2 phosphorylation levels thereby linking mechanical stretch to Jak2 activation in cardiomyocytes (Pan J et al., 1998). In a different model, it was demonstrated that cardiotrophin-1, an IL-6-related cytokine family member and potent inducer of cardiomyocyte hypertrophy, also stimulated Jak2 activation by increasing angiotensinogen mRNA (Pennica et al., 1995; Fukuzawa et al., 2000). Overall, evidence supports that diverse physical and chemical stimuli that generate cardiac hypertrophy also stimulate Jak2 activation.

Jak2 signaling is also connected to cardiac injury resulting from ischemia-reperfusion, a pathological condition characterized by impeded blood flow to an area of tissue followed by the reestablishment of circulation to the same area. Specifically, it was shown that treatment with the Jak2 inhibitor, AG490 resulted in a reduction in cardiac infarct size and a reduction in apoptotic cell death in cardiomyocytes after ischemia-reperfusion in a perfused rat heart

(Mascareno et al., 2001). These results indicate that Jak2 activation is associated with cardiac dysfunction during ischemia-reperfusion.

Approximately ten years ago, it was observed that intracellular reactive oxygen species or exogenous hydrogen peroxide activate the Jak2/STAT pathway in fibroblast cells, vascular smooth muscle cells as well as in human lymphocytes by behaving as intracellular signaling molecules (Simon et al., 1998; Carballo et al., 1999; Madamanchi et al., 2001). The activation of Jak2 by reactive oxygen species is important in the progression of atherosclerosis. It has been shown that oxidative stress-induced Jak2 activation plays a role in vascular smooth muscle cell proliferation, an important factor in the progression of atherosclerosis. Specifically, Madamanchi et al. demonstrated that hydrogen peroxide stimulates heat-shock protein 70 (HSP70) expression in vascular smooth muscle cells via activation of the Jak2/STAT signaling pathway (Madamanchi et al., 2001). The outcome of HSP70 activation in vascular smooth muscle cells could provide these cells a proliferative advantage by protecting cells from undergoing apoptosis (Madamanchi N et al., 2001). Aside from vascular smooth muscle cell proliferation, apoptosis also plays an important part in the progression of atherosclerosis. It is thought that vascular smooth muscle cell apoptosis in atherosclerotic plaques leads to plaque instability and eventual plaque rupturing (Hsieh et al., 2001). Several years ago, our laboratory defined a direct link between Jak2 and oxidative stress-induced apoptosis in vascular smooth muscle cells by demonstrating that inhibition of Jak2 results in an elimination of hydrogen peroxide-mediated apoptosis in these cells (Sandberg and Sayeski, 2004b).

Collectively, there is evidence to support that Jak2 plays an important function in cardiovascular disease states such as cardiac hypertrophy and ischemia-repurfusion. In addition, Jak2 plays a vital role in oxidative stress-mediated cell proliferation and apoptosis which are

important components in atherosclerotic development. These findings suggest that Jak2 could be a potential therapeutic target in vascular diseases.

Role of Jak2 in Hematological Disorders

In addition to its critical role in a number of cardiovascular diseases, somatic Jak2 mutations have been linked to hematological malignancies. These mutations include Jak2 amino acid substitutions, deletions, insertions, and chromosomal translocations that cause constitutive Jak2 kinase activity. For example, TEL-Jak2 is a well characterized fusion protein resulting from a translocation event between Jak2 on chromosome 9 and TEL on chromosome 12 that is found in some T-cell leukemia. Jak2 has also been linked to other chromosomal translocations. These include the REL-Jak2, BCR-Jak2, PCM1-Jak2 and Pax5-Jak2 fusions, which have been associated with myeloid or lymphoid leukemia. In addition, activating Jak2 mutations such as Jak2-T875N, Jak2-K607N, Jak-L611S and Jak2-Δ^{682}IREED have been reported in acute leukemia as well.

In recent years, interest has focused on the gain-of-function Jak2-V617F mutation since it has been detected in almost all polycythemia vera patients and a substantial proportion of individuals with essential thrombocythemia and primary myelofibrosis (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Zhao et al., 2005). Moreover, the Jak2-V617F mutation has been shown to play a critical role in the pathogenesis of myeloproliferative disorders as expression of this mutation in murine hematopoietic cells leads to a polycythemia vera-like disease in mice (Lacout et al., 2006; Xing et al., 2008). As a consequence of the association of aberrant Jak2 activity with diverse hematological disorders, researchers are increasingly focused on identifying Jak2 inhibitors that suppress constitutive Jak2 kinase activity. Some of these inhibitors may one day become therapeutically beneficial for

individuals with Jak2 related hematologic malignancies or myeloproliferative disorders. Here, we will describe in detail the various Jak2 mutations that are associated with hematological disorders and assess some of the inhibitors of Jak2 that are either in the pre-clinical phase or in clinical trials. By examining these specific areas, we hope to have a better understanding regarding the role of Jak2 in hematological disorders and shed light on the utility of Jak2 inhibitors.

Jak2 chromosomal translocations and hematological malignancies

The first study that indicated that a mutant Jak kinase could result in a hematological malignancy was in 1995 when Luo et al. demonstrated that a glycine to glutamic acid substitution at position 341 in the Drosophila hopscotch gene caused a leukemia-like hematopoietic defect (Luo et al., 1995). Two years later, studies linked Jak2 chromosomal translocations to human neoplastic growth. Specifically, a translocation event between the kinase domain of Jak2 and the helix-loop-helix domain of the ETS family transcription factor, Tel, was identified in a child with early B-precursor acute lymphoid leukemia and in an adult with atypical chronic myeloid leukemia (Lacronique et al., 1997; Peeters et al., 1997). The basis for the diverse phenotype detected in these two patients is due to two distinct translocation events within Jak2 and the Tel genes that consequently give rise to distinct chimeras. Nevertheless, these TEL-Jak2 fusion proteins caused increased oligomerization of the Jak2 proteins that lead to growth factor-independent Jak2 activation and subsequent NF-κB signaling (Lacronique et al., 1997). Moreover, creation of TEL-Jak2 transgenic mice revealed a causal relationship between the TEL-Jak2 gene product and leukemogenesis, as over expression of this fusion protein resulted in the development of T-cell leukemia in these animals (Carron et al., 2000).

Apart from TEL-Jak2, studies have shown Jak2 to be implicated in other chromosomal translocations observed in various hematological malignancies. Myamoto et al. showed that the Jak2 inhibitor, AG490, reduced the growth of human B-precursor leukemic cells (Miyamoto et al., 2001). Specifically, they found that AG490 significantly down-regulated Jak2 phosphorylation in these cells at a concentration that had little effect on normal hematopoiesis. Consequently, this study correlated an l1q23 translocation or Philadelphia chromosome with constitutive Jak2 activation in human lymphoid leukemic cells. In addition, Joos and colleagues analyzed four Hodgkin's lymphoma cell lines and identified chromosomal rearrangements of the short arm of chromosome 2 involving REL, a transcription factor belonging to the NF-κB family of transcription factors (Joos et al., 2003). This resulted in a copy number increase of Jak2 (9p24) in three of the four cell lines. These results suggested that REL and Jak2 may play an important role in the pathogenesis of Hodgkin's lymphoma. Recent studies have demonstrated that human auto-antigen pericentriolar material (PCM1) is a Jak2 translocation partner associated with chronic and acute leukemias including chronic eosinophilic leukemia, acute myeloid leukemia and acute lymphoblastic leukemia (Murati et al., 2005; Reiter et al., 2005). In all cases, the PCM1-Jak2 fusion involved a t(8;9)(p22;p24) translocation event. The chimeric gene product was predicted to encode a protein that maintains several of the coiled-coil domains of PCM1 and the kinase domain of Jak2. The PCM1 coiled motifs possibly serve as a dimerization motif to bring about constitutive activation of Jak2. Furthermore, BCR-Jak2 fusions have been identified in patients with typical and atypical chronic myeloid leukemia (Griesinger et al., 2005; Lane et al., 2008). In each case, *in situ* hybridization revealed a t(9;22)(p24;q11.2) translocation in these patients as opposed to the typical t(9;22)(q34;q11.2) translocation. Although the breakpoints were variable in each patient, the rearrangement resulted in a BCR-Jak2 chimera

rather than the classical BCR-ABL fusion protein. A common finding in these patients was that they exhibited relatively early blast crisis. Lastly, PAX5, a regulator of B-cell development, has been recently shown to form a fusion with the Jak2 gene in childhood acute lymphoblastic leukemia (Nebral et al., 2009)

Jak2 somatic mutations and hematologic malignancies

Activating Jak2 somatic mutations such as amino acid substitution mutations and deletions have also been identified in hematological malignancies. Mercher et al. identified a novel Jak2-T875N mutation in an acute megakaryoblastic leukemic cell line using a combination of mass spectrometry and growth inhibition assays via the use of a selective tyrosine kinase inhibitor (Mercher et al., 2006). The authors demonstrated that the Jak2-T875N was constitutively active *in vitro* and induced a myeloproliferative disease with characteristics of megakaryoblastic leukemia in a murine bone marrow transplantation assay. Other novel mutations have been reported in the JH2 domain of Jak2, which confer constitutive activation of the Jak-STAT signaling pathway. These include the Jak2-K607N (Lee et al., 2006) and Jak2-L611S (Kratz et al., 2006) mutations found in acute myeloid leukemia and acute lymphoblastic leukemia, respectively. Finally, a deletion of amino acids 682-686 (Jak2-Δ^{682}IREED) has been observed in a patient with Down Syndrome and B-cell precursor acute lymphoblastic leukemia (Malinge et al., 2007).

In summary, the above studies indicate that the Jak2 locus is susceptible to chromosomal rearrangement, point mutations and deletions; all of which are associated with hematological malignancies. These Jak2 gene aberrations are summarized in Table 1-1. Jak2 translocation chimeras appear to increase Jak2 oligomerization and result in growth factor-independent Jak2

auto activation whereas Jak2 point mutations and deletions lead to hypersensitivity to growth factors through impaired Jak2 autoregulation. Nevertheless, the end result is that the aberrant Jak2 protein has constitutively active tyrosine kinase activity that results in a neoplastic phenotype.

Jak2-V617F and myeloproliferative disorders

In 2005, five independent studies reported the identification of a Jak2 somatic mutation (Val 617 to Phe) in several myeloproliferative disorders at a high frequency (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Zhao et al., 2005). Studies employing sensitive detection methodologies indicated that the Jak2-V617F mutation on exon 14 can be detected in almost all polycythemia vera (PV) patients and approximately 50% of essential thrombocythemia (ET) and primary myelofibrosis patients (PMF) (Levine et al., 2006). These myeloproliferative disorders are characterized by the clonal over-production of normally differentiated hematopoietic lineages. For instance, PV is characterized by the over-proliferation of erythrocytes while ET is defined as the unregulated expansion of megakaryocytes. The V617F substitution leads to constitutive activation of Jak2 and downstream effector signaling pathways including the STAT transcription pathway, PI3-kinase and ERK signaling networks, which in turn induce inappropriate cytokine-independent proliferation of cells (Levine et al., 2005; Shannon and Van Etten, 2005). The nature of this gain-of-function mutation is that Val 617 lies in the JH2/pseudokinase auto inhibitory domain of Jak2. Current molecular models of the pseudokinase domain suggest that it interacts with the activation loop of the kinase domain (Giordanetto and Kroemer, 2002). Moreover, structure/function studies have shown that amino acids located between positions 619 to 970 are critical for maintaining the inhibitory property of the pseudokinase domain (Saharinen et al., 2003). Therefore, it is hypothesized that the V617F

mutation impedes the pseudokinase domain from acting as an internal inhibitory regulator of the adjacent kinase domain, resulting in aberrant Jak2 tyrosine kinase activity.

Other Jak2 mutations associated with myeloproliferative disorders

Although the Jak2-V617F mutation is predominantly associated with myeloproliferative disorders, it is evident that other activating alleles of Jak2 are also involved in these disorders. For example, Scott et al. identified a set of novel somatic Jak2 mutations on exon 12 in Jak2-V617F negative polycythemia vera or idiopathic erythrocytosis patients (Scott et al., 2007). Specifically, these mutations mapped to amino acid residues 537-543, which is a region that links the SH2 and JH2 domains of Jak2. Patients harboring these mutations displayed isolated erythrocytosis, reduced serum erythropoietin and factor-independent erythrocyte colony formation. Moreover, characterization of BaF3 cells expressing the erythropoietin receptor and carrying the exon 12 mutations revealed that these cells could proliferate in the absence of interleukin-3 and expressed increased Jak2 phosphorylation relative to cells transduced by wild-type Jak2 or Jak2-V617F.

A separate group also identified Jak2 exon 12 mutations in 5 of 6 Jak2-V617-negative PV patients from a total of 220 PV cases (Pardanani et al., 2007). They found that approximately 80% of these patients harbored either the F537-K539delinsL or N542-E543del in the bone marrow and/or peripheral blood cells. In addition, they found that bone marrow derived from the PV patients carrying the Jak2 exon 12 mutations displayed erythroid hyperplasia, megakaryocyte abnormalities, and reticulin fibrosis, similar to Jak2-V617F harboring PV patients. Finally, Pietra and colleagues searched for Jak2 exon 12 mutations in 17 patients with Jak2-V617F-negative PV and found that 2 of the 8 mutations were novel, with the most frequent ones being N542-E543del and E543-D544del (Pietra et al., 2008). In addition, they reported that most PV patients carrying an exon 12 mutation had isolated erythrocytosis at clinical onset. Collectively,

the above reported findings imply that Jak2 exon 12 mutations are detected mainly in Jak2-V617F negative PV cases and suggest that exon 12 could also be an active site for gain-of-function mutations.

Other mutations have been identified in the JH2 pseudokinase domain of Jak2. Schnittger et al. detected a novel Jak2-D620E mutation in a 56 year old myeloproliferative syndrome patient with leukocytosis (Schnittger et al., 2006). Simultaneously, another group identified the same Jak2-D620E mutation in a 27 year old PV patient who was also positive for the Jak2-V617F mutation (Grunebach et al., 2006). Their study revealed the Jak2-D620E mutation was found in all peripheral blood subsets including B and T lymphocytes, suggesting that in this patient the D620E mutation arose at the multipotent hematopoietic stem cell level. Finally, Zhang et al. reported a novel Jak2-C616Y point mutation in a PV patient who was Jak2-V617F-negative (Zhang et al., 2007).

In summary, there is evidence to support that other somatic gain-of-function Jak2 mutations, aside from Jak2-V617F, could play an important role in myeloproliferative disorders. These mutations include deletions and insertions on exon 12 and substitutions in the JH2 domain of Jak2.

Inhibitors of Jak2 Tyrosine Kinase

Justification for Jak2 Inhibitors

The discovery of the Jak2-V617F mutation in nearly all polycythemia vera and a large subset of essential thrombocythemia and primary myelofibrosis patients has prompted researchers to closely study the Jak2 gene and its role in myeloproliferative disorders. In addition, constitutive activation of Jak2 kinase activity by chromosomal translocations has been reported in various types of hematological malignancies (Lacronique et al., 2007; Reiter et al., 2005). Currently however, no FDA approved Jak2 inhibitor therapies are available for use in the

clinic, although a few are being examined for their efficacy and safety in Phase I/II clinical trials. Thus, the continual identification of novel activating Jak2 mutations, and their correlation with hematological malignancies, highlights the requirement for the development of potent and therapeutically effective Jak2 inhibitors.

Jak2-Selective Inhibitors

The causal relationship between constitutive Jak2 tyrosine kinase activity and neoplastic growth has prompted researchers to identify potent and selective Jak2 small molecule inhibitors. In 1995, Meydan et al. employed a high throughput screen of potential tyrosine kinase inhibitors and identified tyrphostin B42 (AG490) as the first Jak2 inhibitor (Meydan et al., 1996). Their important finding was that AG490 blocked the growth of leukemic cells derived from patients who expressed constitutive Jak2 tyrosine kinase activity. The compound induced cellular apoptosis, without any deleterious effect on normal hematopoiesis. However, subsequent reports revealed that while AG490 is a potent inhibitor of Jak2, it suffered from a general lack of specificity (Gu et al., 2001).

To circumvent this problem, researchers have utilized different approaches to identify novel Jak2 selective inhibitors. In 2004 for example, Flowers et al. developed a short peptide inhibitor of Jak2 termed, Tkip, that mimics the actions of the Jak2 inhibitor protein, SOCS-1. They reported that the inhibitor peptide mimicked SOCS-1 in that it specifically inhibited Jak2 tyrosine 1007 phosphorylation and suppressed IFN-γ signaling (Flowers et al., 2004). In 2005, our group published a paper whereby we constructed a homology model of the Jak2 kinase domain and utilized a high-throughput program called DOCK to identify novel small molecule inhibitors of Jak2 tyrosine kinase (Sandberg et al., 2005). Specifically, we tested 6451 compounds of known chemical structure *in silico* for their ability to interact with a pocket

positioned adjacent to the activation loop of Jak2. The top seven scoring compounds were obtained from National Cancer Institute and tested for their ability to inhibit Jak2 autophosphorylation *in vitro*. We found that one compound, C7, directly inhibited Jak2 tyrosine kinase activity. Characterization of C7 revealed that this compound suppressed Jak2 tyrosine autophosphorylation in both a dose- and time-dependent manner. C7 significantly reduced growth hormone-dependent Jak2 autophosphorylation, but had no effect on epidermal growth factor receptor tyrosine phosphorylation. Moreover, C7 was not cytotoxic to cells at doses as high as 100 μM as measured by the ability of cells to exclude propidium iodide. All together, the results suggested that C7 may be a relatively specific Jak2 inhibitor and proposed that it may be useful for elucidating signaling mechanisms of Jak2.

The discovery of the Jak2-V617F mutation in 2005 and its identification in a high percentage of myeloproliferative disorders has further spurred interest in the development of small-molecule inhibitors that selectively target Jak2. Moreover, the resolution of the crystal structures of portions of the kinase domains of Jak3 and Jak2 in 2005 and 2006, respectively, have provided a valuable tool for the design of potent and specific Jak2 small molecule inhibitors (Boggon et al., 2005; Lucet et al., 2006). Recently, a group developed several novel Jak2-selective small molecule compounds (TG101209 and TG101348) while taking into consideration the crystal structures of the kinase domains of both Jak2 and Jak3 (Pardanani et al., 2007; Wernig et al., 2008). They showed that TG101209 and TG101348 potently inhibited Jak2 tyrosine kinase, with considerably less activity against other tyrosine kinases such as Jak3. These compounds suppressed the proliferation of human erythroleukemia cells, which express the Jak2-V617F mutation. Furthermore, they demonstrated that both compounds effectively treated Jak2-V617F-induced hematopoietic disease in mice and reduced the growth of

hemopoietic colonies from primary progenitor cells harboring Jak2-V617F mutations. Currently, the TG101348 compound has been assigned as a lead drug for clinical development for the potential treatment of Jak2-V617F-induced myeloproliferative disorders.

Another Jak2-selective inhibitor, INCB18424, is presently under phase I/II clinical trials at MD Anderson Cancer Center for the treatment of primary myelofibrosis patients. While it has reduced splenomegaly, it unfortunately has not diminished the marrow fibrosis (Verstovsek et al., 2008a).

In 2008, Verstovsek et al. demonstrated that a novel analogue of AG490, WP1066, potently suppressed proliferation and induced apoptosis in erythroid human cells harboring the Jak2-V617F mutation (Verstovsek et al., 2008b**)**. In addition, WP1066 inhibited the expansion of peripheral blood hematopoietic progenitors of PV patients who were positive for the Jak2-V617F mutation. Interestingly, WP1066 was previously shown to inhibit phosphorylation of Jak2 in acute myelogenous leukemia cells, but unlike AG490, this compound also degraded the Jak2 protein (Ferrajoli et al., 2007). Collectively, the data suggests that WP1066 is a potent Jak2 inhibitor *in vitro* and *ex vivo* and warrants further development for the treatment of myeloproliferative disorders and other hematological malignancies that are associated with constitutive Jak2 activity.

Our laboratory has recently contributed to the continuing development of small molecule inhibitors that target aberrant Jak2 activity by using a rapid structure-based approach combining molecular docking with cell-based functional testing. Like others, we took into consideration the crystal structure for portions of the Jak3 kinase domain in order to generate an atomic model of the kinase domain of murine Jak2 and then employed the DOCK program to predict the ability of 20,000 small molecules to interact with a structural pocket adjacent to the ATP-binding site.

Consequently, we identified a Jak2-selective inhibitor termed Z3 (Sayyah et al., 2008). We found that it bound to Jak2 with a favorable energy score and inhibited Jak2-V617F autophosphorylation in a dose-dependent manner, but was not cytotoxic to cells at concentrations that inhibited kinase activity. Z3 selectively inhibited Jak2 as it had no effect on Tyk2 and c-Src kinase activity. Furthermore, Z3 significantly inhibited proliferation of the Jak2-V617F expressing HEL cells and this Z3-mediated reduction in cell growth correlated with reduced Jak2 and STAT3 tyrosine phosphorylation levels, as well as marked cell cycle arrest. Finally, Z3 inhibited the growth of hematopoietic progenitor cells isolated from the bone marrow of an essential thrombocythemia patient carrying the Jak2-V617F mutation and a polycythemia vera patient harboring a Jak2-F537I mutation. All together, our results suggest that Z3 is a specific inhibitor of Jak2 tyrosine kinase.

Non-Jak2 Selective Inhibitors

In addition to the drugs that were targeted specifically for Jak2, there exists a group of drugs that were developed for the treatment of non-myeloproliferative disorders, but are now considered to have therapeutic potential in myeloproliferative because of their significant off-target Jak2 inhibitory activity. Some of these drugs are even in phase I/II clinical trials. For example, MK-0457 (formerly, VX-680), a potent inhibitor of Aurora kinases effectively inhibits BCR-ABL, FLT3 and Jak2 (Pardanani, 2008). A phase I/II clinical trial of MK-0457 was initiated in patients with chronic myelogenous leukemia or Ph+ acute lymphoblastic leukemia who carried the T315I BCR-ABL resistance mutation, as well as in patients with refractory Jak2-V617F positive myeloproliferative disease. This compound showed encouraging anti-neoplastic growth activity and a good safety profile (Wang and Serradell, 2007). Another off-target Jak2 inhibitor is CEP-701 (Lestaurtinib), which was originally developed to suppress tropomyosin receptor kinase A activity for possible use in prostate cancer, but later discovered to exhibit

FLT3 inhibitory activity as well. CEP-701 has been shown to inhibit Jak2 tyrosine kinase activity and inhibit the proliferation of progenitor cells obtained from myeloproliferative disorder patients (Hexner et al., 2008). Unfortunately, in Phase II clinical studies, CEP-701 has shown little to no activity in treating primary myelofibrosis. Finally, AT9283, another Aurora kinase as well as potent Jak2 inhibitor, is in phase I/II clinical trials for the treatment of acute leukemias, chronic myelogenous leukemia and primary myelofibrosis (Ravandi et al., 2007).

There are other non-Jak2 selective inhibitors that are still in pre-clinical testing for the treatment of Jak2-associated hematological disorders. One of these inhibitors is Gö6976, which is an inhibitor of the calcium-dependent isozymes of PKC and FLT3 tyrosine kinase. Gö6976 was subsequently found to be a potent inhibitor of Jak2 *in vitro*. This compound also suppressed signaling, survival and proliferation of cells expressing either the TEL-Jak2 fusion protein or the Jak2-V617F mutation (Grandage et al., 2006). These data suggest that Gö6976 may be useful for the treatment of myeloproliferative disorders or other Jak2-associated hematological malignancies. In addition, Erlotinib (Tarceva) which is used for the treatment of patients with locally advanced or metastatic non-small cell lung cancer, inhibited the growth and expansion of Jak2-V617F-expressing polycythemia vera hematopoietic progenitor cells and human erythroleukemia HEL cells while having little effect on normal cells (Li et al., 2007).

Another compound that possesses Jak2-inhibitory property is Atiprimod. Atiprimod is an orally bioavailable agent that has been investigated for its anti-inflammatory and anti-cancer properties. Faderl et al. reported that Atiprimod inhibits Jak2/STAT phosphorylation and blocks clonogenic growth of acute myelogenous leukemia cell lines and patient-derived acute myelogenous leukemic marrow cells by inducing apoptosis (Faderl et al., 2007). Their data suggest that the anti-proliferative and pro-apoptotic activities of Atiprimod towards acute

myelogenous leukemia cells might be attributed to the inhibition of the Jak-STAT signaling pathway. Interestingly, the inhibitory effect of this compound has not been evaluated on Jak2-V617F-dependent pathologic cell growth. Thus, Atiprimod may warrant further evaluation as a drug candidate for the treatment of hematological disorders associated with constitutive Jak2 activation.

Finally, CP-690,550, a selective JAK3 inhibitor, also exhibits Jak2 inhibitory properties. Manshouri et al. demonstrated that this compound exerts potent anti-proliferative activity against cells expressing the Jak2-V617F mutation (Manshouri et al., 2008). In fact, CP-690,550 suppressed Jak2-V617F-dependent cell growth *in vitro* (IC_{50} = 0.2 μM) ten fold more potently than wild-type Jak2 (IC_{50} = 2.1 μM). It induced a marked pro-apoptotic effect on cells harboring the Jak2-V617F mutation whereas a smaller effect was observed for cells carrying wild type Jak2. Furthermore, CP-690,550 selectively inhibited the growth of Jak2-V617F positive cells in *ex-vivo* expanded progenitors from polycythemia vera patients, which correlated with a decrease in Jak2-V617F mutant allele frequency. Taken together, the data suggest that CP-690,550 is a putative inhibitor of Jak2-V617F both *in vitro* and *ex vivo*.

Collectively, work by many groups, including our own, have identified various small molecule inhibitors that suppress Jak2 tyrosine kinase activity. Some of these small molecule compounds may be classified as Jak2-selective (class I) because they specifically target Jak2. Alternatively, a number of these compounds may be categorized as non-Jak2 selective (class II) since they were initially developed for non-myeloproliferative disorders, but subsequently shown to have considerable Jak2 inhibition. These inhibitors are summarized in Table 1-2.

Purpose and Rationale of Studies

Activating Jak2 mutations are found in almost all individuals with polycythemia vera and a substantial proportion of individuals with essential thrombocythemia and primary myelofibrosis. An increasing number of Jak2 aberrations such as substitution mutations, deletions, insertions and gene translocations are also being found in a number of hematopoietic malignancies. The expanding compendium of Jak2 aberrations found in hematological disorders justifies the need for quantitative Jak2 mutation testing in the clinic and validates their candidacy for targeted therapy. As previously mentioned, we have contributed to the identification of novel Jak2 small molecule inhibitors that target aberrant Jak2 tyrosine kinase activity. In the subsequent Chapters 3 and 4, we will describe the characterization of our second and third generation inhibitors named Z3 and G6, respectively. Additionally, since Jak2 structure is intimately tied to its function, the identification and characterization of novel Jak2 phosphorylation sites will give us new insights regarding how this signal transduction pathway operates. In particular, we will define the role of tyrosine 372, a novel Jak2 phosphorylation site, on Jak2 tyrosine kinase function.

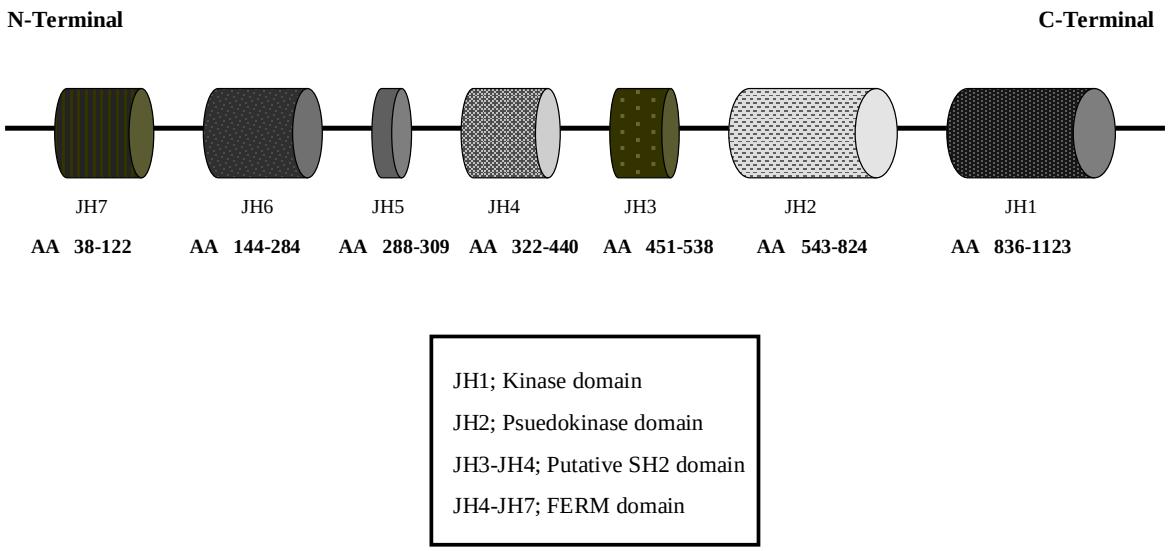

Figure 1-1. Illustration of Jak2 structural domains. The seven Jak homology (JH) domains and their relative positions within Jak2 are presented. The corresponding amino acid sequence of each domain is also shown. The JH1 domain represents the kinase activity site. The JH2 domain corresponds to the pseudokinase domain and exhibits an inhibitory effect on the kinase domain. The JH3 and half of the JH4 domain encode a putative SH2 domain. The FERM domain extends from the second half of the JH4 to the JH7 domain and assists Jak2 association with the receptor.

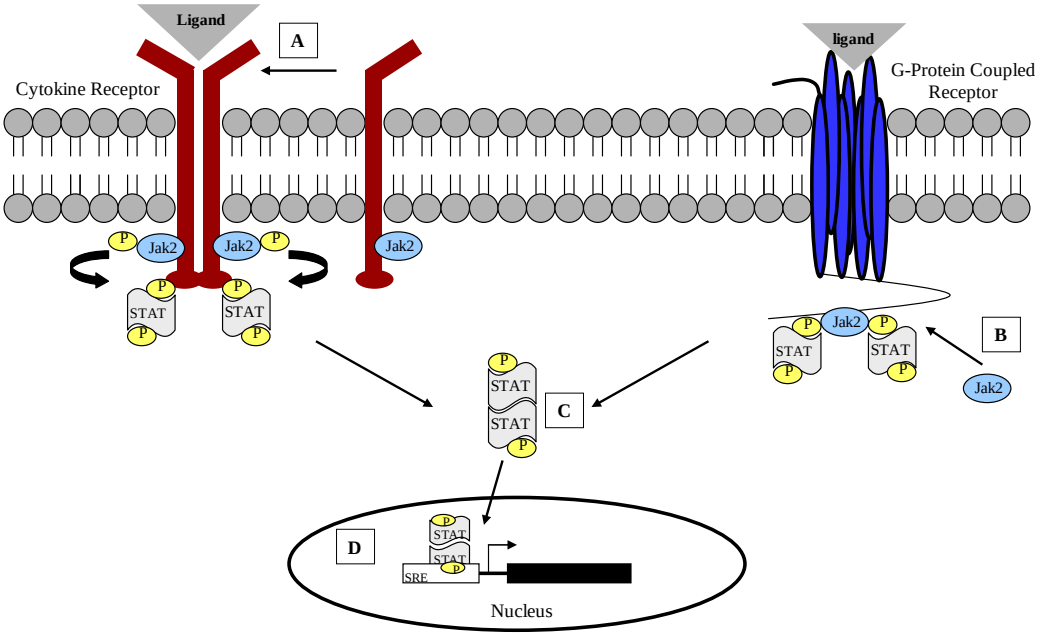

Figure 1-2. Jak/STAT signaling paradigm. Jak2 signals through cytokine, tyrosine kinase or G protein-coupled receptors. A) With respect to cytokine receptors, Jak2 is non-covalently associated with the receptor. The binding of ligand to the extracellular surface of the cytokine receptor results in receptor dimerization and subsequent Jak2 activation. Activated Jak2 then phosphorylates the cytoplasmic tail of the receptor at particular tyrosine residues. The STAT proteins associate with phosphorylated tyrosine residues and are themselves phosphorylated by Jak2. B) In the case of G protein-coupled receptors, Jak2 is not ubiquitously bound to the receptor, but becomes activated and associates with the cytoplasmic tail of the receptor after ligand binding. STAT proteins then bind to phosphorylated tyrosine residues on Jak2 and are themselves activated by Jak2. C) The phosphorylated STAT proteins dissociate from the receptor, form dimers and translocate into the nucleus. STAT proteins then bind specific STAT response elements (SRE) within gene promoters and initiate transcription.

Table 1-1. A complete list of Jak2 gene aberrations reported in hematological disorders. These malignant mutations include Jak2 amino acid substitutions, deletions and chromosomal translocations that were identified from 1997 to 2008.

Mutation Type	Mutation	Phenotype	Year Identified
Translocation	TEL-Jak2	ALL, aCML	1997
Translocation	REL-Jak2	aCML, Hodgkin's Lymph.oma	2003
Translocation	PCM1-Jak2	aCML, AML, ALL	2005
Translocation	BCR-Jak2	CML	2005
Translocation	BCR-Jak2	AML	2008
Substitution	Jak2-V617F	PV, ET, PMF	2005
Substitution	Jak2T875N	Megakaryoblastic Leukemia	2006
Substitution	Jak2K607N	AML	2006
Substitution	Jak2L611S	ALL	2006
Substitution	Jak2K539L	PV, Idiopathic Erythrocytosis	2007
Deletion	Jak2ΔIREED	ALL	2007

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Table 1-2. A complete list of class I and II Jak2 inhibitors identified since 1996. The class I Jak2 inhibitors are considered as Jak2-selective compounds whereas the class II inhibitors are categorized as non-Jak2 selective. The listed Jak2 small molecule inhibitors are either in pre-clinical or phase I/II clinical trials.

Inhibitor	Class	Current Status	Date Identified
AG490	I	Pre-Clinical	1996
Tkip	I	Pre-Clinical	2004
C7	I	Pre-Clinical	2005
TG101209	I	Pre-Clinical	2007
WP1066	I	Pre-Clinical	2008
Z3	I	Pre-Clinical	2008
TG101348	I	Phase I/II	2008
INCB018424	I	Phase I/II	2008
Go6976	II	Pre-Clinical	2006
Erlotinib	II	Pre-Clinical	2007
Atiprimod	II	Pre-Clinical	2007
CP-690,550	II	Pre-Clinical	2008
AT9283	II	Phase I/II	2006
CEP-701	II	Phase II	2007
MK-0457	II	Phase I/II	2007

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CHAPTER 2
METHODS

Small Molecule Database

The small molecules were obtained from the National Cancer Institute/Developmental Therapeutics Program (NCI/DTP), which maintains a repository of approximately 225,000 compounds.

In Silico Molecular Modeling of Jak2

The Swiss Model program was utilized to generate a homology model structure of murine Jak2 based on the human Jak2 crystal structure (PDB code: 2B7A). The Definition of Secondary Structure of Proteins (DSSP) program was used to calculate solvent accessible surface areas of tyrosine 372 (Kabsch and Sander, 1983).

Mass Spectrometry

Wild-type Jak2 protein was expressed in BSC-40 cells using a vaccinia virus over-expression system and the protein was subsequently purified as previously described (Ma X 2004). The purified Jak2 protein was then subjected to SDS-PAGE, coommassie stained, excised from the gel and subjected to nano-HPLC/μESI ionization on an LTQ mass spectrometer as previously described (Godeny et al., 2007).

Cell Lines

The cell lines that were employed to carry out transient transfection experiments were COS-7, BSC-40, and MEF cells derived from Jak2 -/- mice. The COS-7 and BSC-40 are epithelial cell lines that originate from the monkey kidney. Since all these cells express very little to no endogenous Jak2 levels, they provide suitable conditions to carry out transient transfections. The BSC-40 cells are more permissive to vaccinia virus infection, as such, they were preferentially used for virus-mediated over-expression assays. The mouse embryonic

fibroblast cells (MEF) originate from Jak2-deficient mice and were a kind of Dr. Jim Ihle (Parganas et al., 1998).

For our Jak2 inhibitor studies, HEL, Raji and CMK cells were employed. HEL cells are a human erythroleukemia cell line which expresses the V617F mutation on both Jak2 alleles (ATCC). Therefore, these cells provided a suitable background to determine the efficacy of our novel Jak2 small molecule inhibitors. Raji cells are a human Burkitt's lymphoma cell line (ATCC) whose mechanism of aberrant growth is due to a translocation event between the c-Myc gene and the heavy chain locus on chromosome 14 (Hamlyn and Rabbitts, 1983). Finally, CMK cells (DSMZ, Braunschweig, Germany) are a human megakaryoblastic leukemia cell line that expresses constitutively activated Jak3 (Verstovsek et al., 2008b). Thus, the Raji and CMK were appropriate control cell lines to use in our proliferation assays to determine the specificity of our Jak2 inhibitors.

Cell Culture

BSC-40 cells were maintained in high glucose (4.5 g/L) DMEM supplemented with 10% newborn calf serum. COS-7 and MEF cells were grown in high glucose DMEM supplemented with 10% fetal bovine serum. HEL,Raji and CMK cells were maintained in RPMI-1640 medium (Mediatech, Herndon, VA) containing 10% fetal bovine serum and L-glutamine (2 mM final). All cells were cultured at 37^{o}C in a 5% CO_2 humidified atmosphere. Cells treated with interferon-gamma, epidermal growth factor or hydrogen peroxide were growth-arrested with serum-free DMEM for 18 hours prior to treatment.

Site-Directed Mutagenesis

The pRC-CMV-Jak2-Y372F, pBOS-Jak2-Y372F, pRC-CMV-Jak2-Y373F, and pRC-CMV-Jak2-V617F plasmids were generated using the Stratagene QuickChange Mutagenesis protocol. The sense primer for the Jak2-Y372F plasmids is 5'-

TTAATTGACGGGTTTTACAGACTAACT. The antisense primer sequence for these plasmids is 5'-AGTTAGTCTGTAAAACCCGTCAATTAA. The sense primer for the Jak2-Y373F plasmids is 5'-ATTGACGGGTATTTTAGACTAACTGCGDNA. The antisense primer for this plasmid is 5'-CGCAGTTAGTCTAAAATACCCGTCAAT. For the Jak2-V617F plasmid, the sequence for the sense primer is 5'-TATGGTGTCTGTTTCTGTGAAGAGGAG. The sequence for the antisense primer is 5'-CTCCTCTTCACAGAAACAGACACCATA. DNA sequencing was used to verify all mutations.

BSC-40 Cell Transfection/Infection

Jak2 autophosphorylation assays were performed in BSC-40 cells using the vaccinia virus transfection/infection procedure which results in high level Jak2 expression and subsequent tyrosine phosphorylation independent of exogenous ligand addition (Ma and Sayeski, 2004). Specifically, cells were transfected with 10 μg of a plasmid encoding either the wild-type murine Jak2 cDNA (pRC-CMV-Jak2-WT), V617F mutant murine Jak2 cDNA (pRC-CMV-Jak2-V617F), Y372F mutant murine Jak2 cDNA (pRC-CMV-Jak2-Y372F) or Y373F mutant murine Jak2 cDNA (pRC-CMV-Jak2-Y373F) under the control of the bacteriophage T7 promoter, using Lipofectin per the manufacturer's instructions (Invitrogen, Carlsbad, CA). After 4 hours of transfection, the cells were infected with the recombinant vaccinia virus, vTF7-3, at a multiplicity of infection (MOI) of 1.0 for 1 hour. The media containing Lipofectin/DNA/vTF7-3 was then removed from the cells, replaced with fresh serum-containing media and incubated overnight.

Transient Cell Transfections

For lipofectin-mediated transfections, plasmid DNA and lipofectin were incubated separately in 0.5 mL of serum-free DMEM at room temperature for 30 minutes. Plasmid DNA and lipofectin were then combined and incubated at room temperature for 10 minutes.

Afterwards, an additional 2 mL of serum-free DMEM was added to DNA/Lipofectin complex and the 3 mL transfection mixture was added on to the plate of cells. The cells were incubated at 37^{o}C for five hours. After five hours, the transfection mixtures were removed from the cells and replaced with 5 mL of serum-containing DMEM. The cells were allowed to recover for 48 hours.

For Targefect-mediated transfections, plasmid DNA, Targefect (Targeting Systems, CA) and virofect enhancer (Targeting Systems) were combined in a total volume of 1 mL of serum-free DMEM and incubated at 37^{o}C for 20 minutes. Afterwards, 1 mL of serum-containing DMEM was added to the transfection complex and the 2 mL mixture was added onto a plate of cells. The cells were then incubated for 3 hours and after that, 3 mL of serum-containing media was added onto the cells. The cells were allowed to recover for 48 hours.

Cell Lysis and Immunoprecipitation

Cells were washed with two volumes of ice-cold PBS containing 1 mM Na_3VO_4. BSC-40, COS-7 and MEF cells were lysed in 0.8 mL of ice cold RIPA buffer (20 mM Tris pH 7.5, 10% glycerol, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 2.5 mM EDTA, 50 mM NaF, 10 mM $Na_4P_2O_7$, 4 mM benzamidine, and 10 μg/mL aprotinin) while HEL cells (1.0 x 10^7) were lysed in 0.8 mL of ice cold Gentle Lysis Buffer (25 mM Tris, pH 7.4, 10% glycerol, 1% IGEPAL and 137 mM NaCl, 4 mM benzamidine, and 10 μg/mL aprotinin). Cleared protein lysates were incubated with 2 μg of antibody and 20 μL of protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at 4^{o}C. Protein complexes were washed three times with wash buffer (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) and resuspended in SDS sample buffer. Bound proteins were boiled, separated by SDS-PAGE, and transferred onto nitrocellulose membranes.

Western Blotting

Western blots were performed at room temperature. Nitrocellulose membranes were blocked in 25 mL of either 5% BSA/TBST or 5% milk/TBST for 30 minutes. The membranes were then incubated in 25 mL of primary antibody solution for 2 hours and subsequently washed in TBST for 1 hour. Membranes were incubated in secondary antibody solution (1:4000, GE Healthcare) for 30 minutes and then washed in TBST for 30 minutes. Western blots were visualized using the enhanced chemiluminescence system (NEN Life Science Products, Waltham, MA). Densitometry was performed using the automated digitizing software, Un-Scan-It, Version 5.1 (Silk Scientific, Orem, Utah). All phosphorylation levels were normalized to total protein levels.

c-Src *In Vitro* Kinase Assay

Approximately 4 μL (12 units) of catalytically active recombinant $pp60^{c\text{-}Src}$ (Millipore) was incubated in 50 μL of *in vitro* kinase reaction buffer as described (13). Reactions were terminated via the addition of SDS-containing sample buffer and subsequently Western blotted with either anti-ACTIVE c-Src or total c-Src antibody as described.

Luciferase Assay

Using either Lipofectin or Targefect, COS-7 cells were transiently transfected with the appropriate DNA plasmid and 2 μg of a luciferase reporter plasmid consisting of four tandem copies of the interferon-gamma activating sequence (pLuc-GAS). Following 5 hours of transfection, approximately 7×10^5 cells were seeded onto six well culture dishes. The cells were allowed to recover in DMEM for 48 hours. The cells were then lysed in 1X Reporter Lysis buffer (Promega) for 5 hours. During lysis, the lysates were exposed to one freeze-thaw cycle

between room temperature and -80^{o}C. A 20 μL sample of lysate was combined with 100 μL of luciferase substrate and relative light units were assessed by a Monolight 3010 luninometer.

Propidium Iodide Staining of Cells

BSC-40 cells were seeded in 6-well Lab-Tek Chamber Slides (Nunc, Rochester, NY). After adherence, the cells were treated with either DMSO or Z3 at concentrations of 25, 100 or 250 μM for 16 hours. Live cells were treated with 1 μg/mL propidium iodide and then visualized using a Bio-Rad MRC-1024 confocal microscope. Same field images were captured under phase contrast and fluorescent conditions.

Cell Proliferation Assay

HEL cells were plated on 96-well dishes at 5 x 10^4 cells per well and treated with either DMSO or 25 μM Z3 for the indicated times. Alternatively, 5 x 10^4 HEL, Raji or CMK cells were plated on 96-well dishes and treated with either DMSO or G6 at the indicated concentrations for 48 hours. The number of viable DMSO or inhibitor-treated cells was determined by trypan blue exclusion using a hemocytometer.

Cell Cycle Analysis

The CycleTEST^{TM} Plus DNA Reagent Kit (Becton Dickinson, San Jose, CA) was utilized to analyze nuclear DNA from HEL cell suspensions following the manufacturer's instructions. Approximately 5 x 10^5 cells were treated with either DMSO or 25 μM of Z3 for 16, 24, 48 and 72 hours. Cell suspensions were treated with the reagents stated in the protocol, filtered through a 50-μm nylon mesh and analyzed on a FACSCalibur Flow Cytometer (Becton Dickinson).

Apoptosis Assays

HEL cells were treated with either DMSO or 25 μM Z3 or G6 for 16, 24, 48 or 72 hours. Cells were subsequently measured for Annexin V staining via a FACSCalibur Flow Cytometer

(Becton Dickinson). The percent of DMSO or inhibitor-treated cells undergoing apoptosis was plotted as a function of time.

Jak2 Mutation Analysis

Residual bone marrow aspirates were obtained from patients diagnosed with ET or PV (WHO criteria) following approval by the University of Florida Institutional Review Board (approval # 428-2006). Patient de-identified mononuclear cells were isolated by separation over Ficoll-Paque (Pharmacia, Peapack, NJ). Genomic DNA was isolated from 10^6 cells using the Easy-DNA kit (Invitrogen) following the manufacturer's instructions. Exons 12 and 14 were amplified via PCR using previously published exon specific primers and annealing conditions (Scott et al., 2007). Each amplified exon was then subjected to direct DNA sequence analysis.

Colony Formation Assay

Marrow derived mononuclear cells were washed three times in IMDM media and plated at 4×10^5 cells/mL in 1 mL methylcellulose media (0.9% methylcellulose, 30% heat inactivated FCS, 0.1 mM 2-mercaptoethanol, 0.9% BSA, 0.05% $NaHCO_3$, 2 mM/L glutamine, penicillin, streptomycin, 50 ng/mL SCF, and 20 ng/mL IL-3 (Stem Cell Technologies, Vancouver, BC). Z3/G6 (25 μM) and TPO (50 ng/mL) or EPO (1 U/mL) were either added or omitted as described in the legend. The cultures were then incubated at 37°C and 5% CO_2 until assessment of colony formation at day 14. Results were expressed as the average number of colonies from duplicate cultures per 4×10^5 cells.

Statistical Analysis

For IC_{50} determination, data was analyzed using one-way analysis of variance (ANOVA). For proliferation assays and cell cycle analysis, statistical significance between each group was analyzed using a two-way ANOVA. For all other experiments, data were analyzed via Student's *t*-test. Significance was set at $p<0.05$.

CHAPTER 3
Z3, A NOVEL JAK2 TYROSINE KINASE SMALL-MOLECULE INHIBITOR THAT SUPPRESSES JAK2-MEDIATED PATHOLOGIC CELL GROWTH

Summary

Jak2 tyrosine kinase is essential for animal development and hyper-kinetic Jak2 function has been linked to a host of human diseases. Control of this pathway using Jak2-specific inhibitors would therefore potentially serve as a useful research tool and/or therapeutic agent. Here, we used a high throughput program called DOCK, to predict the ability of 20,000 small molecules to interact with a structural pocket adjacent to the ATP binding site of murine Jak2. One small molecule, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one (herein designated as Z3) bound to Jak2 with a favorable energy score. Z3 inhibited Jak2-V617F and Jak2-WT autophosphorylation in both a dose- and time-dependent manner, but was not cytotoxic to cells at concentrations that inhibited kinase activity. Z3 selectively inhibited Jak2 kinase function with no effect on Tyk2 or c-Src kinase function. Z3 significantly inhibited proliferation of the Jak2-V617F-expressing, human erythroleukemia cell line, HEL 92.1.7. The Z3-mediated reduction in cell proliferation correlated with reduced Jak2 and STAT3 tyrosine phosphorylation levels as well as marked cell cycle arrest. Finally, Z3 inhibited the growth of hematopoietic progenitor cells isolated from the bone marrow of an essential thrombocythemia patient harboring the Jak2-V617F mutation and a polycythemia vera patient carrying a Jak2-F537I mutation. Collectively, the data suggest that Z3 is a novel specific inhibitor of Jak2 tyrosine kinase.

Introduction

Aside from its essential role in embryonic development (Parganas et al., 1998), Jak2 plays an important role in pathophysiology. Jak2 has been linked to several hematological malignancies including acute lymphoid leukemia and chronic myeloid leukemia (Lacronique et

al, 1997, Peeters et al., 1997; Shuai K et al., 1996). Recently, a Jak2-V617F somatic mutation has been identified in approximately 98% of patients with polycythemia vera (PV) and in a substantial proportion (50%) of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) (Levine et al., 2005). This valine to phenylalanine substitution (V617F) occurs in the JH2 pseudokinase domain of Jak2. These myeloproliferative disorders are characterized by the clonal overproduction of normally differentiated hematopoietic lineages. The V617F substitution leads to constitutive activation of Jak2 and downstream effector signaling pathways including the STAT transcription pathway and the phosphatidylinositol 3-kinase and extracellular signal-regulated kinase signaling networks, which in turn induce inappropriate cytokine-independent proliferation of cells (Levine et al., 2005; Shannon and Van Etten, 2005). In addition, the Jak2-V617F mutation contributes to myelofibrosis by constitutively phosphorylating STAT3 and diminishing myeloid cell apoptosis (Mesa, 2007). Collectively, these results implicate dysregulated Jak2 signaling in various hematological and myeloproliferative disorders.

As components of the Jak-STAT signaling pathway are hyper-activated in some leukemias and myeloproliferative disorders, control of this pathway using pharmacological inhibition is highly desirable. As a research tool, AG490 is by far the most extensively used commercially available Jak2 inhibitor. Although it has been valuable in identifying Jak2 as a therapeutic target, it suffers from a general lack of specificity. For example, AG490 inhibits calf-serum inducible cell growth and DNA synthesis (Kleinberger-Doron et al., 1998) and is a partial blocker of c-Src activity (Oda et al, 1999). Most importantly, AG490 inhibits epidermal growth factor receptor autophosphorylation more potently than it inhibits Jak2 activity (Osherov et al.,

1993, Gu et al., 2001). Thus, identification and characterization of novel Jak2-specific inhibitors may serve as useful research tools and therapeutic agents.

Here, we utilized a rapid structure-based approach combining molecular docking with cell based functional testing in order to identify a Jak2-selective inhibitor. One small molecule, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one (herein designated as Z3) bound to a pocket that was adjacent to the ATP binding site of Jak2 with a favorable energy score. Our functional testing data suggest that Z3 is a novel Jak2 tyrosine kinase specific, small molecule inhibitor.

Results

Z3 Inhibits Jak2 Tyrosine Autophosphorylation by Interacting with a Solvent Accessible Pocket Adjacent to the ATP Binding Pocket of Jak2

Taking the crystal structure for portions of the Jak3 kinase domain (pdb code 1YVJ) into consideration, a comparative structural modeling approach was employed to generate an atomic model of the kinase domain of murine Jak2 using ProModII as described previously (Sandberg et al., 2005).

The program SPHGEN, which identifies potential ligand binding sites based on chemical and shape characteristics, was employed to select the target pocket on Jak2 for small molecule docking. To prepare the site for docking, all water molecules were removed. Protonation of receptor residues was performed with the program Sybyl. The sphere set utilized for molecular docking was based on the position of a Jak3 inhibitor in the crystal structure of the kinase domain of Jak3 (Figure 3-1A). The position of a pan Jak2 inhibitor that was subsequently crystallized in the kinase domain of human Jak2 is also shown (Figure.3-1A). The site selected for molecular docking was adjacent to the ATP binding site of murine Jak2 and the number of spheres in the site (50-100) was in the ideal range for binding to small molecules.

Utilizing the DOCK program, we tested 20,000 compounds of known chemical structure, *in silico,* for their ability to interact with a structural pocket adjacent to the ATP binding site of the murine Jak2 kinase domain. The top six scoring compounds (Table 1) were obtained from the NCI/DTP and tested for their ability to inhibit Jak2-WT and Jak2-V617F autophosphorylation.

A Jak2 over expression system was used to first determine the effect of each inhibitor on Jak2 autophosphorylation. Specifically, BSC-40 cells were transfected with an expression vector encoding either empty vector control (no Jak2), the mutant murine Jak2 cDNA (Jak2-V617F) or the wild type Jak2 cDNA (Jak2-WT) under the control of T7 RNA polymerase. This resulted in high-level Jak2 expression and subsequent tyrosine phosphorylation independent of exogenous ligand addition. The six inhibitor compounds (Z1-Z6) were each added at a concentration of 100 μM and incubated overnight. Sixteen hours following addition of the compounds, cellular lysates were immunoprecipitated with an anti-Jak2 polyclonal antibody and then immunoblotted with an anti-phosphotyrosine monoclonal antibody to detect tyrosine phosphorylated Jak2 (Figure 3-1B). The results show that compound Z3 (methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one) most effectively inhibited both Jak2-V617F and Jak2-WT tyrosine autophosphorylation. Clearly however, the ability of Z3 to inhibit Jak2-WT autophosphorylation was greater than when compared to Jak2-V617F autophosphorylation. The membrane was then stripped and reprobed with an anti-Jak2 polyclonal antibody to demonstrate equal protein loading (Figure 3-1C).

The structure of the Z3 compound is shown in Figure 3-1D. Based on the data in Figure 3-1B, we returned to our molecular model of Jak2 and utilized the DOCK program to determine the position of Z3 on the Jak2 structure. Based on contact points and energy scores, the Z3

inhibitor was predicted to bind to the solvent accessible pocket adjacent to the ATP binding site as we had intended (Figure 3-1E). Collectively, the data in Figure 3-1 indicate that Z3 inhibits both Jak2-WT and Jak2-V617F tyrosine autophosphorylation (ie, kinase function) via an interaction with a critical solvent accessible pocket adjacent to the ATP binding site.

Z3 Inhibits Jak2 Tyrosine Autophosphorylation in a Dose-Dependent Manner that is Independent of Cellular Cytotoxicity

In order to obtain a better understanding of the inhibitory properties of Z3, we examined whether it could inhibit Jak2-WT and Jak2-V617F tyrosine autophosphorylation in a dose-dependent manner. Specifically, we sought to determine whether Z3 inhibits phosphorylation of the critically important tyrosine 1007 residue, as phosphorylation of this residue within the activation loop of Jak2 is necessary for maximal Jak2 activation (Feng et al., 1997). To meet this end, BSC-40 cells were transfected/infected with either a Jak2-WT or a Jak2-V617F plasmid and then treated for 16 hours with Z3 at the indicated concentrations. For the Jak2-WT expressing cells, cell lysates were first immunoprecipitated with a Jak2 polyclonal antibody and then Western blotted with a phospho-specific (pY1007/pY1008) Jak2 antibody to detect phosphorylated Jak2 at this residue (Figure 3-2A). The results indicate that Z3 inhibited phosphorylation of the critically important tyrosine 1007 residue on Jak2-WT, in a dose-dependent manner. The membrane was subsequently stripped and re-probed with an anti-Jak2 polyclonal antibody to determine Jak2 expression levels amongst all samples (Figure 3-2B).

In order to quantitate Jak2-WT tyrosine 1007 phosphorylation levels in the presence of increasing amounts of Z3, densitometry analysis on four representative western blots was performed (Figure 3-2C). We found that Jak2-WT tyrosine autophosphorylation was reduced by approximately 60 percent between the doses of 10 to 30 μM of Z3. A 100 μM dose of Z3 maximally suppressed Jak2 phosphorylation by about 92 percent. The estimated IC_{50} of Z3 for

Jak2-WT was 15 μM. For the Jak2-V617F expressing samples, cell lysates were immunoprecipitated with a Jak2 antibody and then immunoblotted with the phospho-specific (pY1007/pY1008), as we did previously (Figure 3-2D). Similar to the Jak2-WT expressing cells, Z3 inhibited Jak2-V617F tyrosine 1007 phosphorylation in a dose-dependent fashion. The membrane was then stripped and reprobed with an anti-Jak2 antibody in order to determine relative Jak2 expression levels across all samples (Figure 3-2E). Densitometry analysis on four representative Western blots showed that Jak2-V617F tyrosine 1007 phosphorylation was inhibited by approximately 60 percent between the concentrations of 10 to 100 μM of Z3 (Figure 3-2F). The IC_{50} of Z3 for Jak2-V617F was approximately 28 μM.

To rule out the possibility that the affects of Z3 were due to non-specific cellular toxicity rather than specific Jak2 inhibition, we treated these same BSC-40 cells with either DMSO or with 25, 100 or 250 μM Z3 for 16 hours. The live cells were then stained with propidium iodide. Propidium iodide selectively stains necrotic cells and fluoresces red, but is excluded from the plasma membrane of intact cells. The results show that cells treated with 25 or 100 μM of Z3, showed propidium iodide staining similar to that of DMSO-treated cells (Figure 3-2G). However, cells treated with 250 μM Z3 displayed a marked increase in propidium iodide staining, indicating that this dose of Z3 is cytotoxic. The same propidium iodide fields were also visualized by phase contrast microscopy to determine total cell numbers and overall cellular morphology. Overall, the data indicate that the range of Z3 that inhibits Jak2 tyrosine kinase autophosphorylation by 50% - 100% (25-100 μM), does so in a manner that is independent of cellular cytotoxicity. Taken together, our results indicate that the Z3 compound blocks Jak2 autophosphorylation in a dose-dependent manner, but is not cytotoxic to cells at concentrations

that inhibit Jak2 tyrosine kinase activity. Moreover, the ability of Z3 to inhibit Jak2-WT (IC_{50} = ~15 μM) is greater when compared to Jak2-V617F (IC_{50} = ~28 μM).

Z3 is a Specific Inhibitor of Jak2 Tyrosine Kinase

To determine whether Z3 is selective for suppressing Jak2 autophosphorylation when compared to other Jak family members, we employed an autophosphorylation assay to allow for robust Jak family kinase activation independent of ligand treatment (Ma and Sayeski, 2004). Here, COS-7 cells were transiently transfected with an expression vector encoding the wild type human Tyk2 cDNA. The following day, the cells were treated with either DMSO or 25 μM of Z3 for 16 hours. The cells were then lysed and Tyk2 was immunoprecipitated from the harvested lysates by the addition of a Tyk2 specific antibody. The immunoprecipitated protein was then immunoblotted with an anti-phosphotyrosine antibody to evaluate the level of Tyk2 tyrosine autophosphorylation (ie, catalytic activity). We found that Z3 did not inhibit Tyk2 tyrosine kinase autophosphorylation when compared to DMSO control (Figure 3-3A), bearing in mind that this same 25 μM dose reduced Jak2-WT tyrosine kinase autophosphorylation by approximately 70% (Figure 3-2C). The membrane was subsequently stripped and reprobed with anti-Tyk2 antibody to demonstrate equal Tyk2 expression for both samples (Figure 3-3B).

We next investigated the effect of Z3 on the ubiquitously expressed nonreceptor tyrosine kinase, c-Src. Similar to Jak2, c-Src has been shown to activate several effectors that are also involved in aberrant cell growth. Here, catalytically active recombinant c-Src protein was incubated in kinase reaction buffer, either in the presence of DMSO, 25 μM of Z3, or 25 μM of the Src kinase inhibitor, PP2. The reactions were incubated for 20 minutes at room temperature and then terminated by the addition of SDS-containing sample buffer. The samples were separated by SDS-PAGE and subsequently Western blotted with an anti-active c-Src (pY418)

antibody to determine c-Src catalytic activity (Figure 3-3C). We found that Z3 had no effect on c-Src tyrosine kinase activity when compared to DMSO control. However, in the presence of the known c-Src inhibitor, PP2, c-Src tyrosine kinase activity was completely abolished. To show equal c-Src protein content among all conditions, the membrane was stripped and reprobed with anti-c-Src antibody (Figure 3-3D). Collectively, the results demonstrate that while Z3 suppresses Jak2 tyrosine kinase activity, it does not inhibit Tyk2 and c-Src. This suggests that Z3 is therefore reasonably specific for Jak2.

Z3 Selectively Inhibits Jak2-V617F Dependent Cell Proliferation and this Correlates with Suppression of Jak2 and STAT3 Tyrosine Phosphorylation

It is known that the human erythroleukemia cell line, termed HEL, is homozygous for the Jak2-V617F mutation (Quentmeir et al., 2006). The single point mutation leading to a V617F substitution in the JH2 domain of Jak2 has been associated with its proliferative phenotype (Quentmeir et al., 2006). In addition, it has been shown that expression of the constitutively activated Jak2-V617F mutation is required for transformation of HEL cells. Mechanistically, the Jak2-V617F mutation promotes G1/S phase transition in HEL cells and subsequent increases in cellular proliferation (Walz et al., 2006).

Due to the presence of the constitutively activated Jak2-V617F mutation in HEL cells, we wanted to determine whether Z3 could suppress Jak2-V617F dependent cell proliferation in this cell line. Here, 5×10^4 HEL cells were treated with either DMSO or 25 μM of Z3 for 0, 16, 24, 48 and 72 hours. At the end of each time point, the number of viable DMSO- or Z3-treated cells was determined by trypan blue exclusion using a hemocytometer (Figure 3-4A). The results show that in the presence of DMSO, HEL cells were in rapid growth. However, treatment with Z3 reduced cell numbers when compared to DMSO. Analysis of variance indicated that the Z3 growth curve was significantly different from that of DMSO ($p= 1.06 \times 10^{-14}$).

To determine whether Z3 specifically suppresses Jak2-V61F dependent cell growth as opposed to non-specific inhibition of cell proliferation, we examined the inhibitory effect of Z3 on Raji cells. The molecular mechanism responsible for the aberrant growth of Raji cells is a translocation event between the c-Myc gene on chromosome 8 and the heavy chain locus on chromosome 14 (Hamlyn and Rabbitts, 1983). Here, 5×10^4 HEL or Raji cells were treated for 72 hours with either DMSO or Z3 at the indicated concentrations. The number of viable cells was then determined (Figure 3-4B). The results indicate that HEL cells were more sensitive to the inhibitory effects of Z3, over a wide range of doses, when compared to Raji cells ($p= 5.28 \times 10^{-11}$).

We next examined whether Z3-dependent inhibition of cell proliferation correlated with suppression of Jak2 tyrosine autophosphorylation. Specifically, HEL cells were treated with either DMSO or 25 μM Z3 for the indicated times. Protein lysates were first immunoprecipitated with anti-Jak2 antibody and then Western blotted with an anti-phosphotyrosine antibody to detect total tyrosine phosphorylated Jak2 (Figure 3-4C). We found that Z3 dramatically reduced total Jak2 tyrosine autophosphorylation by 48 hours when compared to DMSO control and this correlated with inhibition of HEL cell proliferation at this time point. The membrane was then stripped and reprobed with anti-Jak2 antibody to demonstrate total Jak2 expression amongst all samples (Figure 3-4D).

We next sought to determine whether Z3 could inhibit Jak2 phosphorylation at Tyr 1007 in these same cells. After treatment of the cells with DMSO or 25 μM Z3 for the indicated times, whole cell protein lysates were Western blotted with the phospho-specific (pY1007/pY1008) Jak2 antibody to detect phosphorylated Jak2 at this residue (Figure 3-4E). The results show that Z3 inhibited Jak2 phosphorylation at tyrosine 1007 in a time-dependent manner when compared

to DMSO control. The membrane was subsequently stripped and reprobed with anti-Jak2 antibody to demonstrate total Jak2 protein expression amongst all samples (Figure 3-4F).

STAT3 is a known substrate of Jak2 and previous studies have shown that STAT3 is constitutively phosphorylated in HEL cells (Spiekermann et al., 2001; Faderl et al., 2007). We therefore examined whether Z3-mediated inhibition of Jak2-dependent cell proliferation in HEL cells also correlates with reduced STAT3 phosphorylation. Specifically, protein cell lysates were immunoprecipitated with a STAT3 antibody and then immunoblotted with a phospho-specific (pY705) STAT3 antibody to assess active STAT3 levels (Figure 3-4G). We found that Z3 inhibited STAT3 tyrosine phosphorylation in HEL cells when compared to DMSO control, thus demonstrating that reduced phospho-STAT3 levels also correlate with reduced cell numbers. The membrane was stripped and reprobed with a STAT3 antibody to demonstrate equal STAT3 content amongst all samples (Figure 3-4H).

Overall, the data in Figure 3-4 demonstrate that Z3 selectively blocks Jak2-V617F pathologic cell growth and this corresponds with reduced levels of activated Jak2 and STAT3.

Z3 Exerts its Effect on the Cell Cycle by Increasing the Percentage of HEL Cells in G_1 Phase while Decreasing the Number of Cells in S phase

To determine the mechanism of Z3-mediated inhibition in cell growth, we first examined whether treatment with Z3 corresponded to an increase in apoptosis in the HEL cells. Specifically, HEL cells were treated with either DMSO or 25 μM of Z3 for 16, 24, 48 or 72 hours and cells were measured for Annexin V staining via flow cytometry. Treatment of cells with Z3 did not result in a significant increase in the percentage of Annexin V-positive-stained cells when compared to DMSO-treated cells (data not shown), indicating that Z3 did not induce apoptosis in this cell type. In addition, HEL cells treated with 25 μM of Z3 stained negative for

an early marker of apoptosis, APO2.7, confirming that the mechanism by which Z3 suppresses HEL cell proliferation is independent of apoptosis (data not shown).

We next examined cell cycle distribution as a function of Z3 treatment using the CycleTest^{TM} PLUS DNA Kit. HEL cells were treated with either DMSO or 25 μM of Z3 for 16, 24, 48 or 72 hours and cell cycle variables were determined by fluorescence-activated cell sorting analysis. We found that Z3 significantly increased the percentage of cells in the G_1 phase (Figure 3-5A) and significantly decreased the percentage of cells in S phase (Figure 3-5B) when compared to DMSO control-treated cells. A representative experiment of the cell cycle profile for DMSO- and Z3- treated HEL cells is also shown (Figure 3-5C and 3-5D, respectively). All together, the results in Figure 3-5 reveal that the mechanism by which Z3 reduces cell numbers is by blocking G_1-S transition.

Z3 Reduces Hematopoietic Colony Formatio*n Ex Vivo*

Somatic Jak2 mutations have been described in myeloproliferative disorders (Gruneback et al., 2006; Malinge et al, 2007; Scott et al., 2007). The most common Jak2 mutation, Jak2-V617F, has been shown to play an essential role in the pathogenesis of several myeloproliferative diseases including polycythemia vera, essential thrombocythemia and primary myelofibrosis. We have already shown that Z3 suppresses Jak2-dependent cell growth in the HEL cell line (Figure 3-4A). We next turned our attention to determining whether Z3 could inhibit the growth of hematopoietic progenitor cells isolated from the bone marrow of confirmed myeloproliferative disorder patients. Here, residual bone marrow aspirates were obtained from an essential thrombocythemia patient who was Jak2-V617F positive (Figure 3-6A) and from a polycythemia vera patient who was Jak2-F537I positive (Figure 3-6B). Their hematopoietic progenitor cells were cultured in a semisolid colony assay medium in the presence

of DMSO or 25 μM of Z3. In addition, since hematopoietic progenitors taken from patients with myeloproliferative disorders are known to be hypersensitive to cytokine stimulation (Axelrad et al., 2000), the cells taken from the ET patient were cultured in both the presence and absence of human thrombopoietin, whereas the cells taken from the polycythemia vera patient were cultured in both the presence and absence of human erythropoietin. The results show that, as expected, treatment of the essential thrombocythemia patient's hematopoietic progenitor cells with thrombopoietin markedly increased megakaryocyte colony formation (Figure 3-6A). However, this cytokine-dependent increase in cell growth was significantly blunted when the cells were cultured in the presence of Z3. Similarly, treatment of the polycythemia vera patient's progenitor cells with erythropoietin significantly increased erythrocyte colony formation. However, treatment of the cell with Z3 significantly reduced both cytokine-independent as well as cytokine-dependent erythrocyte colony formation (Figure 3-6B). Collectively, the results in Figure 6 show that Z3 greatly reduces Jak2-V617F as well as Jak2-F537I-mediated, human pathologic cell growth *ex vivo*.

Discussion

Hyperkinetic Jak2 tyrosine kinase activity has been linked to a variety of human diseases including cardiovascular disease, diabetes and cancer (Neubauer H et al., 1998, Wang X et al., 2002, Real PJ et al., 2002). In recent years, the Jak2-V617F gain-of-function mutation in myeloproliferative disorders has also been well described (Van Etten and Shannon, 2004; Levine RL et al, 2005; Shannon and Van Etten, 2005; Mesa, 2007; Wernig et al., 2006). Mutations in Exon 12 of Jak2 which cause dysregulated Jak-STAT signaling have been identified in Jak2-V617F-negative myeloproliferative disorders as well (Pardanani et al., 2007a; Scott et al., 2007;

Pietra et al., 2008). Thus, the continued identification of novel agents that can inhibit aberrant Jak2 tyrosine kinase function will be of great value.

Here, we have used *in silico* homology modeling and high throughput molecular docking to identify a novel small molecule inhibitor of Jak2. Our results are significant for a number of reasons. First, Z3 inhibits Jak2-WT and Jak2-V617F autophosphorylation, but is not cytotoxic to cells at concentrations that inhibit Jak2. Second, Z3 inhibits phosphorylation of tyrosine 1007, a residue whose phosphorylation is concomitant with hyper-kinetic Jak2 function. Third, Z3 selectively inhibits Jak2 tyrosine kinase activity as it has no effect of Tyk2 and c-Src. Fourth, Z3 preferentially suppresses proliferation of HEL cells that express the Jak2-V617F mutation by inducing cell cycle arrest. This arrest in cell growth directly correlates with reduced levels of active Jak2 and STAT3 proteins. Fifth, Z3 significantly blocks the growth of hematopoietic progenitor cells isolated from the bone marrow of myeloproliferative disorder patients carrying Jak2 mutations.

The Z3 compound is yet another step in the continuing development of Jak2 inhibitors. High throughput screening of potential tyrosine kinase inhibitors identified tyrphostin B42 (AG490) as the first Jak2 inhibitor. Initially, AG490 was regarded as a specific inhibitor of Jak2 because it concomitantly inhibited Jak2 tyrosine phosphorylation levels and suppressed acute lymphoblastic leukemia by inducing cellular apoptosis (Meydan et al., 1996). However, other studies suggested that it suffered from a general lack of specificity (Osherov et al., 1993; Oda et al., 1999; Gu et al., 2001). Ensuing work by multiple groups, including our own, has identified various small molecules or in some cases, a protein mimetic, that block Jak2 kinase function (Sandberg et al., 2005; Flowers et al., 2004; Duan et al, 2007, Pardanani et al., 2007). Despite the fact that these agents all inhibit Jak2 kinase function in some manner, differences in both the

structure as well the mechanism of action of the inhibitors is striking. For example, AG490 inhibits Jak2 and promotes cellular apoptosis (Meydan et al., 1996) whereas our Z3 compound inhibits Jak2 and promotes cell cycle arrest. Interestingly, the TG101209 compound described by Pardanani *et al*. inhibits Jak2 and causes both increased apoptosis and cell cycle arrest (Pardanani et al., 2007). Finally, work by Flowers and colleagues characterized a peptide inhibitor of Jak2 that mimics the Jak2 inhibitory protein, SOCS-1 (Flowers et al., 2004). The peptide mimetic was designed to recognize this autophosphorylation site of Jak2 similar to SOCS-1. They found that the peptide mimicked SOCS-1 in that it suppressed Jak2 tyrosine autophosphorylation and subsequent IFN-γ dependent signaling independent of marked changes in apoptosis or cell cycle progression. Collectively, these works suggest that the relationship between blocking Jak2 kinase function and the eventual fate of the cell is complex and warrants further examination.

Although the Jak2-V617F mutation on exon 14 is the predominant disease-associated allele in myeloproliferative disorders, several other Jak2 exon 14 mutations have been identified in Jak2-V617F-negative, myeloproliferative disorder patients. For example, unique C616Y and D620E substitution mutations have been identified in V617F-negative, myeloproliferative disorder individuals (Schnittger et al., 2006; Zhang et al., 2007). Additionally, a number of mutations have been identified in Jak2 exon 12, including an F537 deletion in one individual and F537 duplication in another (Scott et al., 2007). Furthermore, chromosomal translocations between the Jak2 allele and other alleles, including TEL, REL, PCM1, and BCR, have all been linked to a number of hematological malignancies (Peeters et al., 1997; Joos et al., 2003; Griesinger et al., 2005; Reiter et al., 2005). Although each specific translocation gives rise to a unique chimeric protein, they all share one common feature in that they all exhibit hyperkinetic

Jak2 kinase activity and subsequent malignant hyperplasia. Therefore, given the growing number of known Jak2 somatic cell mutations and chromosomal translocations, as well as the diverse human diseases that hyperkinetic Jak2 kinase activity has been associated with, identifying inhibitors that can block multiple Jak2 mutations, such as our Z3 compound, will be of great value.

In summary, our results suggest that Z3 specifically inhibits Jak2 tyrosine kinase function. It suppresses Jak2-dependent pathologic cell growth *in vitro* via a Jak2/STAT3-dependent mechanism that results in cell cycle arrest. Additionally, it blocks *ex vivo* hematopoietic progenitor cell growth from an essential thrombocythemia patient who harbors Jak2-V617F and a polycythemia vera patient who carries a novel Jak2-F537I mutation. As such, this compound may have practical applications in Jak2 related research.

Table 3-1. Top six scoring Z compounds.

Compound #	NSC #	Formula	Name	mol wt
Z1	302088	C8H6O5	5-hydroxybenzene-1,3-dicarboxylic acid	182
Z2	302311	C8H5FO4	5-fluorobenzene-1,3-dicarboxylic acid	184
Z3	42834	C23H24N2O	2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one	344
Z4	43744	C9H10O4	3,5-dimethoxybenzoic acid	182
Z5	70313	C8H9NO4S	4-methanesulfonamidobenzoic acid	215
Z6	113790	C19H24N2O3	3-hydroxy-4-[(4-methylpiperazin-1-yl)methyl]-7,8,9,10-tetrahydrobenzo[c]chromen-6-one	328

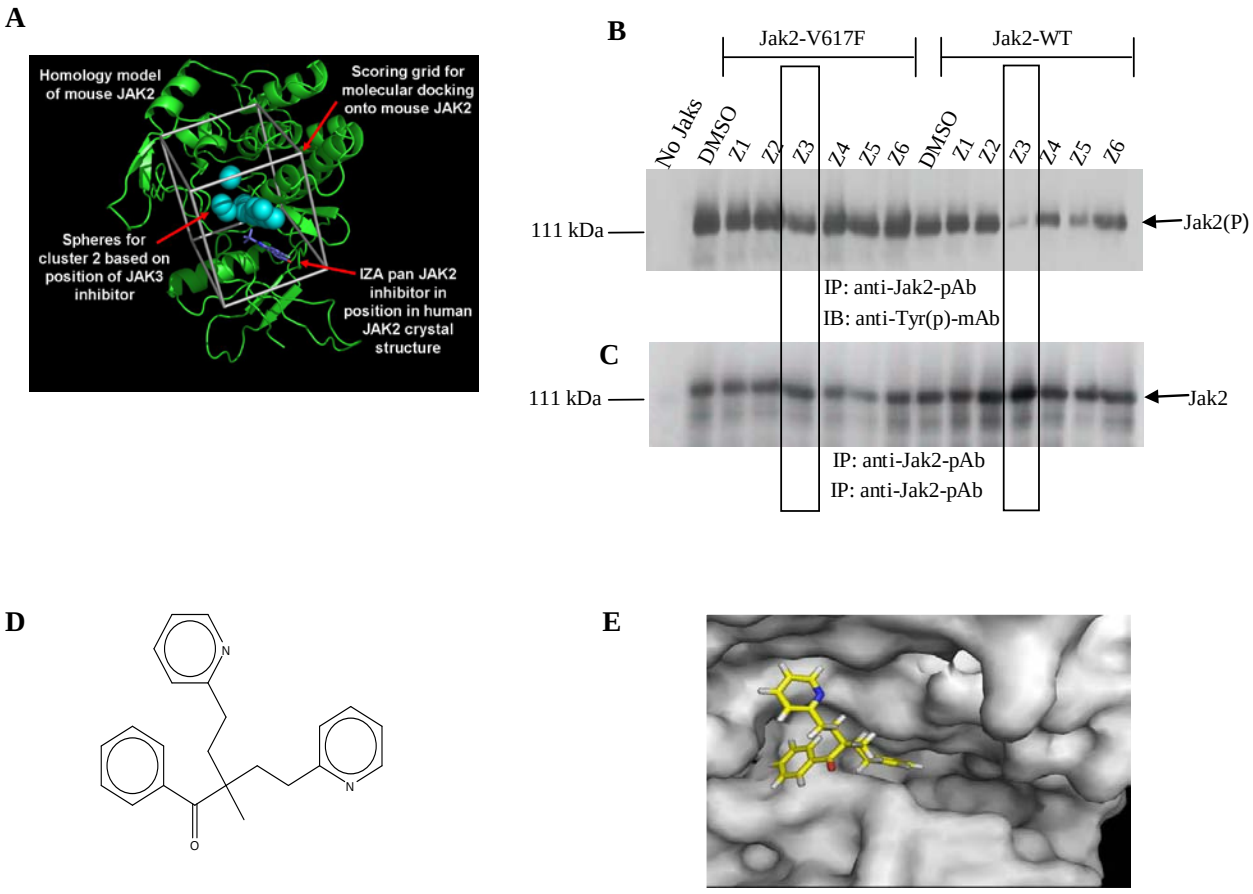

Figure 3-1. 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one (Z3) inhibits Jak2-V617F and Jak2-WT tyrosine autophosphorylation. A) The sphere set used for molecular docking was based on the position of a solvent accessible pocket adjacent to the Jak2 activation loop. For reference, the positions of the resolved structures for portions of both the Jak2 and Jak3 kinase domains are indicated. Also shown is the position of the IZA pan Jak2 inhibitor that was crystallized within the Jak2 kinase domain. B) BSC-40 cells were transfected with empty vector control, Jak2-V617F, or Jak2-WT expression plasmids and then infected with vaccinia virus to drive high-level expression and subsequent Jak2 tyrosine autophosphorylation. The six highest scoring compounds identified by DOCK were incubated with the cells at a concentration of 100 μM each, for 16 hours. Cell lysates were immunoprecipitated with anti-Jak2 antibody and immunoblotted with anti-phosphotyrosine antibody to detect Jak2 tyrosine phosphorylation levels. C) The membrane was stripped and reprobed with anti-Jak2 antibody to demonstrate equal Jak2 expression among all samples. Shown is one of two independent experiments. D) Shown is the molecular structure of the Z3 compound. E) Based on contact points and energy scores, Z3 docks into a structural pocket on the Jak2 kinase domain that is adjacent to the ATP binding site.

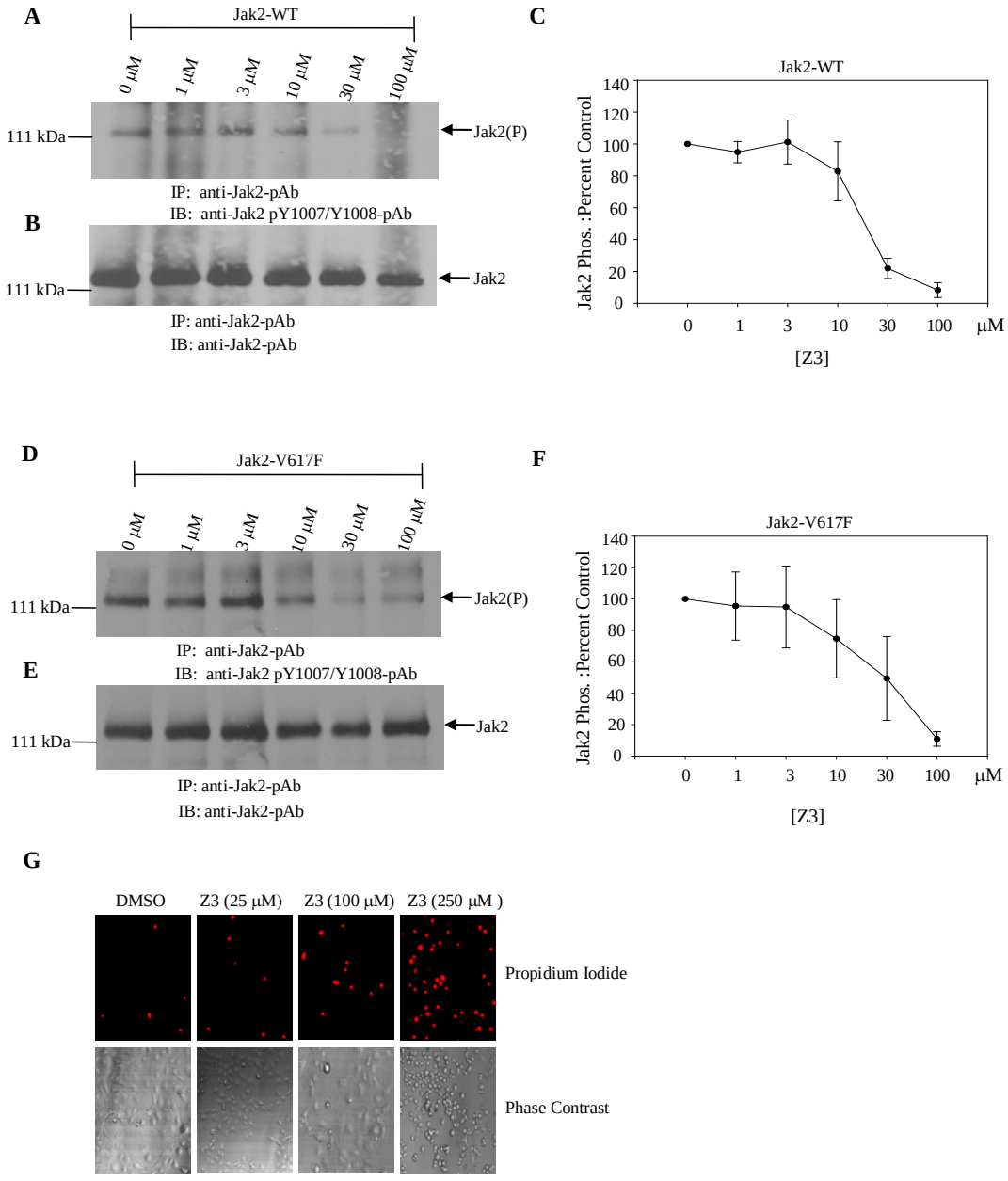

Figure 3-2. Z3 inhibits Jak2 tyrosine autophosphorylation in a dose-dependent manner. A) BSC-40 cells were transfected/infected as described above. Jak2-WT-expressing cells were incubated with either vehicle control (DMSO) or with Z3 at the indicated doses. Protein cell lysates were then immunoprecipitated with anti-Jak2 antibody and immunoblotted with a phospho-specific (pY1007/pY1008) Jak2 antibody to detect phosphorylated Jak2 at this specific residue. B) The membrane was stripped and reprobed with anti-Jak2 antibody to demonstrate equal Jak2 expression among all samples. C) Densitometrical analysis was performed on four representative Western blots to quantify Z3-mediated inhibition of WT-Jak2 phosphorylation at the tyrosine 1007 residue. Data are represented as the ratio of phosphorylated Jak2 to total Jak2

and presented as the mean +/- SEM. The IC_{50} of Z3 for Jak2-WT was approximately 15 μM. Statistical significance between the vehicle control and Z3 treated cells was determined by a one-way analysis of variance (ANOVA) ($p<0.001$). D) For Jak2-V617F expression, cells were first immunoprecipitated with an anti-Jak2 polyclonal antibody and then Western blotted with a phospho-specific (pY1007/pY1008) Jak2 antibody. E) The membrane was then stripped and reprobed with anti-Jak2 antibody. F) Densitometry analysis of four representative Western blots showing Z3-mediated inhibition of Jak2-V617F phosphorylation at the tyrosine 1007 residue. Data are represented as the ratio of phosphorylated Jak2 to total Jak2 and presented as the mean +/- SEM. The IC_{50} of Z3 for Jak2-V617F was approximately 28 μM. There was a statistically significant difference between the vehicle control and Z3 treated cells ($p<0.001$). G) BSC-40 cells were treated for 16 hours with either DMSO or with 25, 100, or 250 μM of Z3. Live cells were then stained with 1 μg/mL propidium iodide to determine whether Z3 was cytotoxic. The cells were visualized using confocal microscopy under fluorescent and phase contrast conditions. Shown is one of two representative results.

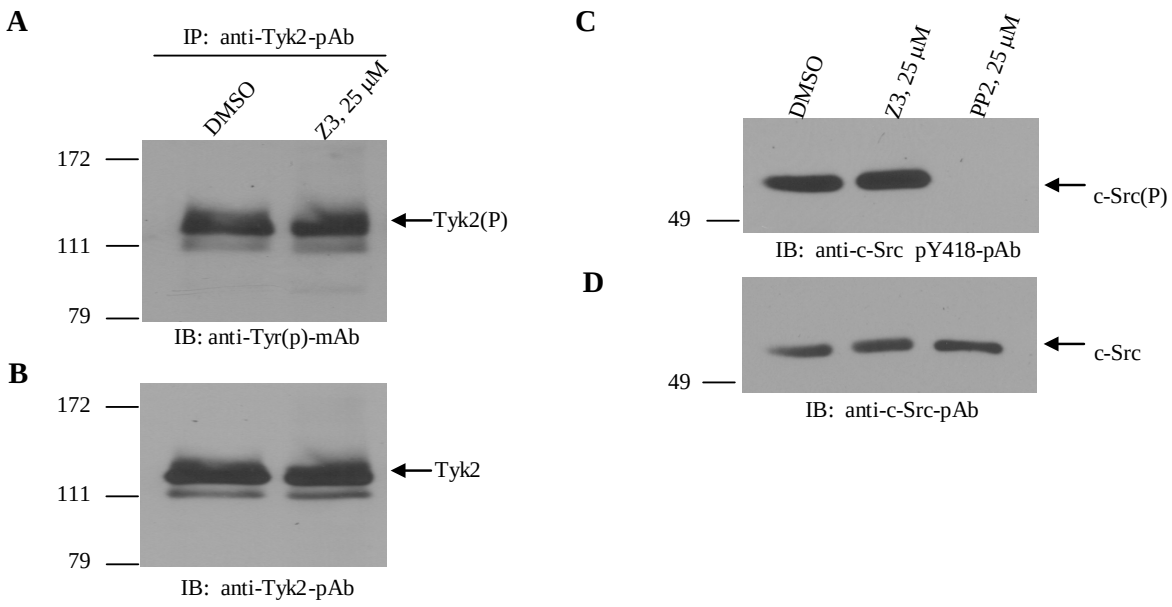

Figure 3-3. Z3 selectively inhibits Jak2 tyrosine kinase activity A) COS-7 cells were transiently transfected with an expression vector encoding wild type human Tyk2 cDNA. Cells were subsequently treated with either DMSO or 25 μM of Z3 for 16 hours. Protein lysates were immunoprecipitated with anti-Tyk2 antibody and then Western blotted with anti-phosphotyrosine antibodies to detect Tyk2 autophosphorylation. B) The membrane was stripped and reprobed with anti-Tyk2 antibody to show equal Tyk2 expression among all samples. C) Catalytically active c-Src protein was incubated in kinase reaction buffer containing either DMSO, 25 μM of Z3, or 25 μM of PP2. The samples were subsequently immunoblotted with anti-phospho c-Src (pY418) antibody to determine relative c-Src tyrosine kinase activity. D) The membrane was then stripped and reprobed with c-Src antibody to assess c-Src protein levels. Shown is one of three representative experiments for each.

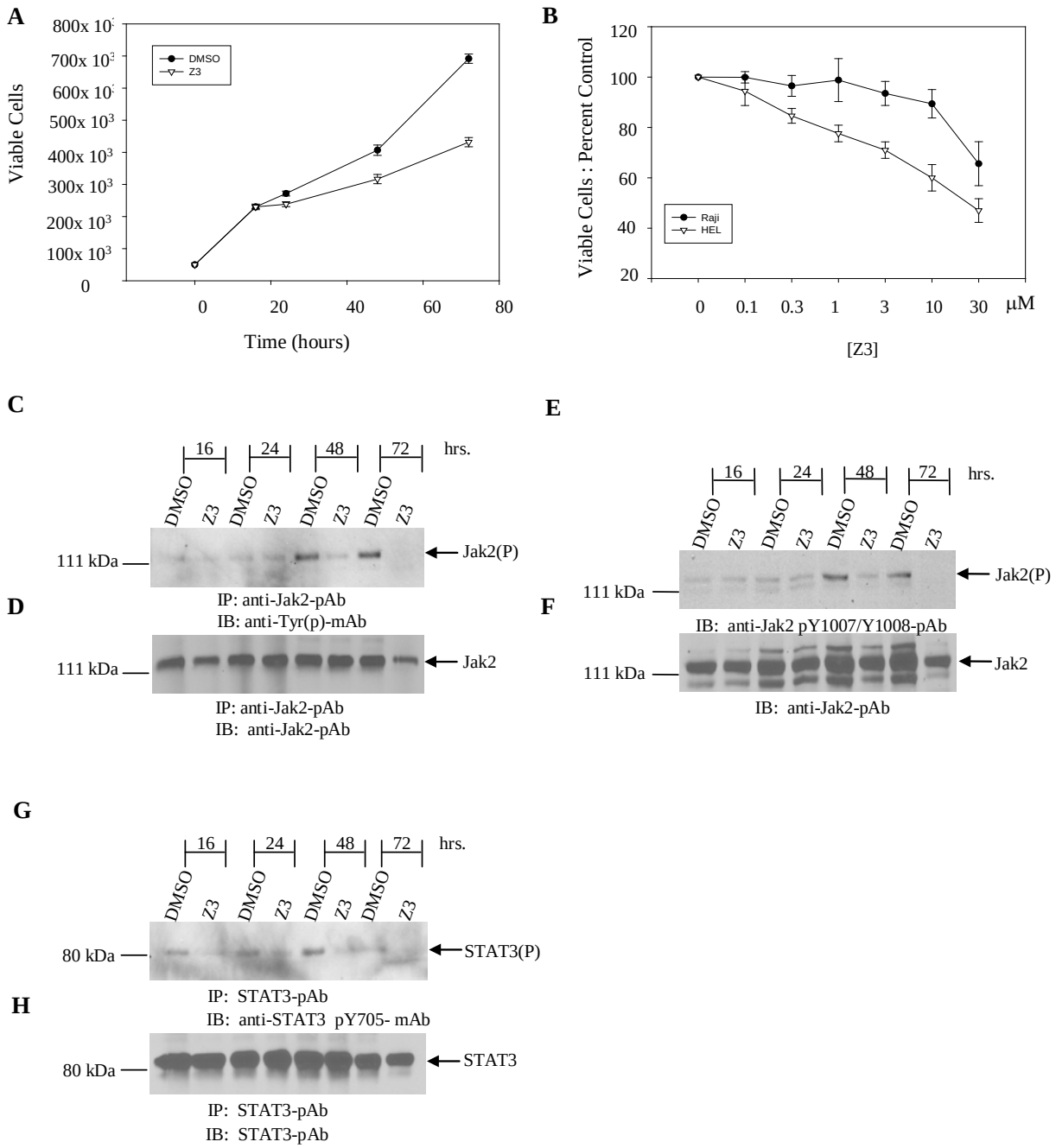

Figure 3-4. Z3 selectively inhibits Jak2-V617F-dependent cell proliferation and this correlates with suppression of Jak2 and STAT3 tyrosine phosphorylation. A) Approximatlely 50,000 HEL cells were treated with either DMSO or 25 μM of Z3 for 0, 16, 24, 48, or 72 hours. The numbers of viable DMSO or Z3 treated cells were assessed by trypan blue exclusion. All data points were measured in triplicate. Statistical significance between each group was analyzed using a two-way analysis of variance (ANOVA) by comparing the entire DMSO curve with the entire Z3 curve. The two conditions were found to be significantly different ($p=1.06 \times 10^{-14}$). B) Approximately 50,000 HEL and Raji cells were treated for 72 hours with either DMSO or with 0.1, 0.3, 1, 3, 10,

or 30 μM of Z3. After 72 hours, the numbers of viable DMSO or Z3 treated cells were assessed as described above. The graph shown is a compilation of three independent experiments. Statistical significance between each group was analyzed using a two-way analysis of variance (ANOVA) by comaring the entire HEL curve with the entire Raji Curve. The two conditions were found to be significantly different ($p=5.28 \times 10^{-11}$). C) Approximately 50,000 HEL cells were treated with either DMSO or 25 μM of Z3 for 16, 24, 48, or 72 hours. Protein lysates were immunoprecipitated with anti-Jak2 antibody and then immunblotted with anti-phosphotyrosine antibody to determine total Jak2 phosphorylation levels. D) The membrane was stripped and reprobed with Jak2 antibody to demonstrate Jak2 expression among all samples. E) Approximately 50,000 HEL cells were treated as indicated. Jak2 Y1007 phosphoryalation levels were determined by immunoblotting with anti-active Jak2 antibody. F) The membrane was stripped and reprobed with Jak2 to show equal Jak2 expression among all samples. G) Approximately 50,000 HEL cells were treated as indicated. STAT3 phosphorylation levels were determined by first immunoprecipitating with STAT3 antibody and then immunobloting with a phospho-specific (pY705) STAT3 antibody. H) The membrane was stripped and reprobed with STAT3 to show total STAT3 protein content among all samples. Shown is one of three (A, B, E, F) or two (C, D, G, H) representative experiments.

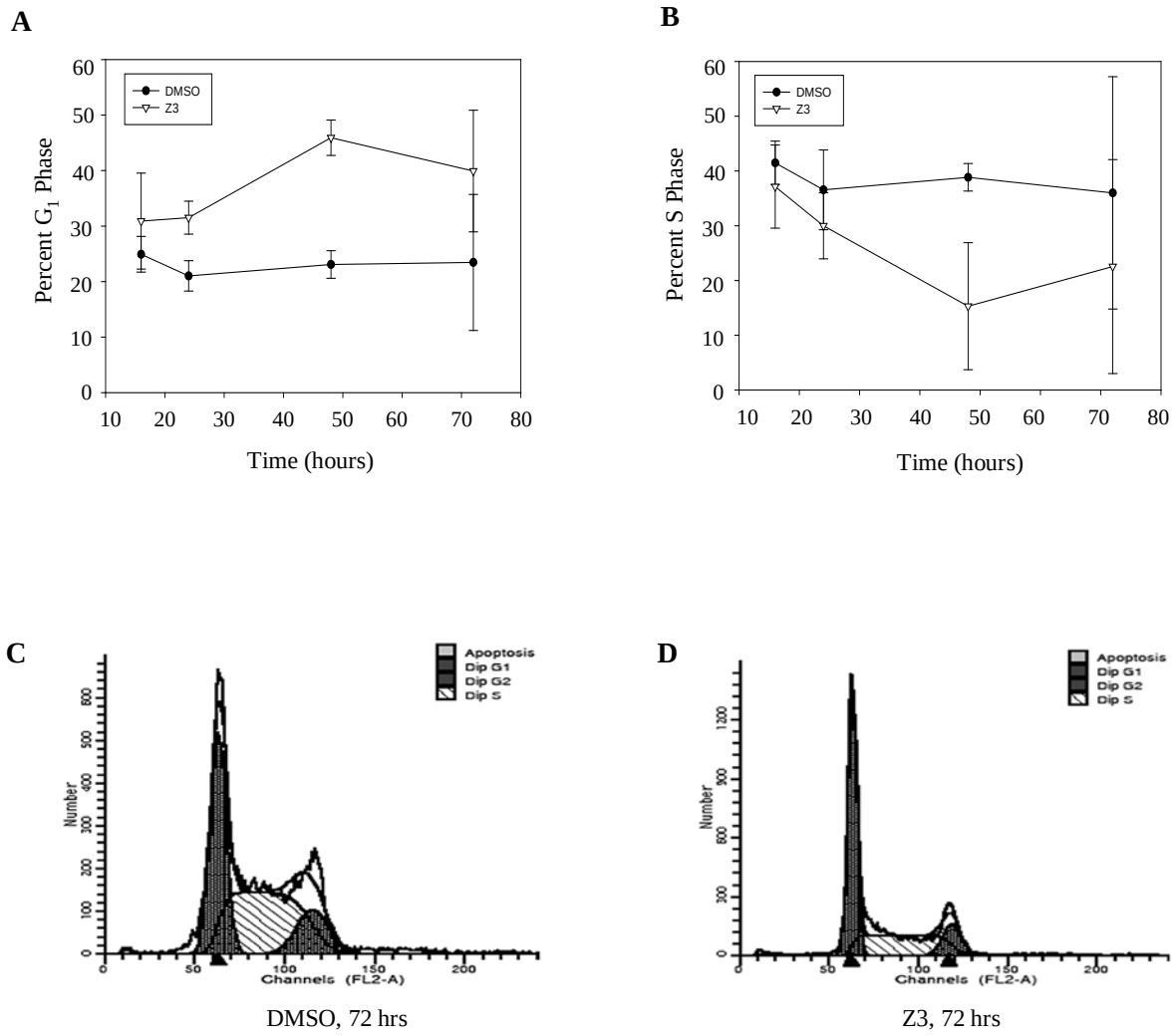

Figure 3-5. Z3 induces cell cycle arrest in Jak2-V617F-transformed human erythroleukemia cells. Aprroximately 50,000 HEL cells were treated with either DMSO or 25 μM of Z3 for 16, 24, 48, or 72 hours and cell-cycle effects were determined. FACSCalibur flow cytometer along with Modfit software (Verity Software) was used to analyze DNA contents. The mean and standard deviation of samples were determined. Statistical significance between each group was analyzed using a two-way analysis of variance (ANOVA) by comaring the entire DMSO curve with the entire Z3 curve. A) Percentage of HEL cells in G_1 phase following treatment with either DMSO or Z3 for the indicated times. The two conditions were considered to be significantly different ($p=0.000407$). B) Percentage of HEL cells in S phase following treatment with either DMSO or Z3 for the indicated times. The two conditions were considered to be significantly different ($p=0.0256$). Shown is one of three independent experiments (A and B). Representative cell cycle analysis profile from one of those three experiments after 72 hours treatment with either DMSO (C) or Z3 (D).

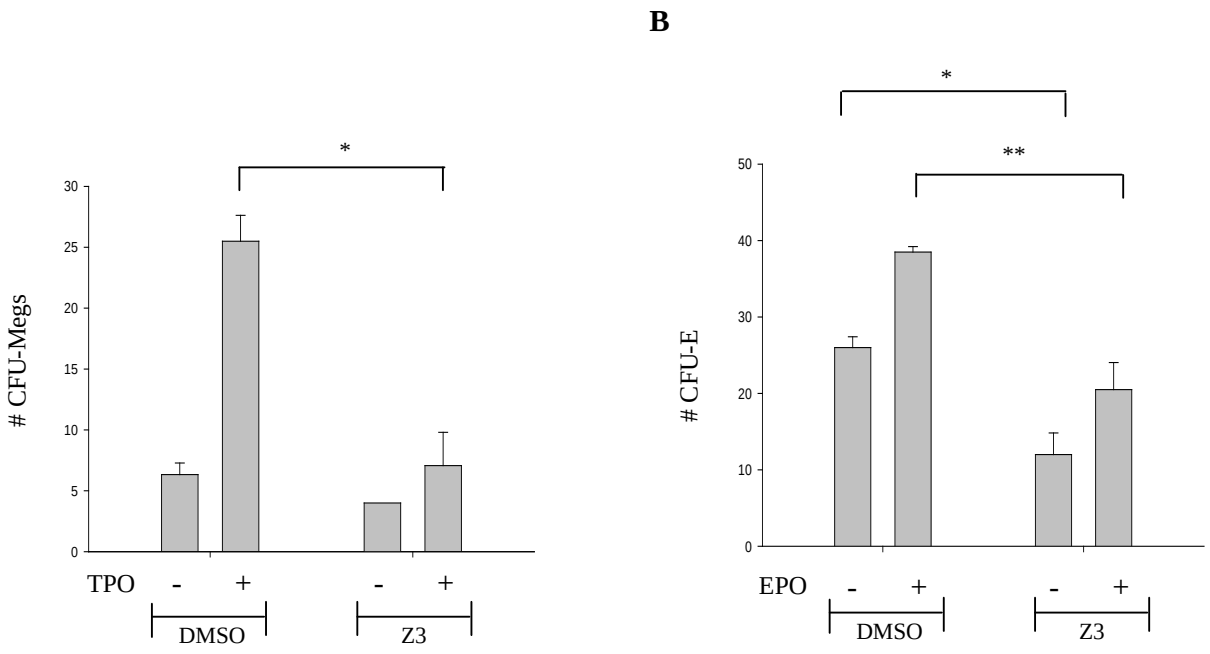

Figure 3-6. Z3 suppresses Jak2-mediated hematopoietic colony formation *ex vivo*. A) Marrow-derived mononuclear cells from an essential thrombocythemia patient who was Jak2-V617F positive were cultured in methylcellulose media containing either DMSO (0.25%, vol/vol) or 25 μM Z3. The number of megakaryocyte colonies was assessed 14 days later. Results are expressed as the average number of colonies from duplicate cultures per 4 x 10^5 cells. Statistical significance between each group was analyzed using Student's *t* test. *, p = 0.017. B) Marrow derived mononuclear cells from a polycythemia vera patient who was Jak2-F537I positive were cultured in methylcellulose media containing either DMSO (0.25%, vol/vol) or 25 μM Z3. The number of erythrocyte colonies was assessed 14 days later. Results are expressed as the average number of colonies from duplicate cultures per 4 x 10^5 cells. Statistical significance between each group was analyzed using Student's *t* test. , *p = 0.0245, ** p = 0.0195.

CHAPTER 4
CHARACTERIZATION OF A SERIES OF NOVEL JAK2 INHIBITORS AND THEIR AFFECT ON JAK2 MEDIATED-DISEASES

Summary

Jak2 tyrosine kinase is important in both physiology and pathophysiology as it plays significant roles in embryonic development, cell signaling, as well as heart disease, diabetes and cancer. In addition, a novel Jak2 gain-of-function, somatic mutation (Jak2-V617F) has been linked to several myeloproliferative disorders including polycythemia vera, essential thrombocythemia and primary myelofibrosis. Control of aberrant Jak2 kinase function through the use of a novel Jak2 selective small molecule inhibitor would therefore potentially serve as a useful research tool and/or therapeutic agent. Recent work from our lab has been aimed at identifying novel Jak2 tyrosine kinase inhibitor molecules. Here, we refined our previous Jak2 molecular model using information obtained from the crystal structures of the Jak2 and Jak3 kinase domains. A total of 223,481 compounds within the NIH small molecule database were then screened *in silico* using FlexX 1.13.2 in order to identify compounds that specifically bind and inhibit Jak2. In what is now our third generation of Jak2 inhibitors, analysis of the highest scoring compounds identified a set of structurally diverse molecules that potently inhibited Jak2 autophosphorylation not only at Tyr 1007, but at other tyrosine residues as well. These compounds significantly inhibited proliferation of the human erythroleukemia (HEL) cells, which express the Jak2-V617F mutation on both alleles. One compound in particular, herein designated as G6, was further examined in greater detail. The mechanism by which G6 inhibits Jak2-V617F dependent cell growth is via a marked increase in cellular apoptosis. We found that G6 does not inhibit c-Src or Tyk2 autophosphorylation at doses that completely inhibit Jak2, therefore suggesting a degree of specificity. In addition, G6 selectively suppressed Jak2-V617F-mediated pathologic cell growth. Finally, we found that the G6 and G13 compounds

significantly reduced *ex vivo* hematopoietic colony formation of cells derived from an ET patient harboring the Jak2-V617F mutation and a PV patient carrying a Jak2-F537I mutation. Collectively, our data demonstrate that these small molecule compounds inhibit Jak2 function *in vitro* and *ex vivo*. As such, they may have therapeutic value in treating diseases that are caused by aberrant Jak2 kinase function.

Introduction

Myeloproliferative neoplasms which include polycythemia vera, essential thrombocythemia and primary myelofibrosis entered the spotlight in 2005 when a somatic acquired Jak2-V617F mutation was described in these disorders. To date, the Jak2-V617F mutation is harbored by virtually all polycythemia vera patients and by more than 50% of essential thrombocythemia and primary myelofibrosis patients (James et al., 2005; Baxter et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Zhao et al., 2005). Subsequent reports have identified Jak2 exon mutations in polycythemia vera patients who do not carry the Jak2-V617F mutation (Scott et al, 2007, Pardanani et al., 2007a, Sayyah et al., 2008). Specifically, several deletions and insertions were noted in the case of exon 12 mutations (Scott et al., 2007; Butcher et al., 2008). A common feature between the exon 12 mutations and the V617F mutation on exon 14 is that they both result in constitutive Jak2 tyrosine kinase activity.

The driving force for the identification of Jak2 inhibitors has been the discovery of the Jak2-V617F mutation in myeloproliferative disorders. However, the continued observation of novel Jak2 somatic cell mutations and chromosomal translocations in hematologic disorders also validates the search for specific inhibitors that target aberrant Jak2 activity (Schnittger et al., 2006; Scott et al., 2007; Zhang et al, 2007; Kearney et al., 2008; Nebral et al., 2009). In fact, several Jak2 inhibitors have been developed. One such molecule, TG101209, was able to inhibit phosphorylation of Jak2-V617F, STAT5 and STAT3 in a Jak2-V617F-expressing acute myeloid

leukemia cell line (Pardanani et al., 2007). In addition, TG101209 suppressed the proliferation of both Jak2-V617F- and TpoR W515L/K-expressing hematopoietic cells (Pardanani et al., 2007a). Another Jak2-specific inhibitor, TG101348, was effective in significantly reducing hematocrit levels in a Jak2-V617F-induced myeloproliferative disorder mouse model and inhibiting the growth of primary hematopoietic cells derived from myeloproliferative disorder patients with Jak2-V617F, MPLW515K and Jak2 exon 12 mutations (Lasho et al., 2008; Wernig et al, 2008). Moreover, this compound inhibited the engraftment of Jak2-V617F-positive hematopoietic stem cells and myeloid progenitors in a bioluminescent xenogeneic mouse transplantation model (Geron et al., 2008). Importantly, TG101348 reduced GATA1 phosphorylation, as phosphorylation of this transcription factor can be associated with erythroid-skewing of Jak2-V617F-positive progenitor differentiation. All together, characterization of the TG compounds reveal that not only do they potently inhibit aberrant Jak2 activity, but they also suppress pathologic cell growth due mutation in the thrombopoietin receptor.

The most extensive clinical trial of a Jak2 inhibitor for the treatment of myeloproliferative disorders has been with ICNB018424. ICNB018424 inhibits Jak1 and Jak2, but not Jak3 or TYK2 at clinically achievable concentrations (Verstovek et al., 2008a). Primary myelofibrosis patients treated with this compound displayed reduced splenomegaly and showed marked improvements in disease-associated symptoms. To date, there have only been modest reductions in Jak2-V617F allele burden. Collectively, a growing number of Jak2 inhibitors are being identified that selectively target aberrant Jak2 tyrosine kinase activity *in vitro, ex vivo* and *in vivo*. In addition, some of these inhibitors have already entered or are expected to go into clinical trials for the treatment of myeloproliferative disorders.

Our laboratory has contributed to the identification of novel Jak2-selective inhibitors that could one day have therapeutic value for the treatment of myeloproliferative disorders or other Jak2-related hematologic malignancies. By using a structure based approach of combining molecular docking with cell-based functional testing, we have identified a series of novel Jak2 inhibitors that potently inhibit Jak2 tyrosine kinase function. In particular, one of these compounds, designated as G6, potently and selectively inhibits Jak2-mediated aberrant cell growth by causing an increase in cellular apoptosis. Furthermore, G6 blunts the growth of hematopoietic progenitor cells derived from myeloproliferative disorder patients displaying Jak2 mutations. All together, our results suggest that G6 is a novel, selective and potent inhibitor of Jak2 tyrosine kinase activity.

Results

G6 Potently and Selectively Suppresses Jak2-V617F-Dependent Aberrant Cell Growth

An aim of our laboratory in recent years has been directed towards the identification of novel Jak2 inhibitors (Sandberg et al. 2005; Sayyah et al., 2008). Here, we refined our earlier Jak2 molecular model using information from the crystal structures of the Jak2 and Jak3 kinase domain. We subsequently screened *in silico* a total of 223, 281 compounds of known structure using FlexX in order to identify compounds that specifically bind to Jak2. The top scoring G compounds were then examined for their ability to inhibit proliferation of a pathologically relevant cell line which expresses the Jak2-V617F mutation. For this, we employed HEL cells which are human erythroleukemia cells that require the constitutively activated Jak2-V617F mutation for their transformation and proliferation (Quentmeir et al., 2006; Walz et al., 2006). To ascertain if the G compounds could block Jak2-V617F-dependent cell proliferation in this cell line, 5×10^4 HEL cells were treated with either DMSO or 25 μM of each of the G compounds for 0, 16, 24, 48, and 72 hours. Our previous work has established that Z3

suppresses HEL cell proliferation (Sayyah et al., 2008). To compare the HEL cell growth inhibitory potential of G6 with that of Z3, HEL cells were also treated with 25 μM Z3 for the indicated times. At the end of each time point, the number of viable DMSO, Z3, or G compound-treated cells was determined by trypan blue exclusion via the use of a hemacytometer (Figure 4-1A). Relative to DMSO control, the G13, G11 and Z3 compounds moderately reduced HEL cell growth by approximately 28%, 30%, and 42%, respectively. However, the G6 compound was more successful in inhibiting cell growth in comparison. By 72 hours, G6 inhibited HEL cell proliferation by over 90% relative to DMSO control. ANOVA indicated that the G6, G13, G11 and Z3 growth curves were statistically significant from that of DMSO.

Although G6 exhibited potent anti-cell growth ability, it was important to determine whether this compound was selectively suppressing Jak2-V617F-dependent cell growth or inhibiting cell proliferation in a non-specific manner. To answer this question, we examined the growth inhibitory effect of G6 on several cell lines such as, Raji, CMK and BSC-40 that proliferate independent of Jak2. The mechanism responsible for the abnormal growth of Raji cells is a translocation event involving the c-myc gene while the abnormal growth of CMK cells is a result of an activating A572V mutation in the Jak3 pseudokinase domain (Hamlyn and Rabbitts, 1983; Walters et al, 2006). BSC-40 cells are monkey kidney epithelial cells transformed with SV40 T antigen. Here, 5×10^4 HEL, Raji, CMK, or BSC-40 cells were treated with G6 at the indicated concentrations. The number of viable cells was subsequently determined (Figure 4-1B). The results reveal that HEL cells were more susceptible to the growth suppressive effects of G6, over a wide range of doses, when compared with Raji, CMK, or BSC-40 cells. This indicates that G6 inhibits Jak2-V617F dependent cell growth in a relatively

specific manner. All together, the results in Figure 4-1 show that G6 is a potent and selective inhibitor of Jak2-V617F-dependent pathologic cell growth.

G6 Suppresses HEL Cell Proliferation by Inducing Cellular Apoptosis

We then wanted to uncover the mechanism by which G6 blocks Jak2-V617F-dependent HEL cell growth by examining whether treatment with G6 corresponds to an increase in apoptosis in HEL cells. Specifically, HEL cells were treated with either DMSO or 25 μM G6 for 16, 24, or 48 hours and cells were analyzed by flow cytometry for Annexin V staining (Figure 4-2). The simultaneous application of propidium iodide allowed for the distinction between necrotic and apoptotic cells since propidium iodide will stain the DNA of only leaky necrotic cells. A representative experiment at 48 hours treatment with G6 showed an increase in the percentage of Annexin V-stained HEL cells (Figure 4-2A) relative to DMSO control (Figure 4-2B), indicating that G6 induced apoptosis in this cell type. The percent of DMSO- or G6-treated cells were then plotted as a function of time (Figure 4-2C). The results show that G6 increased the percentage of HEL cells undergoing apoptosis over time. By 48 hours treatment cells with G6, there was approximately a 45% increase in cellular apoptosis. Collectively, the results in Figure 4-2 show that the mechanism by which G6 reduces Jak2-V617F-dependent pathologic cell growth is by promoting apoptosis.

G6 is a Specific Inhibitor of Jak2 Tyrosine Kinase Activity

While our results showed that G6 selectively inhibits Jak2-V617F-dependent HEL cell growth, another way to establish the specificity of G6 for Jak2 would be to examine the effect of this compound on the phosphorylation of other Jak family tyrosine kinases, such as Tyk2. To achieve this goal, we employed an autophosphorylation assay to allow for strong Jak family kinase activation independent of ligand treatment, as we had done previously (Ma and Sayeski, 2004; Sayyah et al., 2008). Here, COS-7 cells were transiently transfected with an expression

vector encoding the wild-type human Tyk2 cDNA. The cells were subsequently treated with either DMSO, 100 μM Z3, or 100 μM G6 for 16 hours. The cells were then lysed and Tyk2 was immunoprecipitated from the lysates through the use of an anti-Tyk2 antibody. The immunoprecipitated protein was then immunoblotted with an anti-phosphotyrosine antibody to assess the level of Tyk2 tyrosine kinase autophosphorylation (catalytic activity). The results show that G6, similar to Z3, had little to no effect on inhibiting Tyk2 tyrosine kinase autophosphorylation when compared to DMSO control (Figure 4-3A). The membrane was then stripped and reprobed with an anti-Tyk2 antibody to demonstrate equal Tyk2 expression among samples (Figure 4-3B)

To further confirm the specificity of G6, we investigated the effect of this compound on c-Src tyrosine kinase activity. Here, we incubated catalytically active recombinant c-Src protein either in the presence of DMSO, 25 μM Z3, 25 μM G6, or 25 μM of the Src kinase inhibitor, PP2 and incubated the reactions for 20 minutes at room temperature. The reactions were then terminated by the addition of SDS-containing sample buffer, separated by SDS-PAGE and subsequently Western blotted with an anti-active c-Src (pY418) antibody to assess c-Src tyrosine kinase activity (Figure 4-3C). We observed that G6, similar to Z3, had no significant effect on c-Src catalytic activity when compared to DMSO control, but in the presence of PP2, c-Src tyrosine kinase activity was completely eliminated. Equal c-Src protein content among all samples was verified by stripping and reprobing the membrane with anti-c-Src antibody (Figure 4-3D). All together, the results confirm that G6 is a relatively specific inhibitor of Jak2 tyrosine kinase activity as demonstrated by the fact that this compound has little to no activity against Tyk2 or c-Src.

The G Compounds Effectively Block Jak2-Mediated Hematopoietic Colony Formation, *Ex Vivo*

Our previous report shows that Z3 suppresses the *ex vivo* growth of Jak2-mediated hematopoietic colonies (Sayyah et al., 2008). We have already demonstrated that G6 potently and specifically inhibits Jak2-dependent pathologic cell growth *in vitro*. We next wanted to determine if G6 could block the *ex vivo* growth of hematopoietic progenitor cells isolated from the bone marrow of confirmed myeloprolilferative disorder patients. We also wanted to compare the *ex vivo* cell growth inhibitory potential of G6 with that of Z3. Specifically, residual bone marrow aspirates were obtained from an essential thrombocythemia patient who was Jak2-V617F positive (Figure 4-4A) or from a polycythemia vera patient who displayed a Jak2-F537I exon 12 mutation (Figure 4-4B). The hematopoietic progenitor cells isolated from these patients were subsequently cultured in a semisolid growth medium in the presence or absence of the appropriate cytokine and in the presence of DMSO, 25μM Z3 or 25 μM G6. As expected, treatment of the essential thrombocythemia patient's hematopoietic progenitor cells with thrombopoietin dramatically increased megakaryocyte colony formation (Figure 4-4A). However, G13 and Z3 significantly reduced this thrombopoietin-dependent increase in cell growth. In addition, G6 completely blocked cytokine-independent megakaryocyte colony formation (Figure 4-4A). In a similar manner, erythropoietin treatment of progenitor cells obtained from the polycythemia vera patient considerably increased erythrocyte cell growth, but G6 and Z3 significantly suppressed cytokine-dependent as well as -independent erythrocyte colony formation (Figure 4-4B). However, the ability of G6 to block cytokine dependent erythrocyte colony formation was greater relative to Z3 ($p=0.049$). All together, the results in Figure 4-4 show that the G-compounds significantly reduce Jak2-mediated hematopoietic cell

growth *ex vivo*, with G6 blunting Jak2-F537I-mediated, human pathologic cell growth more effectively.

Discussion

Myeloproliferative disorders, which include polycythemia vera, essential thrombocythemia, and primary myelofibrosis are disorders of hematopoietic stem cells, where myeloid progenitors become hypersensitive and/or independent of cytokines for survival and proliferation (Dameshek, 1951). The discovery of the Jak2-V617F mutation in almost all individuals with polycythemia vera and about 50% of patients with essential thrombocythemia and primary myelofibrosis suggested that this Jak2 pseudokinase domain mutation plays a crucial role in these disorders. Confirmation on the role of Jak2-V617F in the pathogenesis of myeloproliferative disorders came from animal models. In particular, retroviral transduction of Jak2-V617F in murine hematopoietic stem cells followed by transplantation into lethally irradiated mice resulted in the development of polycythemia vera phenotype (Bumm et al., 2006; Lacout et al., 2006; Wernig et al, 2006). Later on, several Jak2 exon 12 mutations were detected in Jak2-V617F-negative polycythemia vera patients, which also led to constitutive activation of Jak-STAT signaling pathway.

In order to identify therapeutically effective compounds that target aberrant Jak2 kinase function, we revised our previous Jak2 model yet again using information obtained from the crystal structures of the Jak3 and Jak2 kinase domain. In addition, we screened a significantly larger number of compounds *in silico*, 223,481 to be exact, with the intention of identifying small molecules that specifically bind and inhibit Jak2 more potently than Z3. In what is now our third generation of Jak2 inhibitors, our studies showed that a set of structurally diverse molecules potently suppressed Jak2 autophosphorylation not only at tyrosine 1007, but at other tyrosine residues as well. These compounds also significantly inhibited the growth of human

erythroleukemia cells that express the Jak2-V617F mutation on both alleles. One particular compound, G6, was further characterized since it was more effective in inhibiting Jak2-V617F-dependent cell proliferation when compared to the other G compounds. We found that G6 selectively blocked Jak2-V617F pathologic cell growth by causing an increase in cellular apoptosis. In addition, G6 had no effect on Tyk2 or c-Src tyrosine kinase activity at concentrations that completely inhibited Jak2, thus confirming the relative specificity of this compound. Finally, we demonstrated that the G6 and G13 compounds significantly reduced the growth of hematopoietic progenitor cells isolated from the bone marrow of an essential thrombocythemia patient harboring the Jak2-V617F mutation and a polycythemia vera patient carrying a Jak2-F537I exon 12 mutation.

Upon analysis, we found that there is a difference between the G compounds and Z3 in terms of their efficacy. First, G6 reduced Jak2-V617F-dependent HEL cell growth approximately 43% more than Z3. Another direct comparison between the efficacy of the G compounds and Z3 is demonstrated with our *ex vivo* data. Here, we showed that G6 suppressed hematopoietic colony formation of cells derived from a polycythemia vera patient carrying a novel Jak2-F537I mutation more effectively than Z3 ($p=0.049$). Collectively, our results show that the G6 is more effective than Z3 in suppressing Jak2-dependent pathologic cell growth *in vitro* and *ex vivo*.

In summary, our laboratory has invested effort toward the identification and characterization of novel Jak2-specific inhibitors. Our ability to identify novel Jak2 inhibitors has evolved over the years. Specifically, with every generation of Jak2 inhibitor identified by our lab, our Jak2 homology model has become a more accurate structural representation of the Jak2 kinase domain to be utilized for high throughput molecular docking. In addition, due to an

increase in computational ability, we have screened more compounds for their ability to bind and inhibit Jak2. As a result, with each succeeding generation of Jak2 inhibitor indentified, we have observed an increase in Jak2-mediated cell growth inhibition. In what is now our third generation Jak2 inhibitor, we have demonstrated that G6 is an effective inhibitor of Jak2-dependent pathologic cell growth *in vitro* and *ex vivo*. As such, we believe that G6 could be considered as a lead therapeutic agent.

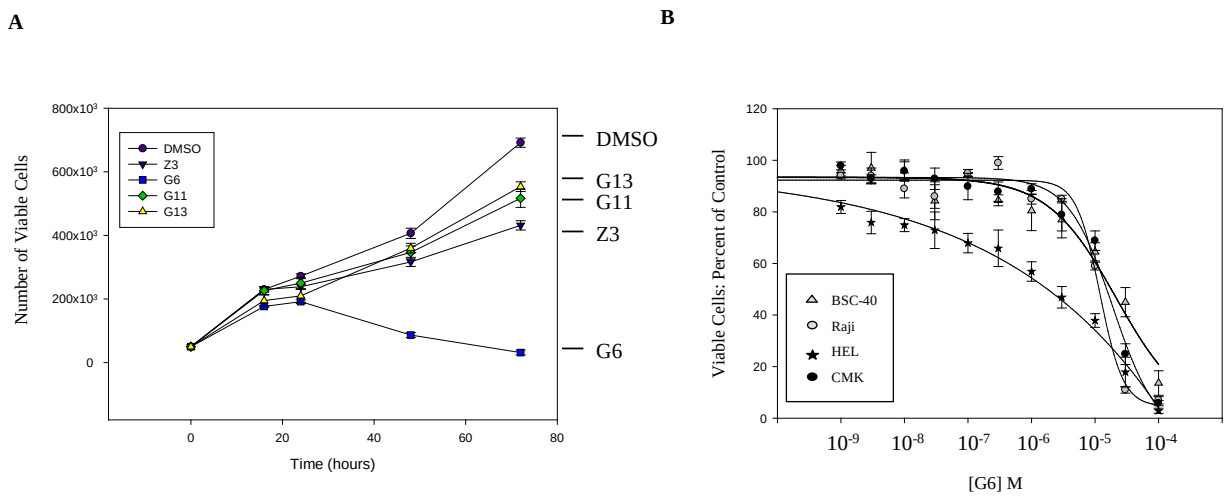

Figure 4-1. G6 potently inhibits Jak2-V617F-dependent cell growth. A) Approximately 50,000 HEL cells were treated with either DMSO or 25 μM of the G compounds for 0, 16, 24, 48, and 72 hours. At the end of each time point, the number of viable DMSO or G compound treated cells was determined by trypan blue exclusion. All data points were measured in triplicate. Statistical significance between each group was analyzed using a two-way statistical analysis of variance (ANOVA). Values of $p=0.025$ (G13), $p=7.52 \times 10^{-9}$ (G11), $p=2.98 \times 10^{-25}$ (G6) were considered to be significant when compared to DMSO control. B) Approximately 50,000 HEL, Raji, CMK, and BSC-40 cells were treated with either DMSO or with G6 at the indicated concentrations. After 48 hours, the numbers of viable cells were assessed as described above. The graph shown is a compilation of three independent experiments. Statistical significance between each group was determined using a two-way ANOVA. Values of $p=2.09 \times 10^{-25}$ (Raji), $p=1.95 \times 10^{-25}$ (CMK), $p=6.4 \times 10^{-23}$ (BSC-40) were regarded as significant relative to DMSO control.

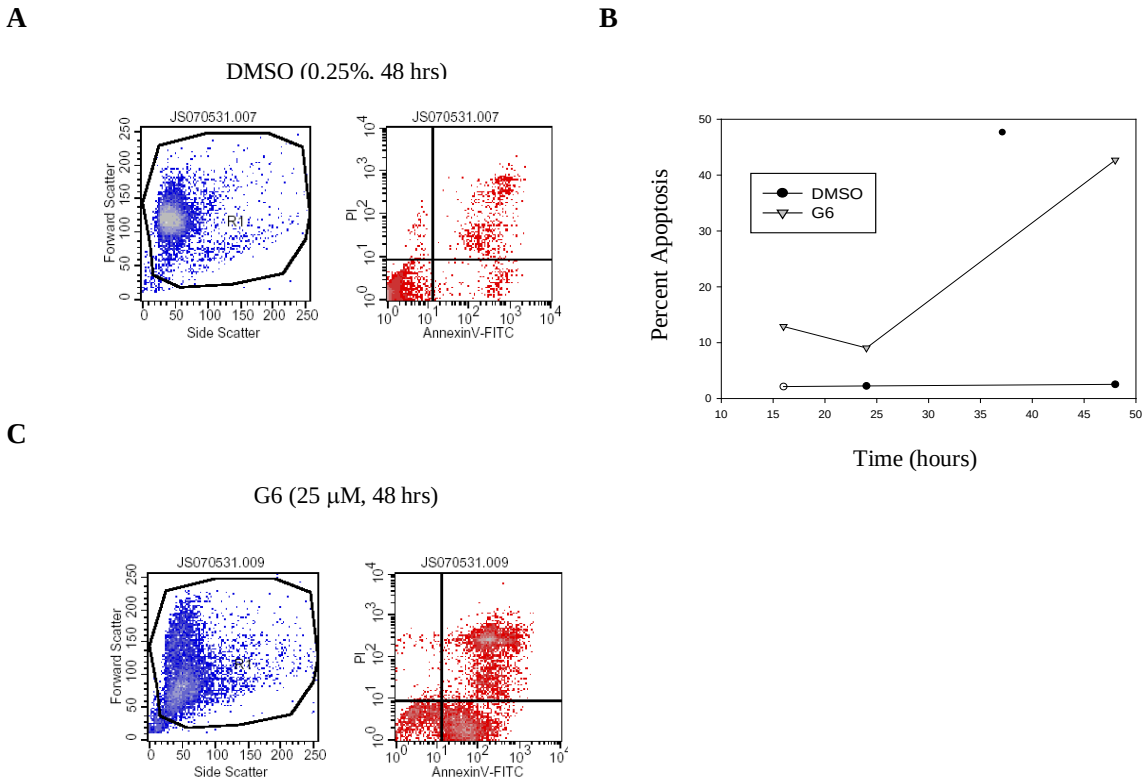

Figure 4-2. G6 reduces HEL cell numbers by increasing cellular apoptosis. Approximately 50,000 HEL cells were treated with either DMSO or 25 μM G6 for 16, 24 or 48 hours and cells were measured for Annexin V staining via flow cytometry. A representative experiment at 48 hours shows the percentage of Annexin V- and propidium iodide-stained cells treated with either 0.25% DMSO (A) or 25 μM G6 B). C). The percent of DMSO or G6-treated cells undergoing apoptosis is plotted as a function of time. Shown is one of two representative experiments.

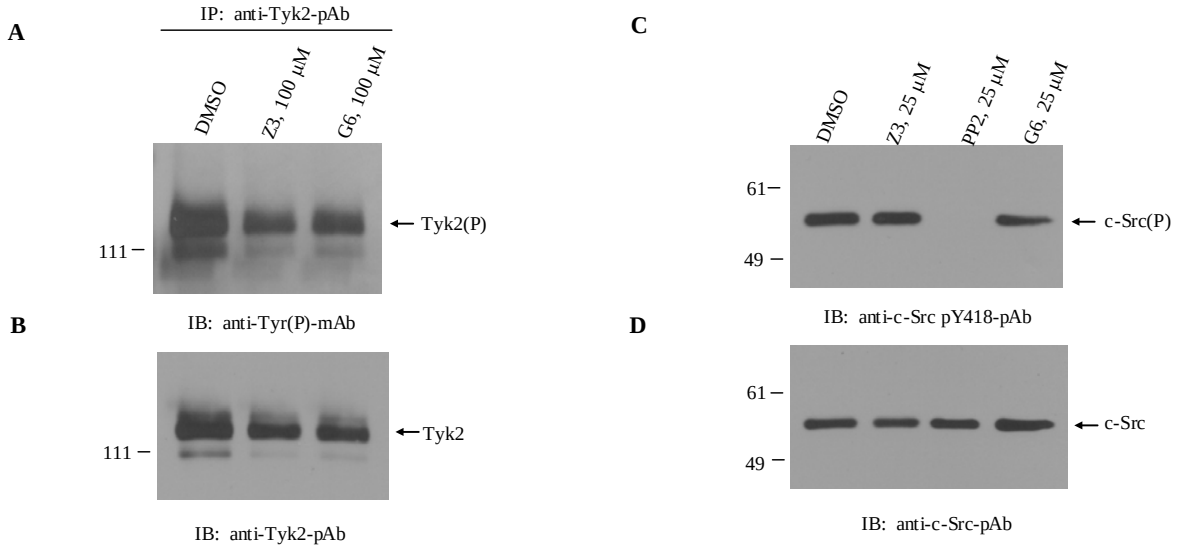

Figure 4-3. G6 has no effect on Tyk2 or c-Src tyrosine kinase activity. A) COS-7 cells were transiently transfected with an expression vector encoding wild-type human Tyk2 cDNA. Cells were subsequently treated with either DMSO, 100 μM Z3 or 100 μM G6 for 16 hours. Protein lysates were immunoprecipitated with anti-Tyk2 antibody and then Western blotted with anti-phosphotyrosine antibodies to detect Tyk2 autophosphorylation. B) The membrane was stripped and reprobed with anti-Tyk2 antibody to demonstrate equal Tyk2 expression among samples. C) A catalytically active recombinant c-Src protein was incubated in kinase buffer containing either DMSO, 25 μM Z3, 25 μM PP2 or 25 μM G6. The samples were subsequently immunoblotted with anti-phospho c-Src (pY418) antibody to assess relative c-Src tyrosine kinase activity. D) The membrane was then stripped and reprobed with c-Src antibody to determine total c-Src protein levels. Shown is one of three representative experiments for each.

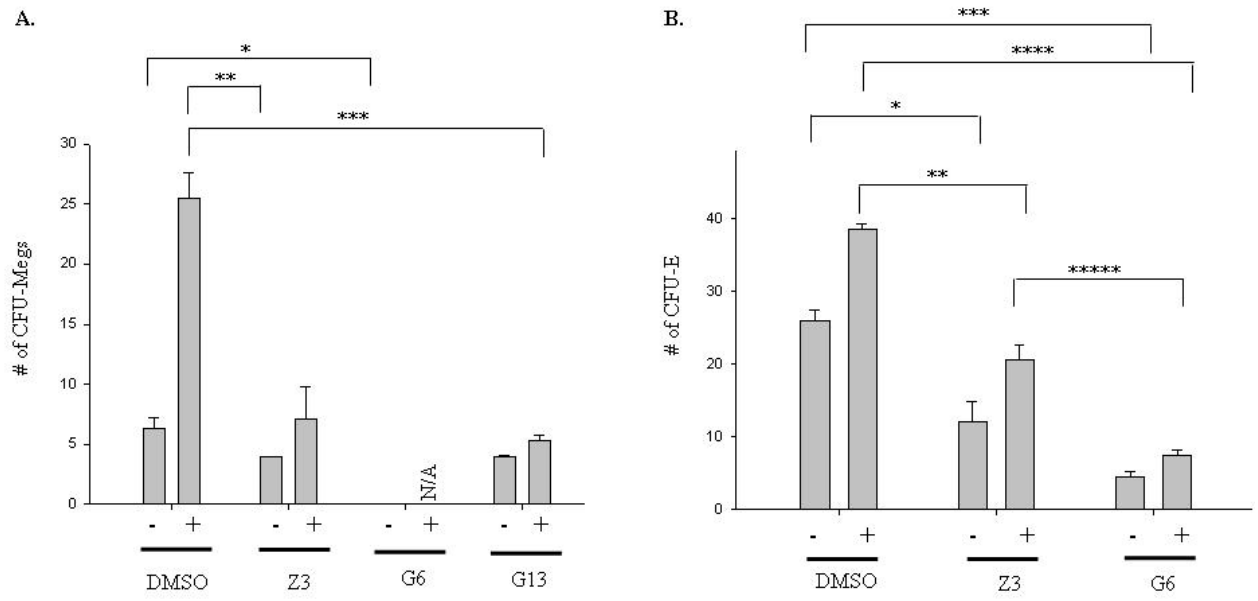

Figure 4-4. The G compounds reduce hematopoietic colony formation *ex vivo*. A) Marrow derived mononuclear cells from an essential thrombocythemia patient who was Jak2-V617F positive were cultured in methylcellulose media containing DMSO (0.25%, vol/vol), 25 μM Z3, 25 μM G6 or 25 μM G13. The number of megakaryocyte colonies was assessed 14 days later. Results are expressed as the average number of colonies of duplicate cultures per 4 x 10^5 cells. Statistical significance between each group was analyzed using Student's *t* test, *p=0.011, **p=0.017, ***p=0.005. B) Marrow derived mononuclear cells from a polycythemia vera patient was who was Jak2-F537I positive were cultured in methylcellulose media containing either DMSO (0.25%, vol/vol), 25 μM Z3 or 25 μM G6. Results are expressed as the average number of colonies from duplicate cultures per 4 x 10^5 cells. Statistical significance between each group was determined using Student's *t* test, *p=0.01, **p=0.0195, ***p=0.01, ****p=0.002, *****p=0.049.

CHAPTER 5
TYROSINE 372 IS CRITICAL FOR JAK2 FUNCTION

Summary

Jak2 is a non receptor tyrosine kinase whose key function is to transduce gene transcription signals from the cell surface to the nucleus through a tyrosine phosphorylation signaling mechanism. Jak2 consists of 49 tyrosine residues and already a handful of these are known to be phosphorylated and play important roles in regulating Jak2 tyrosine kinase activity. Here, we demonstrated by electrospray mass spectrometry that tyrosines 372 and 373 are novel sites of Jak2 phosphorylation. We introduced tyrosine to phenylalanine point mutations at positions 372 and 373 in plasmids encoding wild-type Jak2 protein and expressed these plasmids in cells to determine the effect of loss of tyrosines 372 and 373 phosphorylation on Jak2 tyrosine kinase function. Using a Jak2 autophosphorylation assay, we found that loss of tyrosine 372 phosphorylation not only inhibited Jak2 tyrosine 1007 phosphorylation, but also suppressed phosphorylation of other Jak2 tyrosine residues, indicating that tyrosine 372 is important for Jak2 catalytic activity in the context of a ligand-independent signaling system. Conversion of tyrosine 373 to phenylalanine on the other hand, resulted in more modest reductions in total Jak2 phosphorylation levels and phosphorylation of tyrosine 1007. With particular focus on the more significant tyrosine 372, we also found that loss of phosphorylation at tyrosine 372 suppressed the ability of Jak2 to phosphorylate STAT1 via a mechanism involving reduced Jak2 and STAT1 co-association. Finally, we found that loss of tyrosine 372 phosphorylation blocked interferon-gamma and epidermal growth factor-dependent Jak2 activation relative to wild type Jak2, but had no effect on hydrogen peroxide-mediated Jak2 activation. All together, our results demonstrate that tyrosine 372 phosphorylation plays an important role in Jak2 tyrosine

autophosphorylation. In addition, tyrosine 372 has a significant and differential role in Jak2 activation in response to ligand.

Introduction

Jak2 is a nonreceptor tyrosine kinase belonging to the *Janus* family of tyrosine kinases that also includes Jak1, Jak3 and Tyk2. A key cellular role of Jak2 is to phosphorylate and hence activate members of the Signal Transducers and Activators of Transcription (STAT) family of latent cytoplasmic transcription factors. Once activated, the dimerized STAT proteins translocate to the nucleus, bind DNA promoter elements and modulate gene expression. On an extrinsic level, Jak2 is activated by a variety of cytokine and growth factor receptors as well as by oxidative stress in the form of hydrogen peroxide, resulting in signaling cascades that are involved in the regulation of cell growth, proliferation and death. In addition, Jak2 is intrinsically regulated via specific autophosphorylation of a handful of its tyrosine residues.

The Jak kinases are structurally composed of seven Jak homology (JH) domains. The JH1 domain, located at the C terminus of Jak kinases, corresponds to the catalytically active tyrosine kinase domain (Duhe and Farrar, 1995). The JH2 domain which exhibits sequence similarity with the JH1 domain, but lacks catalytic activity, is termed the pseudokinase domain. The pseudokinase domain has been proposed to negatively regulate Jak kinase activity (Lindauer et al., 2001; Chen et al., 2000; Saharinen et al., 2003). The JH3-JH4 regions of Jak kinases represent the SH2-like domain whose function is unknown to date.

At the amino terminus, the JH4-JH7 regions of Jaks comprise the FERM domain. The FERM domain has been shown to be the region that is involved in receptor-Jak association (Girault et al., 1998; Hilkens et al., 2001; Zhou YJ et al., 2001). The significance of the FERM domain in Jak function was established by the observation of three naturally occurring point

mutations in Jak3 in severe combined immunodeficiency (SCID) patients which resulted in loss of Jak3 kinase activity (Cacalano et al., 1999; Zhou et al., 2001). Specifically it was shown that these mutations reduced the interaction of Jak3 with the common γ-chain of the IL-2 subfamily of cytokine receptors and in parallel inhibited the ability of Jak3 to be activated in response to ligand binding (Cacalano et al., 1999; Zhou et al., 2001). Therefore, it appears that structural changes in the FERM domain brought about by point mutation can alter the activity of Jak kinases.

Of the 49 Jak2 tyrosine residues encoded in murine Jak2, a number have been shown to be phosphorylated and play important roles in overall Jak2 tyrosine kinase regulation. Interestingly, many of these characterized tyrosine residues are situated at the C terminus of Jak2, where the pseudokinase and kinase domains reside. For example, in the activation loop of the kinase domain, phosphorylation of tyrosine 1007 is required for maximal Jak2 activation while phosphorylation of tyrosine 1008 has no effect on Jak2 kinase activity (Feng et al., 1997). Recently, Funakoshi-Tago et al., showed that autophosphorylation at tyrosine 913 in the kinase domain negatively regulates Jak2 by suppressing erythropoietin-induced Jak2 activation (Funakoshi-Tago et al., 2008). In addition, our laboratory recently identified tyrosine 972 as a novel site of Jak2 functional regulation. We showed that phosphorylation of tyrosine 972 is necessary for Jak2 kinase activity in response to angiotensin II (McDoom et al., 2008). Finally, phosphorylation of tyrosine 570 situated in the pseudokinase domain of Jak2 was shown to modulate Jak2 kinase function by suppressing Jak2 tyrosine kinase activity (Argetsinger et al., 2004; Feener et al., 2004). Collectively, the data suggest that the activation or inhibition of Jak2 tyrosine kinase is dependent upon the phosphorylation of its numerous tyrosine residues.

Fewer phosphorylated tyrosine residues have been characterized in the N terminal region of Jak2 which comprise the FERM domain. The characterized phosphorylated tyrosine residues in the FERM domain have different consequences for Jak2 tyrosine kinase regulation based on the presence or absence of ligand activation and the type of ligand-receptor system involved. For instance, Argetsinger et al. have shown that phosphorylation of tyrosine 221 increases ligand-independent Jak2 tyrosine kinase activity while Feener et al have demonstrated that phosphorylation of tyrosine 221 has no effect on Jak2-dependent signaling in the presence of an erythropoietin-leptin receptor chimera (Argetsinger et al., 2004; Feener et al., 2004). In addition, Funakoshi-Tago et al. have reported that phosphorylation of tyrosine 119 in response to erythropoietin down-regulates Jak2 kinase activity by promoting dissociation of activated Jak2 from the erythropoietin receptor while phosphorylation of this residue has no effect on Jak2 regulation in the presence of the interferon-gamma receptor (Funakoshi-Tago et al., 2006). Given the limited knowledge of how the FERM domain regulates Jak2 function, the identification of novel Jak2 tyrosine phosphorylation sites, within this region, will be important for our understanding of Jak2-dependent signaling and its regulation.

In this study, we have identified tyrosine 372 as a novel Jak2 phosphorylation site in the FERM domain of Jak2. We have found that phosphorylation of tyrosine 372 is critical for the maintenance of maximal Jak2 phosphorylation, STAT1 activation and Jak2-dependent gene transcription in the context of a ligand independent system. In addition, tyrosine 372 phosphorylation has an important and differential role on Jak2-dependent signal transduction in response to ligand. In particular, phosphorylation of tyrosine 372 facilitates interferon-gamma and epidermal growth factor-medated Jak2 activation but has no affect on hydrogen peroxide-

mediated Jak2 activation. As such, this work demonstrates the importance of tyrosine 372 in the regulation of the Jak2 signaling pathway.

Results

Tyrosine 372 and 373 are Sites of Jak2 Autophosphorylation

Jak2 protein was expressed at a high level via a vaccinia virus-mediated overexpression system and then purified to homogeneity as previously described (Ma and Sayeski, 2004). The purified Jak2 protein was then subjected to a combination of nano-HPLC/μESI ionization on a LTQ mass spectrometer. The spectra corresponding to the peptide fragment containing tyrosine 372 and 373 was identified and tyrosines 372 and 373 were found to be phosphorylated (Figure 5-1).

Tyrosine 372 is Highly Conserved Among Species Expressing Jak2 and in Different Jak2 Kinase Family Members

To further characterize the significance of tyrosine 372 and 373, we determined whether these amino acid residues were conserved throughout the evolutionary history (Figure 5-2). Comparison of the amino acid sequence of Jak2 from diverse species revealed that tyrosines 372 and 373 are conserved. However, evaluation of the amino acid sequence of the different Jak family members revealed that while tyrosine 372 is well conserved, tyrosine 373 is not. The higher conserved nature of tyrosine 372 relative to tyrosine 373 suggests that tyrosine 372 could play a more critical role in Jak2 function.

Loss of Tyrosine 372 and 373 Phosphorylation Reduce Jak2 Tyrosine Phosphorylation

Published reports have demonstrated that a number of the tyrosine residues of Jak2 are phosphorylated and play important roles in regulating Jak2 tyrosine kinase activity (Ihle et al., 1994; Feng et al., 1997; Ungureanu et al., 2002; Kurzer et al. 2004; Argetsinger et al., 2004; Funakoshi-Tago et al., 2006; Brooks et al., 2007; Godeny et al., 2007; McDoom et al., 2008).

While our mass spectrometry data revealed that tyrosine 372 and 373 are phosphorylated, the next critical step was to determine if these tyrosines are important for Jak2 tyrosine autophosphorylation. To determine how tyrosine 372 and 373 influence Jak2 function, the affect of tyrosine to phenylalanine substitution mutations on total Jak2 tyrosine phosphorylation was assessed. For this, BSC-40 cells were transfected to overexpress either empty vector, wild-type Jak2, Jak2-Y372F, or Jak2-Y373F. Cell lysates were prepared, Jak2 protein was isolated by immunoprecipitating with a Jak2 antibody and total tyrosine phosphorylation was assessed by immunoblotting with anti-phosphotyrosine antibody (Figure 5-3A). Subsequently, total Jak2 phosphorylation levels were quantified and averaged (Figure 5-3D). The results show that the loss of phosphorylation at tyrosines 372 or 373, individually, reduced total Jak2 tyrosine phosphorylation levels relative to wild-type Jak2 in a statistically significant manner. However, mutation at tyrosine 372 suppressed overall Jak2 phosphorylation significantly more than mutation at tyrosine 373. Specifically, the tyrosine 372 mutant reduced total Jak2 phosphorylation by roughly 70% whereas the tyrosine 373 mutant reduced phosphorylation by approximately 40%, when compared to wild-type Jak2 protein.

Since phosphorylation of tyrosine 1007 in Jak2 has been shown to be essential for maximal Jak2 tyrosine kinase activity (Feng et al., 1997), we also investigated whether individually mutating tyrosines 372 and 373 would affect the phosphorylation of this critical residue. For this, the same blot was probed with an anti-active Jak2 antibody to detect tyrosine 1007 phosphorylation levels (Figure 5-3B). Jak2 tyrosine 1007 phosphorylation levels were then quantified and averaged (Figure 5-3E). Similarly, we found that the loss of phosphorylation at tyrosine 372 or 373 significantly decreased Jak2 tyrosine 1007 phosphorylation, when compared to wild-type Jak2 protein. However, the tyrosine 372 mutant completely inhibited Jak2 tyrosine

1007 phosphorylation while the tyrosine 373 mutant reduced phoshporylation by approximately 70%. To verify equal Jak2 expression among samples, the membrane was stripped and re-blotted with anti-Jak2 polyclonal antibody (Figure 5-3C). Collectively, the results indicate that phosphorylation of Jak2 at tyrosines 372 and 373 is important for the regulation of the kinase activity of Jak2 in a ligand independent system. Additionally, the data suggest that phosphorylation at tyrosine 372 is more important for the catalytic activity of Jak2 when compared to tyrosine 373, since mutation of tyrosine 372 reduced Jak2 tyrosine 1007 phosphorylation as well as total Jak2 tyrosine phosphorylation more effectively than mutation at tyrosine 373. As such, for the remainder of the study, we focused on investigating the specific role of tyrosine 372 on Jak2 function.

Mutation of Jak2 at Tyrosine 372 Suppresses the Ability of Jak2 to Phosphorylate STAT1

To determine the outcome of the Jak2-Y372F mutant on the ability of Jak2 to activate substrates, we specifically examined the effect of the Y372F mutant on STAT1 phosphorylation. STAT1 is a signaling protein that is phosphorylated on tyrosine 701 in the presence of active Jak2 (Darnell et al., 1994). Here, BSC-40 cells that endogenously express STAT1 were transiently transfected with either empty vector control, wild-type Jak2, or Jak2-Y372F expressing plasmids. Whole cell lysates were prepared and samples were blotted with an anti-active STAT1 antibody to detect STAT1 phosphorylation at tyrosine 701 (Figure 5-4A). We found that loss of tyrosine 372 phosphorylation significantly reduced the ability of Jak2 to phosphorylate STAT1 at tyrosine 701 by approximately 65%, when compared to wild-type Jak2 ($p=0.004$) (Figure 5-4D). The membrane was stripped and re-probed with a STAT1 polyclonal antibody to confirm equal STAT1 protein expression among samples (Figure 5-4B). To confirm equal Jak2 expression levels, the membrane was stripped again and re-blotted with a Jak2

polyclonal antibody (Figure 5-4C). These results suggest that phosphorylation of Jak2 at tyrosine 372 facilitates Jak2-dependent STAT1 activation.

Loss of Tyrosine 372 Phosphorylation Reduces the Association of Jak2 with STAT1

In the Jak-STAT signaling paradigm, Jak2 associates with receptor bound STAT proteins and subsequently tyrosine phosphorylates them. Here, we examined the effect of loss tyrosine 372 phosphorylation on Jak2-STAT1 co-association. BSC-40 cells were transiently transfected with empty vector, wild-type Jak2 or Jak2-Y372F plasmid. The following day, protein lysates were then immunoprecipitated with anti-STAT1 polyclonal antibody and immunoblotted with anti-Jak2 polyclonal antibody to detect STAT1/Jak2 co-association (Figure 5-5A). We found that elimination of tyrosine 372 phosphorylation hindered the ability of Jak2 to associate with STAT1. In particular, the Jak2-Y372F mutation reduced STAT/Jak2 co-precipitation by approximately 80 % ($*p=3.87 \times 10^{-6}$) (Figure 5-5D). To demonstrate equal STAT1 precipitation across all samples, the membrane was stripped and re-blotted with anti-STAT1 polyclonal antibody (Figure 5-5B). In addition, whole cell lysates were blotted with anti-Jak2 polyclonal antibody to determine Jak2 expression levels (Figure 5-5C).

To verify that the Jak2-Y372F mutation hinders Jak2/STAT1 co-association, the inverse experiment was performed whereby protein lysates were first immunoprecipitated with anti-Jak2 antibody and then immunoblotted with anti-STAT1 antibody (Figure 5-E). Similar to the experiment above, mutation at tyrosine 372 decreased Jak2/STAT1 co-association by approximately 85% ($*p=1.35 \times 10^{-8}$) (Figure 5-5I). To demonstrate equal Jak2 precipitation among samples, the membrane was stripped and re-probed with an anti-Jak2 polyclonal antibody (Figure 5-5F). To assess STAT1 protein levels, whole cell lysates from these same samples were blotted with an anti-STAT1 polyclonal antibody (Figure 5-5G). All together, the results in Figure 5-5 suggest that loss of tyrosine 372 phosphorylation reduces the ability of Jak2 to

associate with STAT1 and thereby provides a mechanistic explanation as to why this Jak2 mutation disrupts STAT1 activation.

The Jak2-Y372F Mutation Abrogates Ligand-Independent Gene Expression

It is known that Jak2 is capable of driving a basal level of gene expression even in the absence of ligand stimulation (Chatti et al., 2004; Wallace et al., 2006). This ligand-independent gene expression corresponds to Jak2's intrinsic functional activity. Here, we sought to determine whether loss of phosphorylation of tyrosine 372 affects the intrinsic functional capacity of Jak2 to induce ligand-independent gene expression by performing a luciferase gene reporter assay. Specifically, COS-7 cells were transiently transfected with a plasmid encoding four tandem repeats of the γ-activating sequence upstream of the firefly luciferase cDNA. In addition, these cells were co-transfected with plasmid encoding either empty vector control, wild-type Jak2, or Jak2-Y372F protein. Two days later, luciferase activity was determined and plotted as a function of Jak2 expression status (Figure 5-6A). We found that the Jak2-Y372F mutation significantly reduced the ability of Jak2 to drive luciferase gene expression when compared to wild-type Jak2 ($p=0.00036$). To confirm equal expression levels of wild-type Jak2 and Jak2-Y372F protein, a portion of the transfected protein lysates were immunoblotted with a Jak2 polyclonal antibody (Figure 5-6B).

Loss of Tyrosine 372 Phosphorylation Hinders Interferon-Gamma-mediated Jak2 Activation

Having shown the importance of tyrosine 372 on ligand-independent Jak2 function, we then examined the functional significance of tyrosine 372 phosphorylation in the context of ligand-dependent Jak2 signaling. Interferon-gamma is a cytokine that has been well characterized to activate the receptor-associated Jak2 tyrosine kinase (Pestka et al., 1997, Parganas et al., 1998). Given the critical role of Jak2 in interferon-gamma receptor signaling, we

sought to determine the effect of tyrosine 372 phosphorylation on interferon-gamma-mediated Jak2 activation. Here, mouse embryonic fibroblast cells (MEF) that endogenously express the interferon-gamma receptor, but that lack Jak2, were transiently transfected with empty-vector, wild-type Jak2 or Jak2-Y372F expressing plasmids. These cells were treated with interferon-gamma for 0 or 10 minutes and then lysed. Jak2 protein was immunoprecipitated from the lysate and Jak2 tyrosine 1007 phosphorylation levels were measured by immunoblotting with an anti-Jak2 pY1007/pY1008 antibody. In agreement with published reports, we found that by 10 minutes of interferon-gamma treatment, wild-type Jak2 was activated (Parganas et al., 1998, Funakoshi-Tago et al., 2006). However, loss of tyrosine 372 phosphorylation blocked the interferon-gamma-mediated increase in Jak2 tyrosine 1007 phosphorylation (Figure 5-7A). The membrane was subsequently stripped and equal Jak2 protein expression was verified via Western blot with an anti-Jak2 antibody (Figure 5-7B). The results in Figure 5-7 suggest that phosphorylation of tyrosine 372 is critical for interferon-gamma-dependent Jak2 activation.

Loss of Tyrosine 372 Phosphorylation Impairs Epidermal Growth Factor-Mediated Jak2 Activation.

Jak2 also becomes activated in response to growth factors, like epidermal growth factor (Shuai et al., 1993; Andl et al., 2004). However, the relevance of Jak2 tyrosine phosphorylation in growth-factor signaling has not been well characterized. Thus, we sought to determine the effect of Jak2 tyrosine 372 phosphorylation on the ability of Jak2 to respond to epidermal growth factor. Mouse embryonic fibroblasts that were derived from Jak2 -/- mice were transiently transfected with empty vector, wild-type Jak2 or Jak2-Y372F expressing plasmids. The cells were then treated with epidermal growth factor for 0 or 10 minutes. To detect Jak2 tyrosine 1007 phosphorylation, protein extracts were immunoprecipitated with anti-Jak2 antibody and then immunoblotted with anti-Jak2 pY1007/pY1008 antibody. By ten minutes of epidermal

growth factor treatment, wild-type Jak2 was increasingly activated, which is in agreement with previously published reports (Shuai et al., 1993; Andl et al., 2004). On the other hand, loss of tyrosine 372 phosphorylation inhibited the epidermal growth factor-mediated increase in Jak2 tyrosine 1007 phosphorylation (Figure 5-8A). The membrane was stripped and re-probed with an anti-Jak2 antibody to verify equal protein loading (Figure 5-8B). The results in Figure 5-8 suggest that Jak2 tyrosine 372 phosphorylation is important for epidermal growth factor-mediated Jak2 activation.

Loss of Tyrosine 372 Phosphorylation Does Not Affect Hydrogen Peroxide-Mediated Jak2 Activation

Although Jak2 is tradionally considered a mediator of cytokine signaling, other stimuli can activate this pathway such as, oxidative stress (Simon et al., 1998; Madamanchi et al., 2001, Sandberg et al., 2004B). Jak2 can be potently activated in a number of cell types by oxidative stress in the form of hydrogen peroxide (Simon et al., 1998; Madamanchi et al., 2001, Sandberg et al., 2004B). We have already shown that tyrosine 372 plays an important role in cytokine and growth factor-dependent Jak2 activation. We next wanted to determine the impact of tyrosine 372 phosphorylation on Jak2 activation in response to a non-traditional ligand such as, hydrogen peroxide. For this, mouse embryonic fibroblasts that lack Jak2 were transiently transfected with empty vector, wild-type Jak2 or Jak2-Y372F expressing plasmids. These cells were treated with hydrogen peroxide for 0 or 10 minutes. Cellular lysates were immunoprecipitated with anti-Jak2 antibody and then immunoblotted with anti-Jak2 pY1007/pY1008 antibody to detect Jak2 tyrosine 1007 phosphorylation. Consistent with previous reports, wild-type Jak2 was potently activated by 10 minutes of treatment with hydrogen peroxide (Simon et al., 1998; Madamanchi et al., 2001; Sandberg et al., 2004B). However, loss of tyrosine 372 phosphorylation had no effect on hydrogen peroxide-mediated Jak2 tyrosine 1007 phosphorylation (Figure 5-9A). To

demonstrate equal Jak2 expression among samples, the membrane was stripped and re-blotted with anti-Jak2 antibody (Figure 5-9B). These results suggest that tyrosine 372 phosphorylation is dispensable for hydrogen peroxide-dependent Jak2 activation.

Discussion

In this study, we identified tyrosines 372 and 373 as sites of Jak2 autophosphorylation via mass spectrometry analysis. Interestingly, comparison of the amino acid sequence of the different *Janus* kinase family members, and the amino acid sequence of Jak2 from various species, indicated that tyrosine 372 is a highly conserved residue through evolution. As such, tyrosine 372 could play a critical role in Jak2 kinase function. To determine the importance of tyrosine 372 in the Jak2 signaling pathway, we utilized site-directed mutagenesis to mutate tyrosine 372 to phenylalanine. We found that loss of tyrosine 372 phosphorylation eliminated the potential of Jak2 to become maximally activated as demonstrated by the loss of tyrosine 1007 phosphorylation. The Jak2-Y372F mutation also suppressed the phosphorylation of other Jak2 tyrosine residues. These results show that phosphorylation of tyrosine 372 is important for Jak2 catalytic activity in the context of a ligand-independent signaling system. Loss of tyrosine 372 phosphorylation also affected the capacity of Jak2 to activate its downstream substrate, STAT1, and hindered the intrinsic ability of Jak2 to drive gene transcription. Moreover, we believe that the Jak2-Y372F-mediated disruption in STAT1 phosphorylation could be due to the impaired ability of Jak2 to co-associate with STAT1.

Tyrosine 372 was also investigated within a signaling context. We found that loss of tyrosine 372 phosphorylation blocked interferon-and epidermal growth factor-dependent Jak2 activation relative to wild type Jak2, but had no effect on hydrogen peroxide-mediated Jak2 activation, suggesting that tyrosine 372 plays a significant and differential role in Jak2 activation

in response to ligand. All together, our results indicate that tyrosine 372 is a novel site of Jak2 functional regulation.

An important finding of this study is that while our results suggest that tyrosine 372 is critical for ligand-independent Jak2 catalytic activity, this residue may not be essential for the catalytic activity of Jak2 in the context of a ligand-dependent system, as demonstrated by the fact that loss of tyrosine tyrosine 372 phosphorylation has no affect on hydrogen peroxide-dependent Jak2 activation. A possible explanation for this disparity is that phosphorylation of tyrosine 372 may facilitate Jak2 dimerization and thus enhance ligand-independent Jak2 catalytic activity, whereas in response to non-traditional ligands like hydrogen peroxide, tyrosine 372 may not play a role in Jak2 dimer formation and therefore have no direct affect on the catlayitic activity of Jak2. Since hydrogen peroxide activates Jak2 by inhibiting phosphatases via their oxidation, it is possible that phosphorylation of Jak2 at sites other than tyrosine 372 could block the association of Jak2 with a phosphatase such as SHP-2, thus maintaining Jak2 in the active state.

The FERM domain of Jak kinases has been shown to be important for the interaction with cytokine receptors (Frank et al., 1995; Chen et al., 2000; Kohlhuber et al., 1997; Zhou et al., 2001). We hypothesize that tyrosine 372, situated in the FERM domain of Jak2, could possibly mediate Jak2 association with various receptors. Another possible mechanistic explanation for the Jak2-Y372F effect on Jak2 function is that loss of tyrosine 372 phosphorlation may promote the dissociation of Jak2 tyrosine kinase from receptor complexes thereby downregulating Jak2-depedent signaling. Therefore, it is possible that phosphorylation of tyrosine 372 causes a conformational change in Jak2 that could stabilize the FERM domain of Jak2 in a more active conformation when bound to receptor, maintaining Jak2 in the active state. Future studies should

be aimed at verifying whether the Jak2-Y372F mutation disrupts the interaction between Jak2 and its associated receptor in response to ligand treatment.

Of the 49 Jak2 tyrosine residues, a number are known to be phosphorylated and play important roles in Jak2 tyrosine kinase function. Interestingly, many of these characterized Jak2 tyrosine phosphorylation sites are situated either in the pseudokinase or kinase domains of Jak2. Less is known regarding the consequences of phosphorylation of tyrosines in the N-terminus of Jak2, where the FERM domain resides. Argetsinger et al. have shown via mass spectrometry and two dimensional peptide mapping that tyrosine 221 in the FERM domain of Jak2 is phosphorylated. When tyrosine 221 was mutated to phenylalanine, Jak2 tyrosine autophosphorylation was reduced suggesting that tyrosine 221 could serve to regulate Jak2 tyrosine kinase activity (Argetsinger et al., 2004). In contrast, Feener et al. reported that when the Jak2-Y221F mutant is overexpressed with an erythropoietin-leptin receptor chimera in HEK 293 cells, Jak2-dependent signaling was unaffected (Feener et al., 2004). The apparent discrepancy in the findings regarding the role of tyrosine 221 could be due to the fact that Feener et al. employed a receptor chimera in their system while there was an absence of coexpressed cytokine receptor in that of the Argetsinger group. These results imply that Jak2 tyrosine 221 phosphorylation could regulate the activity of Jak2 in the absence of cytokine receptor association, or conceivably in association with other cytokine receptors that don't include the erythropoietin or leptin receptors.

The role of the FERM domain in regulating kinase activity has also been suggested by Jak2 tyrosine 119 phosphorylation (Funakoshi-Tago et al., 2006). Using a Jak2-Y119F mutant, Funakoshi-Tago et al. demonstrated this mutation corresponded to a more stable association of Jak2 with the erythropoietin receptor, resulting in prolonged of Jak2 activation (Funakoshi-Tago

et al., 2006). Thus they concluded that phosphorylation of tyrosine 119 within the Jak2 FERM domain downregulates Jak2 tyrosine kinase activity by promoting the dissociation of activated Jak2 from the erythropoietin receptor. Interestingly, by using a phosphorylation mimic Jak2-Y119E mutation, this group further showed that this mutation reduced the interaction of Jak2 with the erythropoietin receptor, while there was no consequence of this mutation on the ability of Jak2 to associate with the interferon-gamma receptor. These results suggest that there are underlying differences in the way Jak2 interacts with erythropoietin receptor and the interferon-gamma receptor.

In addition, in 2006, our laboratory verified another Jak2 phosphorylation site in the FERM domain of Jak2. We showed that phosphorylation of tyrosine 201 served as a binding site for the SHP-2 regulatory protein. This Jak2/SHP-2 interaction allowed the recruitment of Jak2 to the angiotensin II type-1 receptor signaling complex and in turn promoted downstream Jak2-dependent signaling (Godeny et al., 2006).

Finally, Funakoshi-Tago and colleagues have recently investigated the effect of several conserved Jak2 tyrosine residues on Jak2 function, including tyrosine 372. Although they have not provided evidence that tyrosine 372 is phosphorylated, they have shown that a Jak2-Y372F mutant has no effect on Jak2-mediated erythroid progenitor colony formation (Funakoshi-Tago et al., 2008). Their results suggest that phosphorylation of tyrosine 372 is not important for the regulation of Jak2 kinase activity. In contrast, we have shown that phosphorylation of tyrosine 372 is critical for the regulation of Jak2, as loss of tyrosine 372 phosphorylation suppresses ligand-independent Jak2 tyrosine autophosphorylation as well as interferon-gamma and epidermal growth factor-mediated Jak2 activation. The discrepancy between our findings and those of Funakoshi-Tago et al. could be due to a few factors. First, we have examined how

phosphorylation of tyrosine 372 affects the inherent kinase activity of Jak2 in a ligand-independent system, whereas Funakoshi-Tago et al. have observed the consequence of loss of tyrosine 372 phosphorylation on Jak2-dependent cell growth in response to erythropoietin stimulation. Second, while Funakoshi-Tago et al. have examined the effect of the Jak2-Y372F mutation on Jak2 regulation in the context of the erythropoietin signaling pathway, we have studied the effect of the Jak2-Y372F mutant on interferon-gamma and epidermal growth factor signaling. The Jak2-Y372F mutation could differentially influence the manner in which Jak2 interacts with the interferon-gamma/epidmeral growth factor receptors and the erythropoeitin receptor leading to differences in Jak2 activity.

Collectively, studies show that phosphorylation of tyrosine resides in the FERM domain can have differential effects on Jak2 tyrosine kinase activity based on the presence of ligand and the type of ligand-receptor system involved. Undeniably, regulation of Jak2 through the phosphorylation of tyrosines in the FERM domain is quite complex and therefore further insight into the exact mechanism by which ligand binding to a particular receptor activates Jak2 is required.

In conclusion, our laboratory has contributed to the understanding of Jak2 tyrosine kinase regulation by the FERM domain. We have identified tyrosine 372 as a novel site of Jak2 autophosphorylation in the FERM domain and subsequently demonstrated the importance of the phosphorylation of this amino acid residue on Jak2 function. Specifically, we showed that phosphorylation of tyrosine 372 is important for the catalytic activity of Jak2, for the ability of Jak2 to associate with STAT1 leading to STAT1 activation and for Jak2-dependent gene transcription. Additionally, we showed that tyrosine 372 is critical for interferon-gamma and epidermal growth factor-dependent Jak2 activation. Future studies should seek to elucidate the

mechanism by which tyrosine 372 regulates Jak2 function.

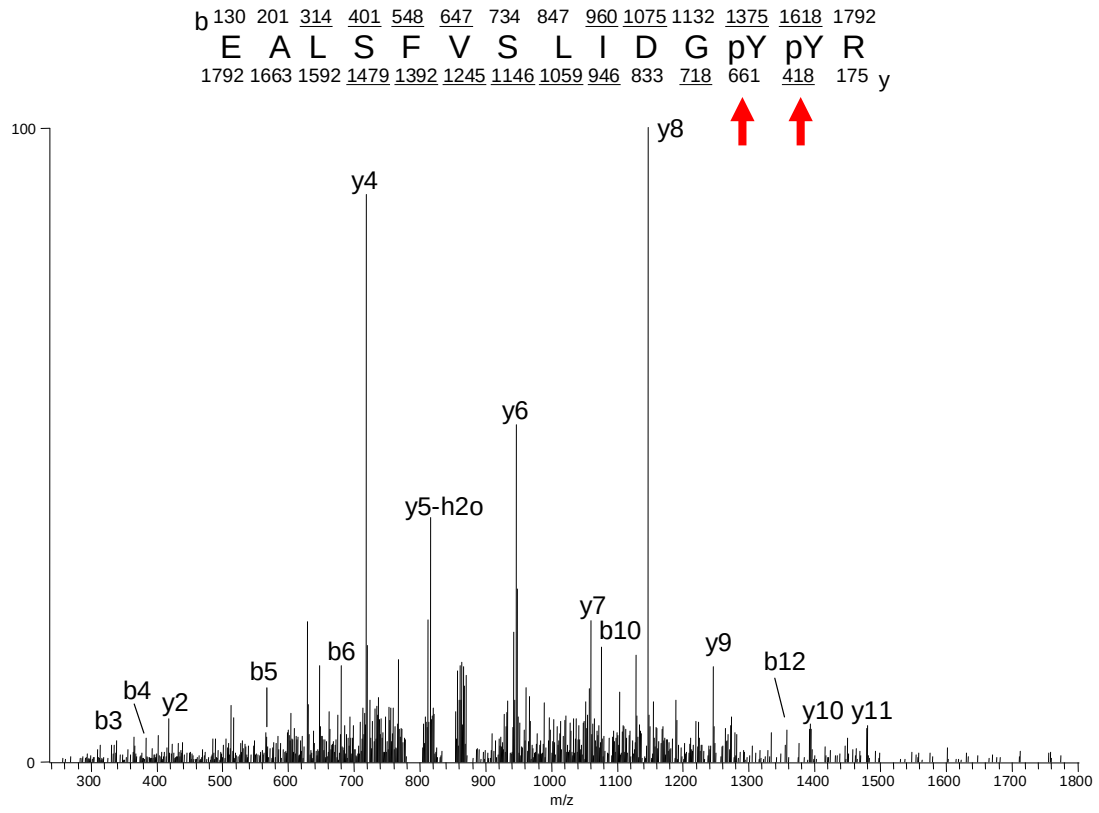

Figure 5-1. Tyrosines 372 and 373 are Jak2 phosphorylation sites. Purified Jak2 protein was subjected to mass spectrometry. Analysis revealed that tyrosines 372 and 373 are phosphorylated. The data in Figure 5-1 was generated by Dr. Jennifer Busby.

In different species:

Mouse Jak2	361	E	A	L	S	F	V	S	L	I	D	G	Y	Y	R	L	T	A	D	A	H	H	Y	L	C	K	E	V	A	P	P	390
Rat Jak2		E	A	L	S	F	V	S	L	I	D	G	Y	Y	R	L	T	A	D	A	H	H	Y	L	C	K	E	V	A	P	P	
Pig Jak2		E	A	L	S	F	V	S	L	I	D	G	Y	Y	R	L	T	A	D	A	H	H	Y	L	C	K	E	V	A	P	P	
Human Jak2		E	A	L	S	F	V	S	L	I	D	G	Y	Y	R	L	T	A	D	A	H	H	Y	L	C	K	E	V	A	P	P	
Puffer fish Jak2		E	A	L	S	F	V	S	L	I	D	G	Y	Y	R	L	T	T	D	A	H	H	Y	L	C	K	E	V	A	P	P	
Zebra fish Jak2		E	A	L	S	F	V	S	L	I	D	G	Y	Y	R	L	T	T	D	A	H	H	Y	L	C	K	E	V	A	P	P	
Chicken Jak2		E	A	L	S	F	V	S	L	I	D	G	Y	Y	R	L	T	A	D	A	H	H	Y	L	C	K	E	V	A	P	P	

In different JAK family members:

Mouse Jak2	E	A	L	S	F	V	S	L	I	D	G	Y	Y	R	L	T	A	D	A	H	H	Y	L	C	K	E	V	A	P	P	
Mouse Jak1	E	G	H	F	G	K	V	E	L	C	R	Y	D	P	E	G	D	N	T	G	E	Q	V	A	V	K	S	L	K	P	
Mouse Jak3	E	A	L	S	F	V	S	L	V	D	G	Y	F	R	L	T	A	D	A	H	H	Y	L	C	T	D	V	A	P	P	
Mouse Tyk2	E	A	L	S	F	V	A	L	V	D	G	Y	F	R	L	T	A	D	S	S	H	Y	L	C	H	E	V	A	P	P	

Figure 5-2. Tyrosine 372 is conserved in different Jak family members and among different species. Comparison of the amino acid sequence of Jak2 from different species indicates that tyrosines 372 and 373 are conserved amino acid residues. In addition, tyrosine 372 is highly conserved among different Janus kinase family members. Figure 5-2 was generated by Sushama Kamarjugadda.

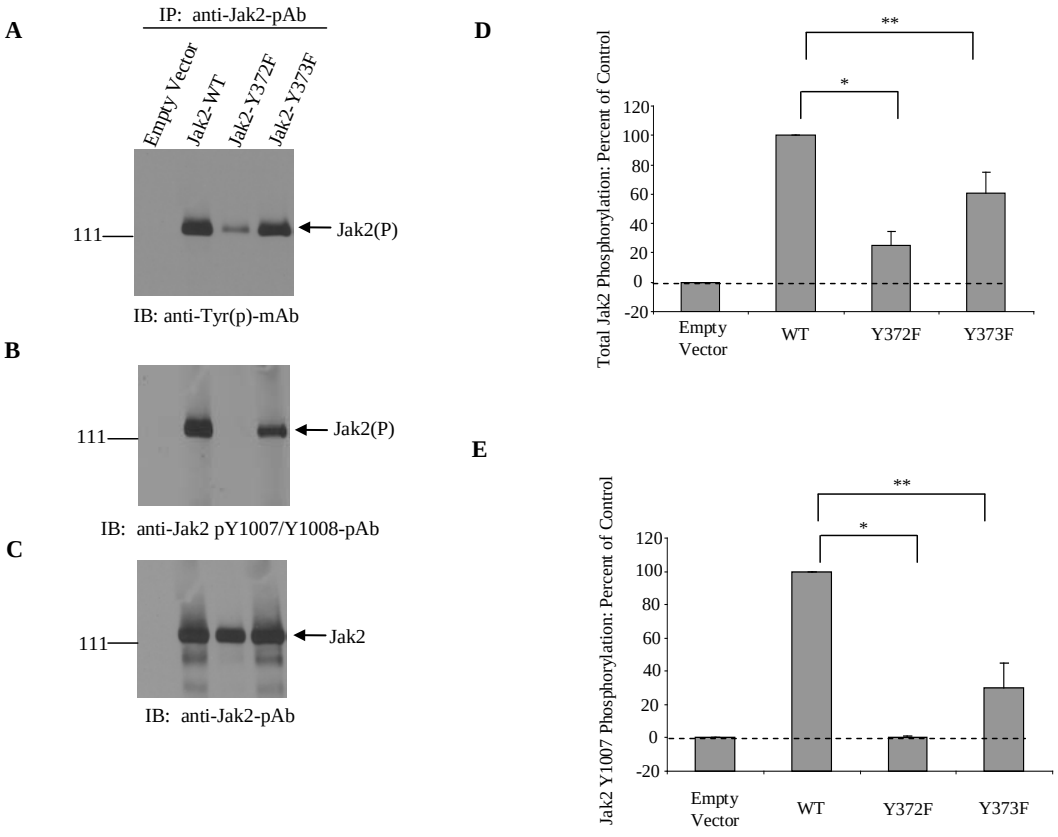

Figure 5-3. Loss of tyrosine 372 and 373 phosphorylation decrease the ability of Jak2 to autophosphorylate. A) BSC-40 cells were transfected with 10 μg of the indicated plasmid DNA and the constructs were overexpressed via a vaccinia-virus-mediated expression system. Proteins were immunoprecipitated using a Jak2 polyclonal antibody and resolved by SDS-PAGE. The immunoprecipitated Jak2 was immunoblotted with an anti-phosphotyrosine monoclonal antibody. B) The membrane was then stripped and re-probed with an anti-pY1007/pY1008 polyclonal antibody. C) To verify equal Jak2 expression among all samples, the membrane was stripped again and re-blotted with an anti-Jak2 polyclonal antibody. D) Densitometry analysis of total Jak2 phosphorylation levels of three representative Western blots. Statistical significance was determined via Student's t-test. Values of p=0.001469 (Jak2-Y372F) and p=0.0454 (Jak2-Y373F) were considered significant relative to Jak2-WT. E) Densitometrical analysis of three Western blots quantitating Jak2 tyrosine 1007 phosphorylation levels. Values of p=2.86 x 10-12 (Jak2-Y372F) and p=.0.0032 (Jak2-Y373F). The data in Figures 5-3A-C were generated by Dr. Peter Sayeski.

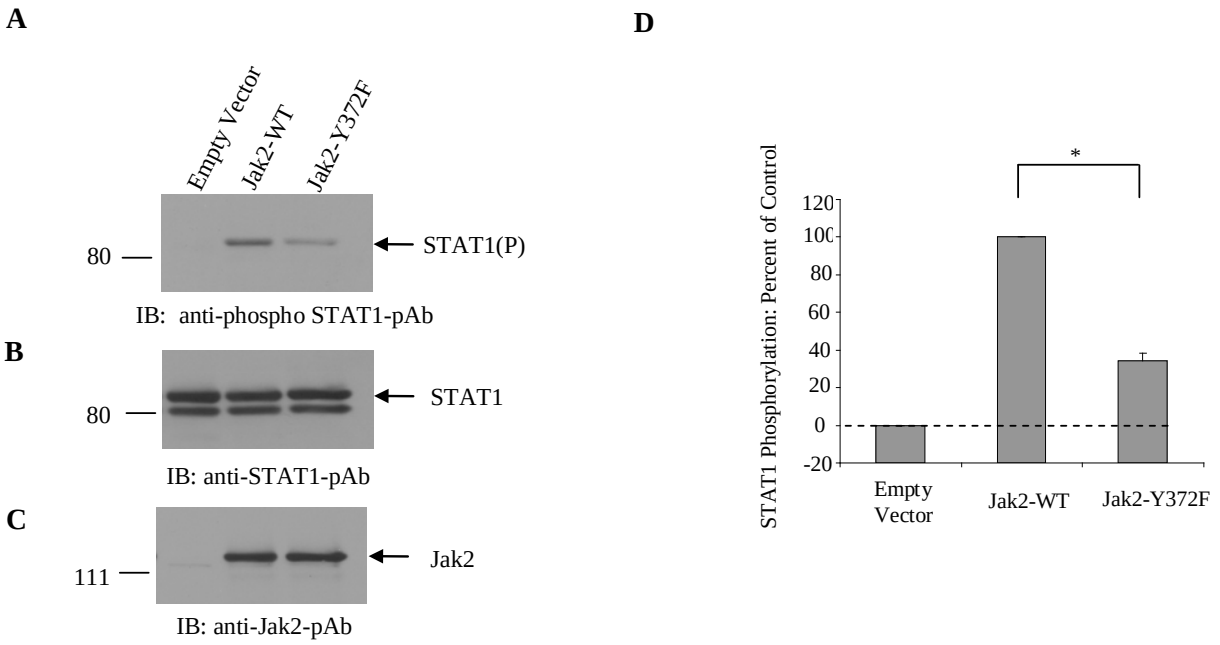

Figure 5-4. Loss of Tyrosine 372 phosphorylation reduces Jak2-mediated STAT1 activation. BSC-40 cells were transiently transfected with 10 μg empty vector, Jak2 wild-type or Jak2-Y372F plasmid. A) STAT1 phosphorylation at tyrosine 701 was determined via western blot using an anti-active STAT1 antibody. B) Equal STAT1 protein levels were verified by stripping membrane and immunoblotting with a STAT1 polyclonal antibody. C)To show Jak2 expression levels among all samples, the membrane was stripped again and probed with a Jak2 polyclonal antibody. D) Densitometry analysis of STAT1 phosphorylation levels of three representative Western blots. Statistical significance ($p=0.004$) was determined using Student's t-test.

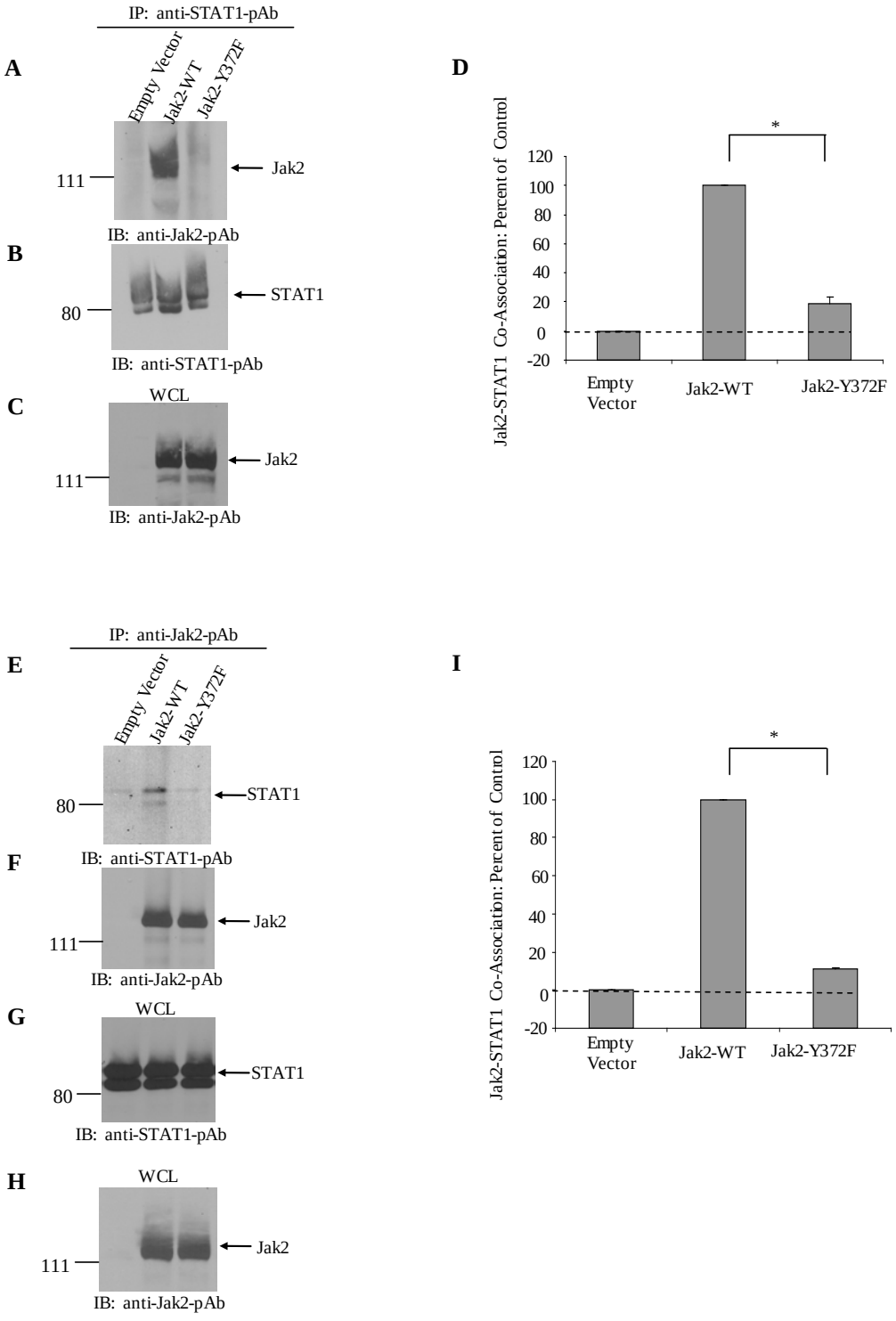

Figure 5-5. The Jak2-Y372F mutant hinders the association between Jak2 and STAT1. BSC-40 cells were transiently transfected with 10 μg of empty vector, Jak2 wild-type or Jak2-Y372F plasmid. A) Lysates were immunoprecipitated with anti-STAT1 polyclonal antibody and then Western blotted with anti-Jak2 antibody to determine STAT1/Jak2

co-association. B) The membrane was stripped and reprobed with STAT1 polyclonal antibody to demonstrate equal STAT1 precipitation among samples. C) Whole cell lysates were probed with anti-Jak2 polyclonal antibody to assess Jak2 protein levels. D) Densitometry analysis of STAT1/Jak2 association levels of three representative experiments. Statistical significance (*$p=3.87 \times 10^{-6}$) was determined using Student's *t*-test. E) Lysates were immunoprecipitated with anti-Jak2 antibody and then immunoblotted with anti-STAT1 polyclonal antibody to determine Jak2/STAT1 co-association. F) The membrane was stripped and re-blotted with Jak2 antibody to demonstrate equal Jak2 precipitation among samples. G) Whole cell lysates were immunoblotted with anti-STAT1 antibody to assess STAT1 protein levels. H) Whole cell lysates were also immunoblotted with anti-Jak2 antibody to assess Jak2 protein levels. I) Densitometry analysis of Jak2/STAT1 association levels. Statistical significance (*$p=1.35 \times 10^{-8}$) was assessed using Student's *t*-test.

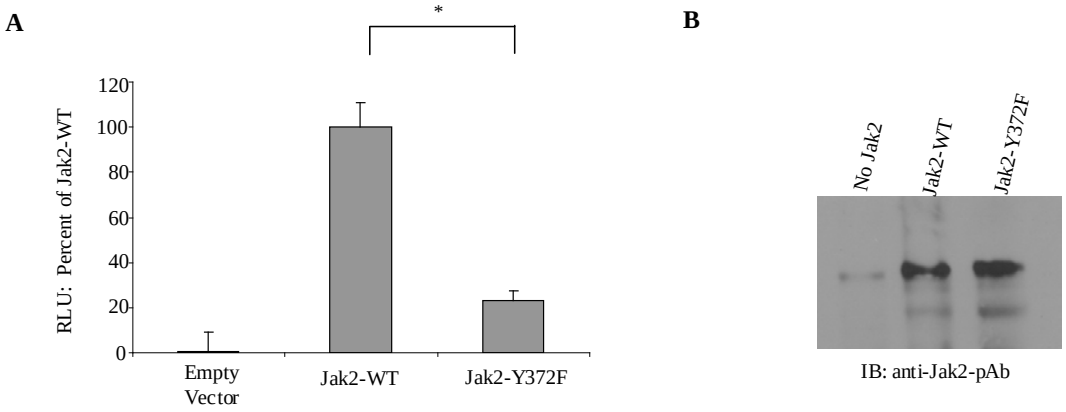

Figure 5-6. Phosphorylation of Tyrosine 372 facilitates Jak2-dependent gene transcription. A) COS-7 cells were transiently transfected with 2 μg of luciferase plasmid and 5 μg of empty vector, wild-type Jak2 or Jak2-Y372F plasmids. Two days later, relative light units (RLU) were read by a luminometer and were taken as a measure of luciferase gene expression. B) Equal Jak2 expression was ascertained via Western blot analysis with an anti-Jak2 polyclonal antibody. Statistical significance ($p=0.00036$) was determined using a Student's *t* test.

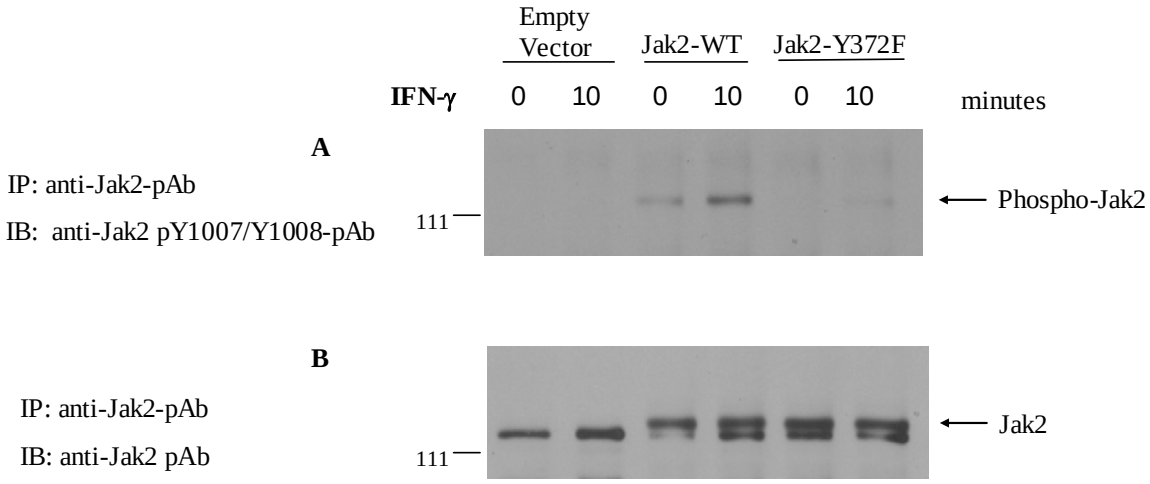

Figure 5-7. Phosphorylation of tyrosine 372 is essential for interferon-gamma-dependent Jak2 activation. MEF cells were transiently transfected with 10 μg empty vector plasmid, Jak2 wild-type plasmid or Jak2-Y372F plasmid. Following transfection, the cells were treated with 1 μg/mL interferon-gamma for 0 or 10 minutes. A) The cells were subsequently lysed and Jak2 protein was immunoprecipitated from the lysates. Jak2 tyrosine 1007 phosphorylation was determined via Western blot analysis with an anti-Jak2 pY1007/pY1008 antibody. B) The membrane was stripped and re-blotted with anti-Jak2 antibody to verify equal Jak2 expression among samples. Shown is one of three independent results.

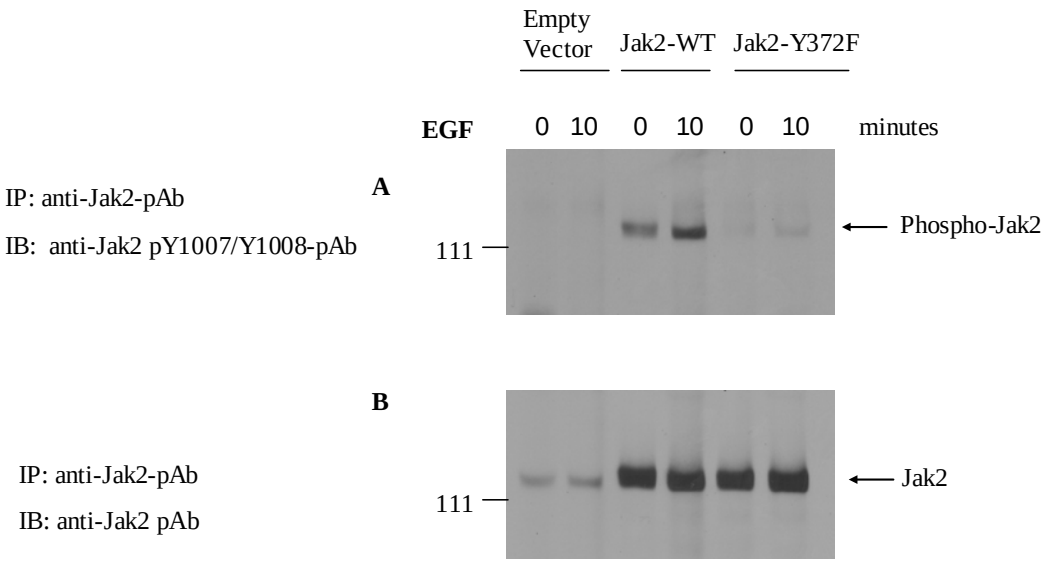

Figure 5-8. Tyrosine 372 is critical for epidermal growth factor-dependent Jak2 activation. MEF cells were transiently transfected with 10 μg empty vector, Jak2 wild-type or Jak2-Y372F plasmids. After transfection, these cells were treated with 200 ng/mL epidermal growth factor for 0 or 10 minutes. A) Lysates were immunoprecipitated with anti-Jak2 antibody and Western blotted with anti-Jak2 pY1007/pY1008 antibody to detect Jak2 tyrosine 1007 phosphorylation. B) The membrane was subsequently stripped and re-probed with anti-Jak2 antibody to confirm total Jak2 protein levels. Shown is one of two independent results.

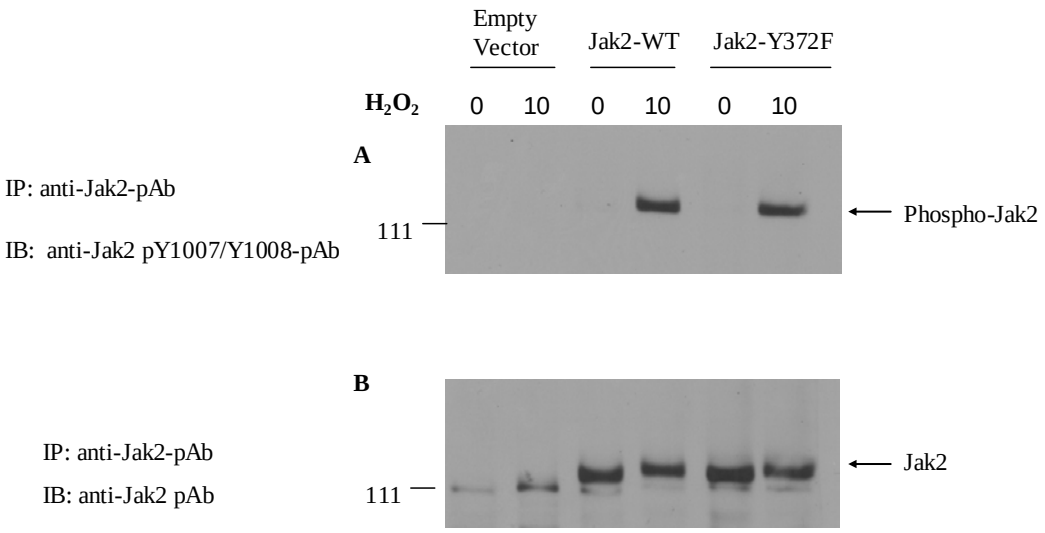

Figure 5-9. Phosphorylation of tyrosine 372 does not affect hydrogen peroxide-dependent Jak2 activation. MEF cells were transiently transfected with 10 μg empty vector plasmid, Jak2 wild-type plasmid, or Jak2-Y372F plasmid. A) Following transfection, cells were treated with 0.5 mM hydrogen peroxide for 0 or 10 minutes and Jak2 protein was immunoprecipitated from the lysates. Jak2 tyrosine 1007 phosphorylation was then assessed by immunoblotting with anti-Jak2 pY1008/pY1008 antibody. B) The membrane was stripped and re-probed with an anti-Jak2 antibody to confirm equal protein loading. Shown is one of two independent results.

CHAPTER 6
DISCUSSION

Overview

Since the discovery of Jak2 seventeen years ago, a wealth of evidence has linked this protein to diverse pathophysiological processes. Specifically, Jak2 has been implicated in cardiovascular disease, autoimmune disorders and cancer. Over the past few years, much attention has been focused on the role of Jak2 in myeloproliferative disorders, as a gain-of-function Jak2-V617F mutation has been found in almost all polycythemia vera patients and a substantial proportion of essential thrombocythemia and primary myelofibrosis patients. The fact that the constitutively active Jak2-V617F mutation is an important contributor to the pathogenesis of myeloproliferative disorders has made this mutation an attractive target for inhibition via small molecule inhibitors. Our laboratory has invested effort toward the identification and characterization of Jak2 inhibitors that target aberrant Jak2 kinase activity. In this process, we identified two novel Jak2 tyrosine kinase small molecule inhibitors, termed Z3 and G6. Z3 and G6 are valuable additions to the growing number of Jak2 inhibitors for a number of reasons. First, both these compounds inhibited Jak2-V617F mediated pathologic cell growth *in vitro*. Second, Z3 and G6 suppressed the *ex vivo* growth of hematopoietic progenitor cells isolated from the bone marrow of a myeloproliferative disorder patient carrying a Jak2 gain-of-function-mutation. Third, Z3 and G6 had no effect on Tyk2 or c-Src tyrosine kinase activity at concentrations that inhibited Jak2, thus suggesting the relative specificity of these compounds for Jak2. Finally, both compounds inhibited the proliferation of Jak2-V617F expressing cells more effectively than cells that don't rely on Jak2 for their aberrant growth, confirming the specificity of these compounds for inhibiting Jak2-dependent aberrant cell

growth. Overall, our results suggest that Z3 and G6 effectively inhibit Jak2 tyrosine kinase function *in vitro* and *ex vivo*.

In addition, the association of Jak2 to a number of different disease states emphasizes the need to fully understand Jak2 function. However, while much progress has been made in trying to comprehend the factors that control Jak2 function, much work still needs to be accomplished. Eight of the ten known tyrosine phosphorylation sites within Jak2 have been linked to regulatory mechanisms, but many more Jak2 phosphorylation sites remain to be identified.

In this dissertation we characterized a novel phosphorylation site in the FERM domain of Jak2 and found that Jak2 tyrosine 372 phosphorylation has important consequences for Jak2 function. Mainly, we found that phosphorylation of tyrosine 372 is important for ligand independent Jak2 tyrosine autophosphorylation, STAT1 activation and Jak2-dependent gene expression. In addition, we showed that tyrosine 372 phosphorylation is critical for interferon-gamma and epidermal growth factor-mediated Jak2 activation.

In Chapter 6, we will discuss the impact of the characterization of Z3 and G6 on Jak2-mediated disease. Moreover, we will discuss the effect of tyrosine 372 on Jak2 function and its potential role in autoimmune disorders and cell growth.

Characterization of the Jak2 Inhibitors, G6 and Z3

A driving force for the identification of Jak2-specific small molecules can be contributed to the identification of the constitutively active Jak2-V617F mutation that has been observed in almost all polycythemia vera patients and a substantial proportion of essential thrombocythemia and primary myelofibrosis cases. Our laboratory has contributed to the growing number of Jak2 small molecules by identifying two novel Jak2-specific small molecules termed, Z3 and G6. Both Z3 and G6 effectively inhibited Jak2-dependent aberrant cell growth.

Although we have clearly shown that Z3 and G6 inhibit Jak2-mediated pathologic cell growth *in vitro* and *ex vivo*, the characterization of these compounds *in vivo* would give us important information about the therapeutic efficacy of these compounds. Work by others in our laboratory has preliminarily shown the efficacy of G6 in a Jak2-dependent erythroleukemia murine model, *in vivo*. By intravenously injecting SCID-NOD mice with HEL cells expressing the Jak2-V617F mutation, we found the pathologic appearance of blast cells in the peripheral blood and this was significantly reduced with G6 treatment. In addition, we established that HEL cell injection alone resulted in an increased spleen weight to body weight ratio and decreased the myeloid to erythroid ratio due to a marked increase in erythroid cells. However, we discovered that treatment with G6 corrected these pathologic effects. Collectively, these results preliminarily show that G6 suppresses Jak2-V617F-mediated pathologic cell growth, *in vivo*.

If Z3 and G6 have the potential to one day treat myeloproliferative disorders in patients, it is imperative that the efficacy of these compounds be tested in a myeloproliferative disorder mouse model. A myeloproliferative mouse model could be generated by reconstituting the bone marrow of mice with hematopoietic stem cells that are retrovirally transduced with the Jak2-V617F mutation. We would anticipate that the Jak2-V617F mutation will induce erythrocytosis and splenomagaly in these mice. We hypothesize that both Z3 and G6 will abrogate these pathologic effects. In addition, since we have demonstrated these compounds to be Jak2-selective, we predict that the reduction in erythrocytosis and spleen size will correlate with suppression in Jak2 and STAT activation in these animals.

We have shown that Z3 inhibits the growth of Jak2-V617F-expressing human erythroleukemia cells by inducing cell cycle arrest while we have demonstrated that G6 inhibits

the growth of these cells by promoting apoptosis. To enhance our understanding regarding the mechanisms by which Z3 and G6 inhibit aberrant Jak2 activity, it would be useful to know what cell survival-associated proteins are downregulated by G6 and Z3 and what apoptotic related proteins are upregulated by G6 in Jak2-V617F expressing human erythroleukemia cells. To achieve this goal, human erythroleukemia cells that are homozygous for the Jak2-V617F mutation can be treated with DMSO, G6 or Z3 and then protein lysates can be separated by 2-dimensional gel electrophoresis to compare the protein expression profile between DMSO and Jak2 inhibitor-treated cells. Subsequently, the differentially expressed proteins would be isolated and subjected to mass spectrometry for their identification. This information could potentially inform us what key proteins are involved in the Jak2-V617F signaling pathway that leads to aberrant cell growth.

Comparison of Z3 and G6

In this dissertation, we characterized two novel Jak2 tyrosine kinase inhibitors, termed Z3 and G6. Upon analysis, we found that Z3 and G6 shared general similarities, but also displayed differences in their inhibitory properties. The similarities and differences between Z3 and G6 could impact their potential to inhibit Jak2-dependent aberrant cell growth.

A main difference between Z3 and G6 is that G6 was more effective than Z3 in suppressing Jak2-V617F-mediated pathologic cell growth In addition, G6 was more effective than Z3 in blocking the *ex vivo* growth of hematopoietic progenitor cells isolated from the bone marrow of a myeloproliferative disorder patient. These results suggest that the structure of G6 could be more favorable for binding to the ATP pocket of Jak2 resulting in a greater inhibition of Jak2-V617F tyrosine kinase activity when compared to Z3.

Interestingly, the mechanism by which Z3 and G6 inhibited Jak2-V617F mediated pathologic cell growth was also different. Z3 inhibited the proliferation of Jak2-V617F

expressing cells by inducing cell cycle arrest while G6 suppressed the growth of these cells by promoting apoptosis. Perhaps we could take advantage of the fact that Z3 and G6 inhibit Jak2-V617F function via different mechanisms by treating Jak2-V617F expressing cells with both Z3 and G6 to determine if the combination of both drugs inhibits Jak2-V617F mediated pathologic cell growth more effectively than either drug alone.

Despite the differences between Z3 and G6, they nevertheless share similarities. Both Z3 and G6 had no effect on Tyk2 or c-Src tyrosine autophosphorylation at concentrations that completely inhibit Jak2. In addition, HEL cells expressing the Jak2-V617F mutation were more sensitive to the inhibitory effects of Z3 and G6, when compared to Raji cells that rely on a c-Myc translocation for their aberrant growth. Overall, these results suggest that Z3 and G6 are relatively selective inhibitors of Jak2 tyrosine kinase. Additionally, the fact that Z3 and G6 have no effect on Tyk2 kinase function *in vitro* is encouraging given that Tyk2 deficiency has been linked to immune functional defects and increased tumor susceptibility (Nakamura et al., 2008; Minegishi et al., 2006; Stoiber et al., 2004).

Additional Reflections Regarding Jak2 Inhibitors

In response to the growing numbers of Jak2 mutations connected with hematological malignancies, there has been an increase in the identification of Jak2 inhibitors (Figure 6-1). A possible weakness facing the current state of Jak2 inhibitors is that although these compounds are suppressing mutant Jak2 tyrosine kinase activity, they are also inhibiting wild-type Jak2 function. For example, Pardanani et al. demonstrated that a 500 nM dose of TG101209 completely inhibits wild-type Jak2 tyrosine kinase activity (Pardanani et al., 2007). Moreover, our laboratory showed that the Z3 compound inhibited Jak2-WT tyrosine autophosphorylation (IC_{50} = ~15 μM) more effectively relative to Jak2-V617F (IC_{50} = ~28 μM) (Sayyah et al., 2008).

Given that normal Jak2 function is critical for hematopoiesis and for the transmission of the growth hormone signal, one wonders about the possible deleterious effects of blocking wild-type Jak2 function.

The lack of structural information regarding full length Jak2 may currently be an impediment for the design of inhibitors that selectively target aberrant Jak2 kinase activity. In order to overcome this obstacle, the crystal structure of the entire Jak2 protein needs to be resolved so that we may have a better understanding regarding the structural differences between mutant and wild-type Jak2 protein and therefore develop inhibitors that block only mutant Jak2 kinase activity. Once our structural knowledge regarding the entire Jak2 protein increases, we may evolve to develop Jak2 designer drugs based on specific mutations and particular hematological malignancies.

Another concern is that there is evidence suggesting that additional mutations could occur before the acquisition of the Jak2-V617F mutation in some myeloproliferative disorder patients. For example, Nussenzveig et al., found homozygous wild-type Jak2 erythropoietin-independent colonies together with Jak2-V617F-positive erythroid-independent colonies in Jak2-V617F-positive polycythemia vera patients (Nussenzveig et al., 2007). These results suggests that an undefined molecular lesion, preceding the Jak2-V617F mutation, may be responsible for clonal hematopoiesis in polycythemia vera. Therefore, this raises doubts that inhibiting the Jak2-V617F protein, which may not be the founding pathogenic event in myeloproliferative disorders, will be useful.

In summary, activating Jak2 mutations are found in almost all individuals with polycythemia vera and a substantial proportion of individuals with essential thrombocythemia and primary myelofibrosis. Interestingly, since the discovery of the Jak2-V617F mutation in

2005, there has been a steady increase in the number of reported Jak2 gene aberrations in hematological disorders as well as the number of class I and II small molecule compounds that effectively target constitutive Jak2 kinase activity (Figure 6-1). The question that arises is whether inhibitors that inhibit Jak2-V617F tyrosine kinase could also block other Jak2 mutations associated with hematological malignancies. In addition, will a compound's absolute mutant over wild-type selectivity be essential for the effective treatment of hematological malignancies? Or, will we evolve to develop Jak2 designer drugs based on specific mutations and particular disease? Furthermore, will a Jak2-V617F-selective inhibitor be useful in light of the fact that a genetic lesion, preceding the Jak2-V617F mutation, could be responsible for the pathogenesis of myeloproliferative disorders? Hopefully in the coming years we will have the answers to these questions as our knowledge regarding the role of Jak2 mutations in hematological malignancies increases and we acquire more information regarding the efficacy and safety of Jak2 inhibitors that are currently in clinical trials.

The Role of Tyrosine 372 on Jak2 Function

Tyrosine autophosphorylation is a fundamental process in Jak2-dependent signaling. In Chapter 5, we investigated the role of tyrosine 372 on Jak2 function. We found via electrospray mass spectrometry that tyrosine 372 is a novel site of Jak2 autophosphorylation. We demonstrated that tyrosine 372 phosphorylation is important for maximal tyrosine autophosphorylation, STAT1 activation and Jak2-dependent gene expression. Within the context of ligand-dependent Jak2 signaling, we found that phosphorylation of tyrosine 372 is important for interferon-gamma and epidermal growth factor-mediated maximal Jak2 activation. Based on these results, we suggest that tyrosine 372 is critical for Jak2 function.

Since tyrosine 372 plays a significant role in ligand-independent and -dependent Jak2 signaling, we hypothesize that tyrosine 372 could have an impact on Jak2-mediated pathologies and Jak2-dependent cell growth.

The Potential Role of Tyrosine 372 in Autoimmune Disorders

Interferon-gamma is a strong activator of inflammatory response and plays a pivotal role in host defense by increasing antigen presentation of macrophages and promoting natural killer cell activity (Schroder et al., 2004). In addition, its excessive release has been associated with the pathogenesis of chronic inflammatory and autoimmune disease. For example, transgenic mice harboring the interferon-gamma gene linked to the human insulin promoter lead to a deficiency in insulin producing beta cells and develop insulin-dependent type I diabetes (Sarvetnick et al., 1988; Sarvetnick, 2000). In addition, interferon-gamma overexpression in the epidermis or liver of transgenic mice causes lupus nephritis or chronic active hepatitis, respectively (Toyonaga et al., 1994; Seery et al., 1997). Moreover, hyperesponsiveness to interferon-gamma activates the Jak2 signaling pathway and results in sustained STAT1 activation in human inflammatory diseases and autoimmune disorders (Bach et al., 1995; Sampath et al., 1999; Yasukawa et al., 2000; Kuhbacher et al., 2001; Mazzarella et al., 2003; Dong et al., 2007). Interestingly, there is a lack of information regarding the molecular mechanisms leading to interferon-gamma-mediated STAT1 activation in inflammatory diseases and autoimmune disorders. Therefore, understanding how Jak2 tyrosine autophosphorylation regulates the Jak2 signaling pathway in inflammatory disease and autoimmune disorders could increase our knowledge regarding these pathologies.

We have shown in Chapter 5 that loss of tyrosine 372 phosphorylation reduces Jak2 Y1007 phosphorylation, total Jak2 tyrosine phosphorylation and STAT1 activation thereby revealing the importance of tyrosine 372 in the upregulation of Jak2-dependent signaling. Importantly, we

have also shown that phosphorylation of tyrosine 372 is important for interferon-gamma mediated Jak2 activation in mouse embryonic fibroblasts. Since the pathologic effect of interferon-gamma is evident in autoimmune disorders like type 1 diabetes, and is mediated through the Jak/STAT signaling pathway, it would be important to determine whether tyrosine 372 phosphorylation is important for interferon-gamma-mediated Jak2 and STAT1 phosphorylation in immortalized insulin producing pancreatic beta cells. In addition, it would be worthy to examine if interferon-gamma dependent tyrosine 372 phosphorylation leads to beta cell dysfunction. To carry out the above experiments, immortalized pancreatic beta cells that display a normal glucose-dependent insulin secretion could be stably overexpressed with wild-type Jak2 or Jak2-Y372F and then treated with interferon-gamma to examine the consequence of tyrosine 372 phosphorylation on Jak2 and STAT1 activation in these cells (Efrat et al., 1995; Cottet et al., 2001). If interferon-gamma mediated Jak2 tyrosine 372 phosphorylation is critical for Jak2 signaling in insulin secreting beta cells, then we would anticipate the Jak2-Y372F mutation to suppress Jak2 and STAT1 phosphorylation levels in these cells relative to wild type Jak2. These results would therefore suggest that tyrosine 372 is important for interferon-gamma mediated Jak2 signaling in physiologically relevant insulin producing cell system.

Subsequently, it would be important to demonstrate that the requirement of tyrosine 372 phosphorylation for Jak2 signaling in response to interferon-gamma accompanies pathologic events in insulin producing pancreatic beta cells. It has been reported that interferon-gamma diminishes insulin gene expression, insulin cellular content and glucose-stimulated insulin secretion in beta cells and that these effects could be mediated by activation of the Jak/STAT pathway (Baldeon et al., 1998; Cottet et al., 2001). In particular, Cottet et al. have shown that when SOCS-1, a Jak2 negative regulatory protein, is stably expressed in beta cells, it can

effectively block the suppressive effects of interferon-gamma on insulin expression and secretion (Cottet et al., 2001). Therefore, their results suggest that the Jak2 signaling pathway is involved in the interferon-gamma induced defect in insulin expression and secretion. Examining the effect of Jak2 tyrosine 372 phosphorylation on interferon-gamma dependent insulin expression and glucose-stimulated insulin secretion in beta cells would give us more insight on how the Jak/STAT pathway mediates this pathologic process. Specifically, insulin mRNA and protein expression levels could be determined in interferon-gamma treated beta cells stably expressing wild-type Jak2 or Jak2-Y372F. In addition, the amount of glucose-stimulated insulin secretion could be determined in these interferon-gamma stimulated cells that are treated with glucose. We hypothesize that loss of tyrosine 372 phosphorylation would increase insulin expression and glucose-stimulated insulin levels in beta cells relative to wild-type. These results would suggest that Jak2 tyrosine 372 phosphorylation mediates the downregulation of insulin and glucose-induced insulin secretion in beta cells in response to interferon gamma which could lead to beta cell dysfunction.

If interferon-gamma signaling in insulin-secreting beta cells requires Jak2 tyrosine 372 phosphorylation to mediate the defect in insulin expression and secretion that occurs in these cells, then perhaps this pathologic effect can be blocked by developing a pharmacological inhibitor that is targeted toward tyrosine 372. We foresee that a tyrosine 372 specific inhibitor would inhibit interferon-gamma mediated Jak2 activation in beta cells leading to suppression in STAT1 phosphorylation. Furthermore, by pharmacologically blocking Jak2 tyrosine 372 phosphorylation and thus inhibiting interferon-gamma dependent Jak2 signaling, we could perhaps prevent deficits in insulin expression and excretion which lead to beta cell dysfunction in type I diabetes.

The Potential Role of Tyrosine 372 Phosphorylation in Cell Growth

Activation of epidermal growth factor receptor (EGFR) is implicated in diverse cell functions, including regulation of cell proliferation, differentiation and cell survival. In addition EGFR activation is involved in processes that are critical for cancer progression such as metastasis and angiogenesis. Epidermal growth factor receptor signaling has been shown to be mediated by Jak2 tyrosine kinase and result in the activation of STAT3 (Andl et al., 2004; Colomiere et al., 2009). In turn, STAT3 activation has been shown to increase cell proliferation and survival *in vitro* and promote tumor growth rates *in vivo*. Interestingly, not much is known regarding which of the 49 Jak2 tyrosine residues could be critical for epidermal growth factor-mediated activation of Jak2 and STAT3. In addition, there is no information about which of the phosphorylated Jak2 tyrosine residues could have an impact on cell growth and survival in response to epidermal growth factor stimulation. The more we understand about how Jak2 structure influences its cellular function, the more insight we will gain into its essential role in physiology and pathophysiology.

We have shown in Chapter 5 that loss of tyrosine 372 phosphorylation suppresses epidermal growth factor-mediated Jak2 tyrosine 1007 phosphorylation. These results suggest that phosphorylation of tyrosine 372 is critical for epidermal-growth factor-dependent Jak2 activation. Since it is established that activated Jak2 phosphorylates its substrate, STAT3 and activated STAT3 increases cell proliferation and survival, it would be pertinent to examine if Jak2 tyrosine 372 phosphorylation is required for epidermal growth factor-mediated STAT3 activation. Furthermore, it would be important to determine it tyrosine 372 phosphorylation has functional consequences for cells such as, increasing cell proliferation and survival.

To answer the above questions, it would be important to utilize fibroblast cells that stably express wild-type Jak2 or the Jak2-Y372F mutant to ensure long-term Jak2 gene expression in

cells. To determine whether tyrosine 372 is critical for Jak2-dependent-STAT3 activation in response to epidermal growth factor, we would analyze STAT3 phosphorylation levels in these cells in response to epidermal growth factor. We hypothesize that cells stably expressing the Jak2-Y372F mutation would suppress STAT3 activation relative to Jak2-WT expressing cells. These results would imply that Jak2 tyrosine 372 phosphorylation is critical for Jak2-dependent STAT3 phosphorylation in response to epidermal growth factor.

Subsequently, it would be important to investigate if tyrosine 372 is essential for cell growth and survival. To determine the role of tyrosine 372 phosphorylation on cell growth, the number viable stably expressing wild-type Jak2 or Jak2-Y372F cells that have been treated with epidermal growth factor could be compared. In addition, the role of tyrosine 372 in cell survival could be addressed by serum starving cells to introduce cell stress and then determining the number of viable cells. We anticipate that loss of tyrosine 372 phosphorylation could result in a lower number of viable cells relative to wild-type expressing cells. These results would link Jak2 tyrosine 372 phosphorylation to upregulation of cell growth and survival.

Conclusion

In Chapters 3 and 4 of this dissertation, we have identified and characterized two novel Jak2-specific small molecule inhibitors termed, Z3 and G6, which effectively inhibit Jak2-mediated pathologic cell growth *in vitro* and *ex vivo*. Due to the selective nature by which Z3 and G6 inhibit Jak2, these compounds could serve as useful research tools to further understand the Jak2 signaling pathway in the context of physiology and pathophysiology. In addition G6 could potentially serve as a therapeutic agent for the treatment of myeloproliferative disorders based on the encouraging fact that this compound potently inhibits Jak2-mediated aberrant cell growth *in vitro*, ex vivo and preliminarily, *in vivo*.

We have also advanced our understanding of how structural changes in the FERM domain affect Jak2 function by identifying a novel site of Jak2 tyrosine phosphorylation. In Chapter 5, we have shown that phosphorylation of tyrosine 372 is important for the upregulation Jak2 tyrosine autophosphorylation in the context of a ligand-independent system. In addition, we have found that phosphosphorylation of tyrosine 372 has a significant role in ligand-dependent Jak2 function by facilitating interferon-gamma and epidermal growth factor-mediated Jak2 activation. Due to the critical role of tyrosine 372 in Jak2 function, future analysis of this phosphorylation site could give us insight on how to regulate abnormal Jak2 activation in disease states.

Reflections

The identification and characterization of novel Jak2 tyrosine kinase small molecule inhibitors and the elucidation of the role of tyrosine 372 in Jak2 function are important ongoing research components in our laboratory. Characterization of Z3 and G6 contribute to the body of knowledge pertaining to Jak2 inhibition, whereas characterization of tyrosine 372 enhances our understanding regarding the relationship between Jak2 structure and function. Upon reflection, we realize that our investigations could be enhanced and rendered more complete if certain elements or important experiments are considered in the future.

Z3 and G6

Since Jak3 plays a significant role in immune function and is structurally similar to Jak2, it would be important to investigate the specificity of Z3 and G6 for inhibiting the Jak2-V617F mutant protein versus Jak3. A direct approach would be to obtain a recombinant Jak2-V617F along with a recombinant Jak3 protein and perform an *in vitro* kinase assay in the presence or absence of ATP and in the presence of DMSO or Z3/G6. The inhibition of Jak2-V617F and Jak3 tyrosine kinase activities by the Jak2 inhibitors could then be compared. In addition to Jak3,

Tyk2 and c-Src, it would be important to determine if Z3 and G6 directly inhibit other tyrosine kinases so that we may have a broader knowledge regarding the specificity of these compounds for inhibiting Jak2.

We have shown that Z3 and G6 effectively block the *ex vivo* growth of hematopoietic progenitor cells isolated from an essential thrombocythemia patient carrying the Jak2-V617F mutation and from a polycythemia vera patient harboring the Jak2-F537I mutation. Our studies would have more significance if we examine the efficacy of Z3 and G6 for inhibiting the *ex vivo* growth of hematopoietic progenitor cells obtained from a greater number of myeloproliferative disorder patients carrying Jak2 mutations. Importantly, it would be informative to genotype colonies in our *ex vivo* colony formation assays to compare the percentage of Jak2-V617F-postive myeloid and erythroid colonies in the absence or presence of Z3 or G6. These results would reveal whether selective suppression of Jak2-V617F harboring colonies derived from myeloproliferative disorder patients occurs in the presence of Z3 or G6.

Tyrosine 372

While mass spectrometry analysis revealed that tyrosine 372 is a site of Jak2 phosphorylation, acquisition of an antibody that specifically recognizes phosphorylated tyrosine 372 on Jak2 would confirm that tyrosine 372 is a Jak2 phosphorylation site. In addition, the characterization of tyrosine 372 would be augmented by the creation of construct in which tyrosine 372 is mutated to glutamic acid (Jak2-Y372E). Since the Jak2-Y372E mutation would be used to mimic the consequence of phosphorylation, we anticipate that expression of the Jak2-Y372E construct in cells would enhance Jak2 and STAT1 phosphorylation levels, validating the importance of tyrosine 372 in Jak2 functional regulation. Furthermore, given the fact that tyrosine 372 is conserved in different Jak family members, it would be informative to determine whether phosphorylation of tyrosine 372 is important for Tyk2, Jak1 or Jak3 tyrosine kinase

function. Finally, the investigation of tyrosine 372 lacks a mechanistic explanation for the tyrosine 372 mediated effects on Jak2 function. Since tyrosine 372 is situated in the FERM domain of Jak2, we hypothesized that tyrosine 372 phosphorylation may be important for Jak2 association with the cytokine receptor. Our study would greatly benefit from an evaluation of the role of tyrosine 372 in Jak2/receptor co-association.

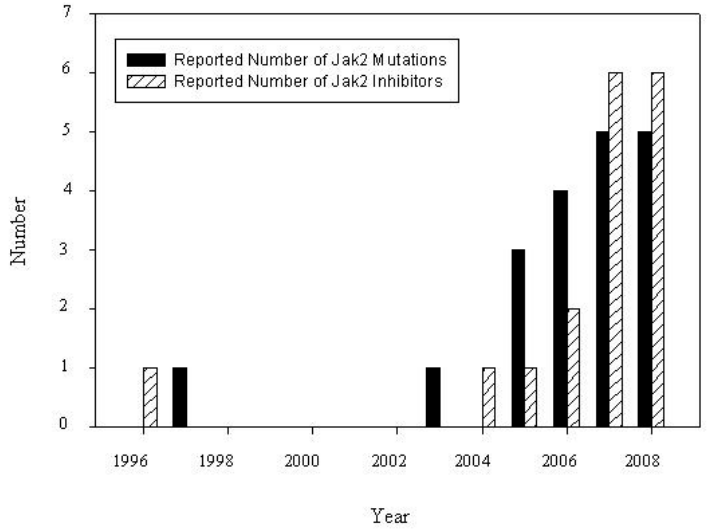

Figure 6-1. The number of reported Jak2 mutations and Jak2 inhibitors discovered by year. The reported Jak2 gene aberrations include Jak2 amino acid substitutions, deletions, insertions and chromosomal translocations that were identified since 1997. The Jak2 inhibitors consist of Jak2 and non-Jak2 selective compounds that are either in pre-clinical or clinical trials. Reprinted with permission from Current Medince Group LLC. Sayyah J and Sayeski PP. (2009) Jak2 inhibitors: Rationale and Role as Therapeutic Agents in Hematologic Malignancies. *Curr Onc Rep* 11:117-124. Figure 1, page 124.

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BIOGRAPHICAL SKETCH

Jacqueline Sayyah grew up in Santa Monica, California and attended the University of California, Los Angeles, where she received a Bachelor of Science degree in biochemistry. While attending UCLA, her interest in science was sparked by taking an introductory cancer biology class. As an undergraduate student, her first exposure to research was in the laboratory of Dr. Gayle Baldwin, where she investigated the effects of tobacco, marijuana and cocaine on human pulmonary immune cell function. Jacqueline subsequently moved to Washington D.C. to earn her M.S. degree in biochemistry and was then awarded a Cancer Research Training Award from NIH to study the role of AKT activation in lung tumorigenesis. In 2004, Jacqueline began pursuing her Ph.D. in biomedical sciences at the University of Florida, under the mentorship of Dr. Peter P. Sayeski in the Department of Physiology and Functional Genomics. She received her Ph.D. from the University of Florida in the spring of 2009.