CHARACTERIZATION OF JAK2 SMALL MOLECULE INHIBITORS FOR CANCER THERAPY AND EXAMINATION OF NOVEL CELL SURVIVAL SIGNALING MECHANISMS

By

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To my grandparents, J.M. and Hazel Rigdon
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<tr>
<td>ET</td>
<td>Essential Thrombocythemia</td>
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<td>FERM</td>
<td>4.1 protein/ezrin/radixin/moesin</td>
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<td>GBM</td>
<td>Glioblastoma</td>
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<td>HEL</td>
<td>Human Erythroleukemia</td>
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<td>MPN</td>
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<td>PV</td>
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<td>SGK1</td>
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<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
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By

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Jak2 is a non-receptor tyrosine kinase that is involved in proliferative signaling through its association with various cytokine receptors. Hyperactive Jak2 signaling has been implicated in numerous hematological malignancies as well as in various solid tumors, including glioblastoma (GBM). Our lab has developed a Jak2 small molecule inhibitor called G6, which exhibits potent efficacy in vitro and in several in vivo models of Jak2-mediated hematological malignancies. In the current studies, we sought to investigate 1) the structure activity relationship properties of G6 and 2) the therapeutic effects of G6 on GBM cells expressing hyperactive Jak2. We hypothesized that the para-hydroxyl structure of G6 would have greater Jak2 inhibitory potential than a meta-hydroxyl structure. We also hypothesized that G6 would inhibit cell growth and would reduce the invasive potential in glioblastoma cells expressing hyperactive Jak2. Using a variety of in vitro, in silico, and ex vivo approaches, we demonstrated the significance of the para-hydroxyl structure of G6. Using the T98G glioblastoma cell line as a model, we further demonstrated that G6 has efficacy against GBM cell growth, colony formation, migratory, and invasive potential. Collectively, these results indicate that the stilbene-
derived Jak2 inhibitor G6 is a potential therapy not only for hematological malignancies, but also for solid tumors such as GBM.

Angiotensin II (Ang II) is the primary signaling molecule of the renin angiotensin system (RAS) and is one of the many signaling molecules capable of regulating expression of the serum- and glucocorticoid-inducible kinase 1 (SGK1). SGK1 is known to regulate a wide variety of cellular processes, including renal sodium retention and cell survival. Here, we examined the role of SGK1 in Ang II-mediated cell survival. We hypothesized that Ang II protects cells from apoptosis by upregulating and activating SGK1. To test this, we examined the effects of Ang II stimulation on SGK1 expression and downstream signaling. We also examined the effects of Ang II treatment and siRNA-mediated SGK1 knockdown on apoptosis after serum starvation. Here, we found that Ang II treatment in fibroblasts caused an increase in SGK1 expression and activity that was correlated with increased cell survival. In these studies, we have identified a novel cell survival signaling mechanism that may be important in Ang II/SGK1-mediated diseases such as fibrosis and cancer. Collectively, these works have described novel Jak2 small molecule inhibitors for cancer therapy and a novel cell survival signaling mechanism.
Janus Kinase 2

Canonical Jak/STAT Signaling Pathway

Janus kinase 2 (Jak2) is a member of the Janus family of cytoplasmic non-receptor tyrosine kinases. Other members of the family include Jak1, Jak3, and Tyk2. Jak2 is ubiquitously expressed and can be activated by a number of cytokines and growth factors. This tyrosine kinase forms constitutive associations with various cytokine receptors, including the erythropoietin receptor (EpoR) and the thrombopoietin receptor (MPL) (Witthuhn et al. 1993; Sattler et al. 1995). Binding of a ligand to its receptor leads to receptor dimerization and allows adjacent Jak2 proteins to trans-phosphorylate each other on specific tyrosine residues, thus activating the protein. The active kinase then phosphorylates specific sites along the cytoplasmic tail of the receptor, which serve as docking sites for proteins containing an SH2 domain. Jak2 phosphorylates bound proteins, such as the Signal Transducers and Activators of Transcription (STATs), which translocate to the nucleus and mediate gene transcription (Figure 1-1A). This kinase plays a key role in signal transduction pathways regulating cell growth, proliferation, and differentiation. Jak2 knock-out mice die at embryonic day 12.5 due to a lack of definitive erythropoiesis, demonstrating that Jak2 is essential during early embryonic development (Neubauer et al. 1998; Parganas et al. 1998).

Jak2 Structure

Jak2 and the other Jak family members contain seven JAK homology (JH) domains, from the C-terminal JH1 through the N-terminal JH7 (Figure 1-1B). JH1 is the functional kinase domain and adjacent to this is JH2, which is known as the pseudo-
kinase domain. JH2 possesses an auto-inhibitory function over the kinase domain in the absence of a ligand. The Jak2 ATP-binding pocket contains several critical regions involved in catalysis, including the hinge region, the glycine loop, the catalytic loop and the activation loop (Figure 1-2A and B). The function of each of these regions is described as follows by Lucet et al. in their 2006 Blood manuscript (Lucet et al. 2006).

The hinge region contains Glu930 and Leu932, which interact with the adenine group of ATP. The glycine loop is important for substrate and nucleotide binding. The activation loop contains the tyrosine 1007/1008 residues, which upon phosphorylation allow this region to undergo a conformational change to accommodate substrate binding. The catalytic loop contains residue Arg980, which is involved in the coordination of magnesium ions. These critical residues are important for normal Jak2 function, but also serve as targets for Jak2 inhibition under pathological Jak2 activation.

**Jak2 Mutations in Hematological Malignancies**

Constitutive activation of Jak2 has been linked to a variety of pathologies including various solid-tumor cancers, hematological malignancies, and the myeloproliferative neoplasms (MPNs). The classical MPNs include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). These diseases arise from clonal expansion of a single transformed hematopoietic stem cell, which causes excessive numbers of terminally differentiated cells of the myeloid lineage to be present in the blood. PV is characterized by an increase in the number of erythrocytes, ET is associated with an excess number of platelets, and PMF is characterized by excess numbers of neutrophils and macrophages leading to fibrosis within the bone marrow. Numerous studies have established clear links between aberrant Jak2
signaling and MPNs (Lacout et al. 2006; Wernig et al. 2006; Bumm et al. 2006; Tiedt et al. 2008; Xing et al. 2008).

Interestingly, a single Jak2 mutation has been identified in all of the MPN pathologies. In 2005, a somatic Jak2 mutation in which Val is converted to Phe at position 617 (Jak2-V617F) was independently identified in MPNs by multiple research groups (Levine et al. 2005; Kralovics et al. 2005; James et al. 2005; Baxter et al. 2005; Zhao et al. 2005). This acquired mutation was identified in approximately 90% of PV patients and 50% of ET and PMF patients. Jak2-V617F occurs in exon 14 within the JH2 domain and is believed to relieve the auto-inhibitory function of this domain, thus leading to constitutive Jak2 kinase activity. Jak2 signaling often occurs through the STAT proteins, and deregulated Jak-STAT signaling leads to excessive proliferation and/or reduced apoptosis.

Not only is the Jak2-V617F mutation observed in MPN patients, but there is also a large body of evidence demonstrating a causative role of Jak2-V617F in the development of MPNs. Specifically, transplantation of retrovirally transduced bone marrow cells expressing Jak2-V617F has been shown to produce a strong PV phenotype in mice (Lacout et al. 2006; Wernig et al. 2006; Bumm et al. 2006). Additionally, transgenic mouse strains expressing Jak2-V617F in hematopoietic tissues displayed PV, ET, and PMF phenotypes (Tiedt et al. 2008; Xing et al. 2008). In one of these studies, it was shown that the severity MPN phenotypes correlated with the ratio of Jak2-V617F to wild type Jak2 expression (Tiedt et al. 2008). The Val to Phe substitution is not the only Jak2 mutation associated with these neoplasms. Several Jak2 mutations have been identified in Jak2-V617F-negative MPN patients, including
the exon 12 mutations K539L and H538Q/K539L (Scott et al. 2007). In addition, a number of V617F-negative ET and PMF patients are positive for W515L mutations in the thrombopoietin receptor (Xu et al. 2008). The common effect shared by all of these mutations is excessive downstream Jak-STAT signaling, demonstrating that Jak2 plays a significant role in the pathogenesis of MPNs.

**Role of Jak2 in Glioblastoma**

The hyper-activation of Jak2 in MPNs is well-established, but recent evidence has also implicated Jak2 signaling in various solid tumors. These include non-small cell lung carcinoma (Looyenga et al. 2012), colorectal cancer (Seavey et al. 2012), breast cancer (Berishaj et al. 2007), prostate cancer (Liao and Nevalainen 2011), and glioblastoma (GBM) (Sciaccaluga et al. 2007). Numerous studies have examined the *in vitro* and *in vivo* efficacy of Jak inhibitors as a means of therapy for solid tumors, but the mechanisms responsible for Jak2-dependent cell growth are still being investigated.

In recent years there has been evidence that Jak/STAT signaling is deregulated in glioblastoma (GBM) (Sciaccaluga et al. 2007; Senft et al. 2011). GBM is the most common and most aggressive form of primary brain tumor, with a median survival of approximately 14 months after optimal therapy. Current therapies for GBM include surgical resection, radiation therapy and chemotherapy. The most commonly used chemotherapeutic agent for GBM is temozolomide, a DNA alkylating agent. However, the success rate is still relatively low and temozolomide resistance has arisen among a large subset of GBM patients, thus requiring development of alternative therapies (Friedman et al. 2000).

Recently, many of the molecular mechanisms contributing to GBM tumor formation have been identified, leading to the development of molecular-targeted
therapies for this disease. Four distinct GBM subtypes have been identified through genomic analysis. These include Classical, Proneural, Neural, and Mesenchymal. Three of the subtypes are associated with distinct somatic mutations and/or changes in gene expression; Classical is associated with EGFR, Proneural with PDGF and IDH1, and Mesenchymal with NF1 (Verhaak et al. 2010). Several pathways that have been targeted to date include VEGF, EGFR, PDGF, PI3K, Akt, and mTOR (Agarwal et al. 2011). Unfortunately, while many of these therapies have shown pre-clinical efficacy, there has been limited success in clinical trials so far (Ohka et al. 2012).

Over the past few years, Jak/STAT signaling has been implicated in GBM, and small molecule Jak/STAT inhibitors have been investigated for GBM treatment in vitro and in vivo. In 2007, constitutive phosphorylation of Jak2 was found in the GL15 glioblastoma cell line, and treatment with the tyrphostin AG490 was shown to induce cell cycle arrest in these cells (Sciaccaluga et al.). More recently, several studies have demonstrated efficacy of AG490 and other more specific Jak2 inhibitors in cell culture and animal models (McFarland et al. 2011; Sai et al. 2012). Given this recent implication of Jak/STAT signaling as a significant factor in glioblastoma pathogenesis, there is great interest in further investigating the use of Jak2 inhibitors as a means of therapy.

Development of Jak2 Inhibitors

Given the important role of Jak2 in hematological malignancies and solid tumors, there has been much effort within the last few years to develop specific Jak2 inhibitors. Interestingly, the history of Jak2 tyrosine kinase inhibitors extends back nearly two decades. The first Jak2 inhibitor to be developed was tyrphostin AG490. It was initially found to inhibit type I Fc epsilon receptor-induced PLC gamma 1 phosphorylation and
ensuing inositol phosphate formation (Schneider et al. 1992). Subsequent work demonstrated that AG490 also inhibited Jak2 tyrosine kinase activity and blocked acute lymphoblastic leukemic cell growth, in vitro and in vivo (Meydan et al. 1996). While this later work underscored the importance of small molecules in the inhibition of Jak2-mediated disease, it simultaneously called attention to the non-specific nature of AG490. As a consequence, several derivative compounds of AG490 were developed with the hope of making a more potent and/or specific Jak2 inhibitor (Gu et al. 2005; Iwamaru et al. 2007; Ferrajoli et al. 2007). While the progress of Jak2 inhibitors moved along at a reasonable pace, the field began growing more rapidly in 2005 when the Jak2-V617F mutation was identified in a large percentage of MPN patients (Levine et al. 2005; Kralovics et al. 2005; James et al. 2005; Baxter et al. 2005; Zhao et al. 2005).

A number of Jak2 small molecule inhibitors have been investigated in clinical trials, and one such compound recently achieved FDA approval for the treatment of myelofibrosis. This compound, ruxolitinib, significantly reduced splenomegaly and reduced the overall symptom score in high-risk myelofibrosis patients in clinical trials (Deisseroth et al. 2012). However, the effects of this drug appear to be primarily palliative rather than curative, and ruxolitinib is associated with high withdrawal rates due to incidence of high grade side effects such as thrombocytopenia and anemia (Tefferi et al. 2011). The goal of Jak2 inhibitor therapy is not only to relieve disease symptoms, but also to cure patients by eliminating the Jak2 mutant clones within the bone marrow. In one study, ruxolitinib was shown to only reduce Jak2-V617F allele burden in the peripheral blood by approximately 10% (Verstovsek et al. 2008a). In addition, the patient response rates to ruxolitinib have been shown to be independent of
Jak2-V617F mutation status (Verstovsek et al. 2008b). Other Jak2 inhibitors in clinical trials include CEP-701 (lestaurtinib), TG101348, SB1518, and CYT387. Unfortunately, many of the Jak2 inhibitors in MPN clinical trials have had little to no bone marrow efficacy thus far (Majumder and Sayeski 2010); therefore, there is still a large unmet need for more efficacious MPN therapies.

Using techniques such as library screening, molecular docking, fragment-based drug discovery, scaffold morphing, and derivatization of lead compounds, numerous laboratories continue to develop Jak2 inhibitors. The majority of these inhibitors target the ATP-binding pocket within the Jak2 kinase domain (JH1). These compounds belong to several different structural classes including pyrazines, pyrimidines, azaindoles, aminoindazoles, deazapurines, stilbenes, benzoxazoles and quinoxalines. Through structure-based optimization, a number of functional groups have been identified that play crucial roles in potent and specific Jak2 inhibition.

Through structure-based in silico screening, our laboratory identified a novel Jak2 inhibitor with a stilbene core (Kiss et al. 2009). This compound, known as G6, forms hydrogen bonds with Leu932, Asp994, Glu930, and Arg980 in the ATP-binding pocket of Jak2 (Majumder et al. 2010). Our group was one of the first to show that Asp994 could be a significant point of interaction for Jak2 inhibition beyond the established hydrophobic interactions with the hinge region of Jak2. We have demonstrated that the stilbene structure is essential for the activity of G6, as non-stilbenoid derivatives show a reduction in Jak2 inhibition in vitro and ex vivo and bind the protein less efficiently in silico (Majumder et al. 2010). We have shown that G6 inhibits Jak2-mediated pathologic cell growth in several cell culture models, three in vivo
MPN models, and in ex vivo MPN patient samples (Kiss et al. 2009; Kirabo et al. 2011a, 2011b, 2012). Additionally, we have shown that G6 reduces the Jak2 mutant allele burden in the bone marrow in mouse MPN models (Kirabo et al. 2011a, 2011b, 2012). This compound also significantly reverses bone marrow fibrosis in PMF mice (Kirabo et al. 2012). Thus, further investigation of this compound as a potential therapy for Jak2-mediated pathologies is of great interest.

**Rationale for Jak2 Inhibitor Studies**

Jak2 plays a critical and causative role in the development and progression of MPNs, and has been implicated in the pathogenesis of numerous solid tumors. To date, molecular targeted therapies to inhibit Jak2 signaling have been effective at relieving disease symptoms in MPN patients, but have not been curative in nature. Therefore, there is still a need for more efficacious Jak2 inhibitors. Our lab has identified a potent and specific Jak2 small molecule inhibitor known as G6. This compound contains a core stilbene structure, and is the first known stilbene compound to show anti-Jak2 activity. Therefore, examining the structure activity relationship (SAR) properties of this compound could be valuable in gaining an understanding of how to enhance Jak2 binding to improve inhibitory potential. In addition, the role of Jak2 in solid tumors such as glioblastoma has only come to light within the past few years. The signaling mechanisms involved are still being investigated and the impact of Jak2 inhibitors has yet to be fully determined. Thus, the ability of Jak2 inhibitors to serve as a viable therapy for solid tumors as well as hematological malignancies is an important scientific question to address. Chapters 2-5 will describe experiments aimed at 1) understanding the structure-function correlations and structural optimization of G6 and 2) examining the efficacy of this compound in a Jak2-dependent glioblastoma tumor model.
Angiotensin II

Signaling Mechanisms

Angiotensin II (Ang II) is the primary signaling molecule of the renin-angiotensin system (RAS). In the systemic RAS, renin is secreted from the kidney into the circulation under low blood volume conditions. Renin then converts the precursor angiotensinogen (AGT) into the decapeptide angiotensin I. Angiotensin I is then cleaved by angiotensin-converting enzyme (ACE) to produce the octapeptide Ang II. Ang II may bind to the angiotensin II type 1 or type 2 receptors (AT1R and AT2R). These two G protein-coupled receptors have been shown to have opposing physiological effects. In the classic RAS, Ang II binds to the AT1R and has potent vasoconstrictor activity, leading to an increase in blood pressure. The AT1R signaling axis involves activation of various heterotrimeric G proteins, leading to the production of a wide variety of second messengers. This signaling axis also exhibits cross-talk with a number of tyrosine kinases and serine/threonine kinases (Saito and Berk 2001; Hunyady and Catt 2006). In addition to the classical systemic RAS, local tissue RAS have been identified in the kidney, heart, brain and vasculature, among other tissues. The tissue RAS contains all of the typical RAS components and acts independently of the systemic components. In addition to its role in vasoconstriction, Ang II signaling through the AT1R is known to be growth stimulating, pro-angiogenic, pro-inflammatory, pro-fibrotic and anti-apoptotic in various tissues. Alternatively, the AT2R is typically associated with vasodilatory action, anti-inflammatory and pro-apoptotic signaling (Nguyen Dinh Cat and Touyz 2011). The AT2R signaling axis involves activation of protein tyrosine phosphatases and nitric oxide production (Berk 2003).
Role of Angiotensin II in Disease

Given the various physiological effects of Ang II acting through the AT1R, it is not surprising that this peptide has been implicated in a variety of physiological and pathological conditions. Its regulation of blood pressure makes Ang II an important factor in hypertension and cardiovascular disease. In addition to the systemic RAS, local tissue RAS have been shown to play important roles in many of these pathological conditions (Westcott et al. 2009; Siragy and Carey 2010; De Mello and Frohlich 2011). Ang II contributes to hypertension-dependent cardiac hypertrophy via multiple mechanisms, including secretion of various factors from cardiac fibroblasts and activation of mitogen-activated protein kinase (MAPK) signaling (Cacciapuoti 2011). Ang II also contributes to hypertension-dependent cardiac remodeling through its activation of Janus kinases (JAKs), platelet-derived growth factor (PDGF), and epidermal growth factor receptor (EGFR) pathways (Mehta and Griendling 2007). The pro-fibrotic effects of Ang II, such as increased connective tissue growth factor (CTGF) expression, contribute to fibrosis in many tissues including the heart, lung, kidney and liver (Pereira et al. 2009; Ruiz-Ortega et al. 2006; Mehta and Griendling 2007; Uhal et al. 2012). In addition, many of the pathological effects of Ang II signaling involve inflammatory cytokine signaling and production of reactive oxygen species (ROS). These mechanisms, for example, have been linked to Ang II-mediated insulin resistance (Mehta and Griendling 2007). Ang II has also been implicated in various cancers, including breast and prostate cancer (Zhao et al. 2010b; Uemura et al. 2011). The role of Ang II in cancer is believed to be at least in part due to its growth promoting and anti-apoptotic signaling through the AT1R.
Role of Angiotensin II in Cell Survival

The role of Ang II in cell survival varies depending on the tissue, physiological state, and receptor expression. Ang II acts through the AT1R to promote cell survival in various cell types, but promotes apoptosis through the AT2R in other cell types. For example, it has been shown that Ang II/AT1R signaling promotes cell survival in breast cancer cells, and that blockade of AT1R using angiotensin AT1 receptor blockers (ARBs) can inhibit prostate cancer cell growth (Zhao et al. 2010b; Uemura et al. 2011). In addition, Ang II has been shown to protect fibroblast-like synoviocytes from apoptosis in an AT1R-dependent manner (Pattacini et al. 2007).

Serum and Glucocorticoid Inducible Kinase 1

Signaling Mechanisms

The serum and glucocorticoid inducible kinase 1 (SGK1) is a ubiquitously expressed serine/threonine kinase that plays a key role in the regulation of renal sodium retention and fluid homeostasis. SGK1 regulates renal sodium retention and potassium excretion by regulating epithelial sodium channel (ENaC) and Na⁺-K⁺-ATPase expression and activity. This kinase also regulates expression and activity of a wide variety of other ion channels and transporters. SGK1 transcription is regulated by glucocorticoids, mineralocorticoids, transforming growth factor-β (TGF-β), PDGF, protein kinase C (PKC), intracellular calcium, and many other signaling molecules. The kinase activity of SGK1 is regulated by phosphorylation at several key residues, including Ser422 within the hydrophobic motif of the protein and Thr256 within the active site (Lang et al. 2006). It has been established that phosphorylation at Thr256 is dependent on PDK1, and mTOR has been implicated in the phosphorylation at Ser422 (Kobayashi and Cohen 1999; Garcia-Martinez and Alessi 2008; Hong et al. 2008; Lu et
SGK1 is highly expressed in the kidney, heart, brain, lung, and gastrointestinal tract. It is important in kidney function, contributes to cellular glucose uptake in many tissues, and is associated with learning and memory in the hippocampus (Lang et al. 2006). Deletion of SGK1 causes impaired salt retention under salt-depleted conditions and decreased glucose uptake to numerous tissues. SGK1 expression in the kidney is regulated by aldosterone binding the mineralocorticoid receptor (Lang et al. 2006).

**Role of SGK1 in Disease**

Due to its important role in sodium retention and fluid balance, SGK1 signaling has an impact on blood pressure and cardiovascular health. Thus, SGK1 has been implicated in the pathology of numerous diseases such as hypertension, obesity, and metabolic syndrome (Lang et al. 2006). A number of SGK1 gene variants have been associated with high blood pressure. Additionally, SGK1 expression is elevated in a number of fibrotic diseases, including liver, kidney, heart, and lung fibrosis (Lang et al. 2006). It has been shown that SGK1 regulates CTGF expression, which may contribute to its pro-fibrotic effects. For example, in salt-sensitive rats, a high salt diet was associated with cardiac fibrosis that correlated with upregulation of SGK1 and CTGF (Matsui et al. 2008). In addition, SGK1 plays a role in inflammatory signaling through upregulation of ICAM-1 (Terada et al. 2008). SGK1 has also been implicated in the pathogenesis of cancers such as non-small cell lung cancer, in which high SGK1 expression correlated with poor prognosis (Abbruzzese et al. 2012).

**Role of SGK1 in Cell Survival**

Numerous studies have demonstrated a role for SGK1 in promoting cell survival in various tissues. One mechanism by which SGK1 is known to promote cell survival is
through the phosphorylation of the transcription factor FOXO3A. SGK1 phosphorylates FOXO3A, thus inhibiting its activity and blocking the downstream expression of pro-apoptotic factors (Brunet et al. 2001). It has been shown that SGK1 promotes cell survival in breast cancer cells (Wu et al. 2004) and colorectal carcinoma cells (Lang et al. 2010). This kinase has also been shown to mediate androgen receptor-dependent cell survival, a mechanism which may be important in prostate cancer (Shanmugam et al. 2007). Loss of SGK1 causes an increase in apoptosis in cardiac endothelial and vascular smooth muscle cells (Catela et al. 2010). SGK1 also contributes to the protective effect of erythropoietin during renal ischemia/reperfusion (Rusai et al. 2010).

**Rationale for Angiotensin II/SGK1 Study**

Angiotensin II and SGK1 have overlapping roles in various pathological conditions, including cardiovascular disease, fibrosis, and cancer. It is known that SGK1 is influenced by Ang II through the classical RAS, since SGK1 is transcriptionally regulated by aldosterone in the kidney. However, the ability of Ang II to influence SGK1 expression and activity independently of aldosterone signaling remains largely under-investigated. One interesting characteristic shared by both of these signaling molecules is their ability to regulate cell survival. Strikingly, both Ang II and SGK1 have been independently implicated in cell survival of breast cancer and prostate cancer cells. Also, Ang II has been shown to protect fibroblast cells from apoptosis, and has separately been shown to upregulate SGK1 expression in fibroblasts. Therefore, it seems relevant to investigate whether there is a direct signaling mechanism linking Ang II and SGK1 to cell survival. Additionally, given the important role of both of these signaling molecules in fibrotic diseases, it seems reasonable to use fibroblast cells as a model to investigate this question. Chapter 6 will describe experiments that were
conducted in order to address the role of Ang II/SGK1 signaling in fibroblast cell survival.
Figure 1-1. Structure and classical signaling pathway of Jak2. A) The classical Jak-STAT signaling pathway involves binding of a ligand to its receptor, leading to Jak2 activation and phosphorylation of downstream targets such as STATs. This pathway typically leads to up-regulation of pro-growth and/or pro-differentiation genes. B) Jak2 contains seven Jak homology domains and four functional domains. From C-terminal: the kinase domain (red), the pseudo-kinase domain (blue), the SH2-like domain (green) and the FERM domain (yellow).
Figure 1-2. Structure of the Jak2 kinase domain. A) Jak2 kinase domain with the ATP-binding pocket shown inside the box. B) Closer view of the ATP-binding pocket showing key residues. Critical regions are shown by color, with the glycine loop in blue, the hinge region in cyan, the catalytic loop in magenta and the beginning of the activation loop in red.
Janus kinase 2 (Jak2) is a member of the Janus family of non-receptor tyrosine kinases and plays a key role in numerous signaling pathways regulating cell survival, proliferation, and differentiation. Jak2 forms associations with growth factor and cytokine receptors, and upon activation, it phosphorylates substrates such as the Signal Transducers and Activators of Transcription (STATs) (Witthuhn et al. 1993; Argetsinger et al. 1993; Sattler et al. 1995; Parganas et al. 1998). Activated STAT proteins then translocate to the nucleus to modulate gene transcription. Mutations in Jak2 can lead to constitutive activation of the kinase and thus cause aberrant downstream Jak-STAT signaling. Specifically, the Jak2-V617F mutation occurs within the pseudo-kinase domain of Jak2 and relieves the auto-inhibitory function of this domain, thus allowing the kinase domain to be constitutively active in the absence of ligand (Zhao et al. 2010a; Gnanasambandan et al. 2010).

In 2005, the Jak2-V617F mutation was discovered in a large number of myeloproliferative neoplasm (MPN) patients (Levine et al. 2005; Kralovics et al. 2005; James et al. 2005; Baxter et al. 2005; Zhao et al. 2005). These patients suffer from an overproduction of blood cells of the myeloid lineage. This heterogeneous group of diseases includes polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). The Jak2-V617F mutation occurs in over 90% of PV patients and a majority of ET and PMF patients.

This mutation has also been shown to cause MPN phenotypes in bone marrow

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1This chapter has been reproduced in part with permission from Identification of novel SAR properties of the small molecule inhibitor G6: Significance of the para-hydroxyl orientation. Bioorg Med Chem Lett 2012; 22(3):1402-07 ©Elsevier
transplant and transgenic mouse models (Bumm et al. 2006; Xing et al. 2008). Currently, there are no effective therapies for these diseases beyond palliative treatments such as hydroxyurea and phlebotomy. Therefore, Jak2 has been investigated in recent years as a potential therapeutic target, and a number of compounds are in pre-clinical and clinical development (Kiss et al. 2010).

Our lab recently used an *in silico* drug discovery approach to develop a Jak2 small molecule inhibitor known as G6 (Kiss et al. 2009). We have shown that G6 inhibits Jak2-mediated pathologic cell growth using *in vitro* cell culture models, three *in vivo* models, and *ex vivo* patient samples (Majumder et al. 2010, Kirabo et al. 2011a, 2011b, 2012). We also demonstrated that the stilbene core structure of G6 is essential for its activity as a Jak2 inhibitor, *in vitro* and *ex vivo* (Majumder et al. 2010). Here, we further examined the structure of G6 to determine if structural modifications of the compound could enhance its Jak2 inhibitory potential. We hypothesized that the structure of G6 could be minimized in order to enhance its Jak2 inhibitory potential and that maintaining the *para*-hydroxyl orientation is required for Jak2 inhibition. To test this, we synthesized a set of G6 derivatives with different structural features (Figure 2-1) and examined their *in silico* Jak2 binding properties and their effects on Jak2-mediated pathologic cell growth *in vitro* and *ex vivo*.

**Materials and Methods**

**Computational Docking**

The program AutoDock 4.2 (Scripps) was used to computationally dock each compound into the Jak2 ATP-binding pocket (Morris et al. 1998; Huey et al. 2007). The Jak2 crystal structure 3E64 was obtained from the RSCB Protein Data Bank (Antonysamy et al. 2009). Ligands were generated and energy minimized using ProDrg
Chimera was used to prepare the protein and ligand structures by adding hydrogens and charges (Pettersen et al. 2004). The protein structure was also prepared by removing the ligand and water molecules. Additionally, phospho-tyrosine residues 1007 and 1008 were converted to tyrosines using Chimera. AutoDockTools (Scripps) was used to prepare the ligand and protein for docking. The grid had a size of 52 x 56 x 52 Å and was centered on the Jak2 ATP-binding pocket (coordinates X 31.343, Y 37.696, Z 35.393). Docking was performed using Lamarckian GA energy scoring. The docking orientation from the most populated, lowest energy cluster was analyzed for each compound. Hydrogen bonds and hydrophobic interactions were analyzed using Chimera.

**Compounds and Cell Culture**

All compounds were synthesized by the laboratory of Dr. Kirpal Bisht (University of South Florida). Human erythroleukemia (HEL) cells were purchased from the American Type Culture Collection. Ba/F3-EpoR-Jak2-V617F cells were created as described previously (Pradhan et al. 2010). Both cell lines were cultured in RPMI 1640 (Mediatech) supplemented with 10% FBS, penicillin, streptomycin, and L-glutamine at 37°C and 5% CO₂.

**Cell Proliferation Assay**

HEL cells or Ba/F3-EpoR-Jak2-V617F cells were plated in 96-well plates and treated with either 0.25% DMSO or varying concentrations of G6 and its derivatives for the indicated periods of time. Cell viability was determined by trypan blue exclusion staining or by MTS (Promega) according to the manufacturer's protocol.
Cell Lysis and Immunoprecipitation

HEL cells were treated with DMSO or 25 μM of the different compounds for 24 h. Approximately $10^7$ cells were then lysed in 0.8 ml of ice-cold radioimmunoprecipitation assay buffer, and protein concentration was determined using a Bradford assay (Bio-Rad). For immunoprecipitation, the cell lysates (~2500 μg) were then incubated with 2 μg of the STAT5 antibody and 20 μl of protein A/G beads (Santa Cruz Biotechnology) for 4 h at 4°C with constant shaking. The protein complexes were washed three times with immunoprecipitation wash buffer (25 mm Tris, pH 7.5, 150 mm NaCl, and 0.1% Triton X-100) and then resuspended in SDS sample buffer. Immunoprecipitated proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. For whole cell protein lysates, ~50 μg of soluble protein was separated via SDS-PAGE and then transferred to nitrocellulose membranes for analysis by Western blotting.

In vitro Jak2 Kinase Assay

Recombinant Jak2 protein encoding amino acids 808-1132 (Abcam) was incubated with a recombinant GST-STAT1 substrate in a reaction buffer containing: 60 mM HEPES, 5 mM MgCl$_2$, 5 mM MnCl$_2$, 3 μM Na$_3$VO$_2$, 12.5 μM DTT, and 1 μM ATP. Reactions were allowed to run for 30 minutes at room temperature in the presence or absence of 0.025% DMSO or 2.5 μM of each compound. Reactions were stopped by the addition of 4x SDS sample buffer and subsequently analyzed by Western blot.

Western Blotting

Nitrocellulose membranes were blocked with 5% milk/TBS-T for 1 h and then probed with primary antibodies. The immunoreactive bands were visualized using the enhanced chemiluminescence system (Western Lightning® Ultra; PerkinElmer Life
The following antibodies were obtained from Santa Cruz Biotechnology and were used at the indicated dilutions: STAT5 (1:1000), STAT1 (1:1000), cyclin A (1:250), CDK1 (1:500). The following antibodies were obtained from Cell Signaling Technology and were used at a 1:500 dilution: phospho-STAT5, poly(ADP-ribose) polymerase, Bid, Bim, caspase-3, and β-actin. The cyclin B1 antibody was obtained from Abcam and was used at a 1:500 dilution. The antibody for phospho-STAT1 was from Millipore and was used at 1:1000. The phospho-Jak2 antibody was from Invitrogen and was used at 1:1000.

**Real Time PCR**

HEL cells were treated with 25 μM of the different drugs for 16 h and RNA was extracted using an RNeasy® mini kit (Qiagen) according to the manufacturer's protocol. 2 μg of each RNA sample was reverse transcribed into cDNA in a final reaction volume of 20 μl using a high capacity cDNA reverse transcription kit (Applied Biosystems). The level of expression of Bcl-xL was determined using the TaqMan® gene expression assay Hs00236329_m1 (Applied Biosystems). The level of glyceraldehyde-3-phosphate dehydrogenase expression was determined as an internal loading control using the assay Hs02758991_g1 (Applied Biosystems). Real time PCR was then performed with TaqMan® universal PCR Master Mix (Applied Biosystems) in a final reaction volume of 20 μl in a StepOne™ real time PCR system according to the manufacturer's protocol (Applied Biosystems).

**Apoptosis Assay**

HEL cell apoptosis levels were determined using the FITC annexin V apoptosis detection kit (BD Pharmigen) according to the manufacturer's protocol. The cells were
incubated with DMSO or 10 μM of each compound for 48 h and then analyzed using a FACSCalibur™ flow cytometer (BD Biosciences).

**Cell Cycle Assay**

HEL cell DNA content was measured using the CycleTEST PLUS™ DNA Reagent Kit according to the manufacturer’s protocol (BD Biosciences). Cells were incubated with DMSO or 10 μM of each compound for 24 h and analyzed using a FACSCalibur™ flow cytometer (BD Biosciences).

**Colony Formation Assay**

Jak2-V617F transgenic mice were generously provided by Dr. J. Zhao (Xing et al. 2008). Bone marrow aspirates were obtained from these mice at 6 months of age. Cells were cultured in Iscove’s modified Dulbecco’s medium in the presence of 25 μM of each compound for 0, 12, or 24 h. The drug was removed via extensive washing and the cells were then cultured in MethoCult® medium in the absence of cytokine at a concentration of $4 \times 10^4$ cells/ml. After 6 days of growth, the number of erythroid colony forming units (CFU-E) and granulocyte/macrophage colony forming units (CFU-GM) were counted.

**Statistical Analysis**

Statistical evaluation was performed using a Student’s $t$-test. Conditions were considered statistically significant when $p < 0.05$.

**Results**

**Molecular Docking**

The Jak2 ATP-binding pocket contains several critical regions involved in catalysis, including the hinge region, the glycine loop, the catalytic loop and the activation loop (Lucet et al. 2006). The glycine loop is important for substrate and
nucleotide binding. The hinge region contains Glu930 and Leu932, which interact with the adenine group of ATP. The activation loop contains the tyrosine 1007/1008 residues that, once phosphorylated, allow this region to undergo a conformational change to accommodate substrate binding. The catalytic loop contains residue Arg980, which is involved in the coordination of magnesium ions.

Each of the G6 derivatives was docked into the Jak2 ATP-binding pocket using AutoDock 4.2 (Scripps). Figure 2-2 shows the most favorable docking orientation for each compound. NB13 and NB15 showed interactions very similar to those of the parent compound, G6 (Majumder et al. 2010). These include hydrogen bonds with residues in the hinge region, the activation loop, and the catalytic loop (Figure 2-2C and D). NB4 and NB6 showed hydrogen bond interactions with several residues, but lacked any interactions with the critical hinge region residues, Glu930 or Leu932 (Figure 2-2A and B). These results indicate that the para-hydroxyl derivatives (NB13 and NB15) most closely mimic the in silico Jak2 binding properties of G6.

**Inhibition of Jak2-V617F-Dependent Cell Growth**

G6 is known to inhibit the proliferation of human erythroleukemia (HEL) cells in vitro and in vivo (Kiss et al. 2009; Kirabo et al. 2011a). The HEL cell line is homozygous for the Jak2-V617F mutation and is highly proliferative due to constitutive Jak2 kinase activity. Therefore, we examined the ability of the four G6 derivatives to inhibit HEL cell growth in both a dose- and time-dependent manner. The cells were treated with increasing concentrations of each compound for 72 hours or with a 10 µM concentration for 24, 48, or 72 hours and cell viability was determined. We found that NB15 had the most potent effect on HEL cell growth in both a dose- and time-dependent manner (Figure 2-3A and B). This compound demonstrated 50% cell death at a concentration of
0.85 µM, and completely blocked cell growth at all time points measured (Figure 2-3A and B). The other derivatives had varying levels of cell growth inhibition, with NB6 consistently showing the poorest level of inhibition (Figure 2-3A and B).

In order to determine the effects of these compounds on another Jak2-V617F-positive cell line, we examined their impact on cell growth in the Ba/F3-EpoR-Jak2-V617F cell line. This murine pro B cell line has been retrovirally transduced with the Jak2-V617F mutant cDNA and demonstrates cytokine independent growth (Pradhan et al. 2010). Again, cells were treated with increasing concentrations of each drug for 72 hours or with a 10 µM concentration for 24, 48, or 72 hours and cell viability was determined. We found that again NB15 was highly effective at inhibiting cell growth in both a dose- and time-dependent manner (Figure 2-3C and D). Also, NB6 consistently had little impact on cell growth, except in this case at the highest concentration (Figure 2-3C and D). Overall, the data in Figure 2-3 demonstrate that NB15 has the greatest cell growth inhibitory potential while NB6 has the lowest, as observed in two independent models of Jak2-V617F-dependent cell growth.

Inhibition of In vitro Jak2 Kinase Activity

In order to determine if the reduced cell growth inhibitory potential observed in Figure 2-3 correlated with reduced Jak2 kinase activity per se, Jak2 in vitro kinase assays were carried out in the presence of a known Jak2 substrate, STAT1. Specifically, recombinant Jak2 protein was incubated with a STAT1 substrate in the presence or absence of a 2.5 µM concentration of each compound. After completion of the enzymatic reactions, the samples were analyzed by Western blot for phosphorylation of both Jak2 and STAT1. We found that NB15 significantly reduced Jak2 autophosphorylation (Figure 2-4A and B). Both NB15 and NB13 significantly
reduced the ability of Jak2 to phosphorylate the STAT1 substrate (Figure 2-A and C). The two *meta*-hydroxyl compounds had no significant effect on Jak2 autophosphorylation or STAT1 phosphorylation (Figure 2-A, B and C). Overall, these results indicate that the *para*-hydroxyl orientation confers greater *in vitro* Jak2 inhibitory potential than the *meta*-hydroxyl orientation, with NB15 demonstrating the highest level of Jak2 kinase inhibition.

**Inhibition of Jak-STAT Signaling in HEL Cells**

Jak2 activation leads to the phosphorylation of the transcription factor STAT5 (Sattler et al. 2005). STAT5 activation plays an important role in HEL cell proliferation, and we have previously shown that G6 reduces STAT5 phosphorylation in HEL cells (Majumder et al. 2010). Therefore, we wanted to determine whether specific structural features in G6 correlate with reduced STAT5 phosphorylation in these cells. Figure 2-5A shows a representative phospho-STAT5 blot and Figure 2-5B shows the densitometric quantification of all experiments. We found that only the *para*-hydroxyl derivative NB15 was able to significantly reduce STAT5 phosphorylation when compared to vehicle treated control cells. The data in Figure 2-5 indicate that the *para*-hydroxyl orientation with a minimized stilbenoid core, correlates with significantly reduced levels of phospho-STAT5 expression in HEL cells.

**Induction of Cell Cycle Arrest and Apoptosis in HEL Cells**

Constitutively active Jak-STAT signaling leads to increased cell proliferation and inhibition of apoptosis. We have previously shown that the mechanism by which G6 decreases HEL cell viability is induction of cell cycle arrest and apoptosis (Kirabo et al. 2011a). Therefore, we next wanted to determine if specific changes in compound structure correlate with specific changes in cell cycle properties and/or apoptosis. For
this, cells were treated with each compound at a 10 µM concentration for 24 hours and then analyzed by flow cytometry for cell cycle properties. We found that NB15 and NB4 caused a significant increase in the number of cells in G2 with a corresponding decrease in G1, indicating a G2/M phase arrest (Figure 2-6A). In order to further confirm the G2/M arrest, cells were again treated with 25 µM of drug for 24 hours and the levels of several cell cycle regulatory proteins were examined via Western blot analysis. We found that treatment with NB15 and NB4 caused a reduction in cyclin A expression, but had no effect on cyclin B1 or CDK1 expression. The other compounds had no effect on cyclin A, cyclin B or CDK1 expression (Figure 2-6B). During cell cycle progression, CDK1 forms associations with both cyclin A and cyclin B1 in order to promote mitotic entry (Knoblich and Lehner 1993; King et al. 1994). Therefore, it appears that NB15 and NB4 may cause G2 arrest due to decreased cyclin A expression and a subsequent block of mitotic entry.

We also wanted to determine any possible structure activity relationships between the derivative compounds and apoptosis. For this, HEL cells were treated with a 10 µM concentration of each drug for 48 hours and analyzed by flow cytometry. We found that NB15, NB13, and NB4 induced apoptosis in HEL cells whereas NB6 was without effect (2-6C). We further confirmed the induction of apoptosis by examining expression of apoptotic markers via Western blot. After treating HEL cells for 24 hours at a 25 µM concentration of compound, we first examined cleavage of poly (ADP-ribose) polymerase, a hallmark of apoptosis. Additionally, we examined the cleavage of Bid, Bim and caspase-3. Bid and Bim are pro-apoptotic factors that have been shown to be cleaved into active forms during apoptosis via the intrinsic pathway, and caspase-3 is
an effector caspase that is also cleaved into its active form during apoptosis (Gross et al. 1999; Gomez-Bougie et al. 2005; Scoltock and Cidlowski 2004). We found that NB15, NB13, and NB4 induced cleavage of PARP while NB6 did not (Figure 2-6D). NB4 also caused cleavage of caspase-3. However, only NB15 induced cleavage of PARP and caspase-3, along with cleavage of Bid and Bim (Figure 2-6D). Overall, these results indicate that the minimized para-hydroxyl derivative, NB15, has the most potent impact on cell cycle progression and apoptosis in HEL cells.

**Inhibition of Jak2-V617F-Mediated Bone Marrow Colony Formation**

It has been shown that Jak2-V617F transgenic mice develop a mixed MPN phenotype, with pathologically high numbers of erythrocytes, white cells and platelets (Xing et al. 2008). We have previously shown that G6 has efficacy in this transgenic model, as it is able to normalize the cell counts and cause a reversal of other disease symptoms (Kirabo et al. 2011b). Therefore, we wanted to determine if specific structural changes to G6 correlate with the ability to reduce the clonogenic growth potential of bone marrow cells obtained from the Jak2-V617F transgenic mice. For this, bone marrow cells from Jak2-V617F transgenic mice were isolated and cultured ex vivo in the presence of each compound for 0, 12, or 24 h, then washed extensively and transferred to methylcellulose media. They were then cultured for an additional 6 days, at which time the number of erythroid colony forming units (CFU-E) and granulocyte/macrophage colony forming units (CFU-GM) were counted. The numbers of observed colonies were then plotted as a function of derivative compound. The results for these experiments are shown in Figure 2-7. We found that NB4 significantly reduced the number of CFU-E and CFU-GM after 12 and 24 hours of treatment (Figure 2-7A). The only observed effect with NB6 was a significant reduction in the numbers of CFU-GM at the 24 hour time
point (Figure 2-7B). NB13 significantly reduced both the numbers of CFU-E and CFU-GM, but only after 24 hours (Figure 2-7C). Lastly, we found that NB15 significantly reduced or completed eliminated all clonogenic growth potential (Figure 2-7D). Overall, these results indicate that NB15 had the most potent effect on ex vivo colony formation of Jak2-V617F-positive bone marrow cells.

**Discussion**

In this study, we identified important structural features of the Jak2 small molecule inhibitor G6. We tested a series of derivatives with a minimized structure containing the native para-hydroxyl orientation or an alternative meta-hydroxyl orientation. We found that the para-hydroxyl structure with a reduced stilbene core conferred more abundant and stronger Jak2 interactions in silico (Figure 2-2) as well as greater Jak2 inhibitory potential in vitro and ex vivo as measured by reduced cell viability (Figure 2-3), reduced Jak2 kinase activity (Figure 2-4), reduced the levels of phospho-STAT5 (Figure 2-5), increased cell cycle arrest and apoptosis (Figure 2-6) and reduced Jak2 clonogenic growth potential (Figure 2-7).

*In silico* derived Jak2 binding properties do not necessarily lend themselves to effective Jak2 inhibition in cell-based assays. Therefore, we further tested these compounds for their ability to inhibit Jak2-mediated cell growth. In these assays, we found that NB15 was superior to the other compounds in its ability to inhibit cell growth, impact Jak-STAT signaling, induce cell cycle arrest, induce apoptosis, and reduce Jak2-mediated clonogenic potential. The other compounds had variable results, but only the other para-hydroxyl compound, NB13, had any impact on Jak2 activity in the *in vitro* kinase assay. However, this compound was still much weaker than NB15 as a Jak2 inhibitor, despite its *in silico* binding properties. Overall, these results indicate that the
Para-hydroxyl orientation alone is not sufficient to confer Jak2 inhibitory activity, but appears to be a necessary component.

Our molecular docking results indicated that the two para-hydroxyl compounds, NB13 and NB15, interact with the Jak2 ATP-binding pocket through hydrogen bonds with the hinge region, the activation loop, and the catalytic loop. In contrast, while the meta-hydroxyl compounds, NB4 and NB6, interacted with the activation and catalytic loops, they lacked an interaction with the hinge region. It has previously been shown that the hinge region is an important area to target for Jak2 inhibition as it interacts with the adenine group of ATP (Lucet et al. 2006). Our structure-function correlations determined here support the importance of the hinge region in Jak2 kinase inhibition as the two meta-hydroxyl compounds exhibited either moderate (NB4) or poor (NB6) Jak2 inhibitory potential. Interestingly, NB4 was able to inhibit Jak2-mediated cell growth and induce apoptosis and cell cycle arrest. However, it was unable to reduce Jak2 kinase activity or reduce STAT phosphorylation. Furthermore, it demonstrated weak binding interactions with Jak2 in silico. Therefore, we believe the effects of this compound on cell growth are via Jak2-independent mechanisms and underscore the importance of the para-hydroxyl structure in not only maintaining potency, but also specificity.

The results obtained with NB6 are also of interest. This meta-hydroxyl derivative had a minimized stilbene core. We found that it had the poorest Jak2 binding properties in silico and was largely without effect in the protein and cell based assays performed in vitro, as well as in the clonogenic assays performed ex vivo. From this, we conclude that minimization of the stilbene core on the meta-hydroxyl scaffold results in poor Jak2 inhibitory potential.
The parent compound, G6, has previously been shown to interact with Jak2 *in silico* via hydrogen bond interactions with the hinge region, the activation loop, and the catalytic loop (Majumder et al. 2010). Additionally, we have previously shown that G6 inhibits HEL cell proliferation and induces apoptosis and cell cycle arrest (Kirabo et al. 2011a). Of the four G6 derivatives tested in the current study, NB15 was clearly the most effective inhibitor as determined in these assays. This compound maintained the *para*-hydroxyl orientation of G6, but had a minimized stilbenoid core. The *in silico* studies revealed strong interactions with the activation loop, the catalytic loop, and the hinge region. These interactions include an additional hydrogen bond with Asn981 that is lacking in G6 (Majumder et al. 2010). NB15 induced cell cycle arrest and apoptosis leading to reduced cell viability. Furthermore, these inhibitory effects correlated with reduced Jak2 kinase activity and decreased STAT phosphorylation. The published HEL cell IC$_{50}$ value for G6 is 4.0 µM (Kirabo et al. 2011a). In comparison, NB15 inhibits HEL cell growth with an approximate IC$_{50}$ of 0.85 µM. Based on these results, we conclude that having a minimized stilbenoid core on the *para*-hydroxyl structure provides the highest degree of Jak2 specific inhibition.

Interestingly, stilbene compounds such as resveratrol and its derivatives have been implicated as potential therapeutic agents for hematological malignancies. These compounds have antioxidant properties, but also induce apoptosis and cell cycle arrest in leukemia cells (Kelkel et al. 2010). It has been shown that the positioning of hydroxyl groups in resveratrol derivatives greatly impacts their antioxidant and cell growth inhibitory potential. Interestingly, the overall cytotoxic effects of *para*-hydroxyl resveratrol derivatives are lower than those of *ortho*-hydroxyl derivatives (Kelkel et al.
This may indicate that using a \textit{para}-hydroxyl stilbene scaffold for the development of tyrosine kinase inhibitors may be beneficial in reducing non-specific cytotoxicity. Also, this type of structure allows for addition of other groups, such as amine moieties, in order to enhance the specificity for the target protein.

In summary, G6 is the only stilbene-derived Jak2 inhibitor to be developed to date. It demonstrates excellent Jak2 inhibitory potential, \textit{in vitro}, \textit{ex vivo}, and \textit{in vivo}. Here, we identified key structural features that may contribute to the potency and specificity of this compound. As such, these results may be useful in the future development of stilbene-derived Jak2 inhibitors for the purpose of treating Jak2-mediated pathologies.
Figure 2-1. Structures and molecular weights of G6 and its four derivatives.
Figure 2-2. Molecular docking of the NB compounds. Each compound was computationally docked into the ATP-binding pocket of the Jak2 kinase domain (PDB code 3E64). The protein structure is represented as a ribbon, with the compounds shown as sticks. Colored regions of the protein are the glycine loop (blue), the hinge region (cyan), the catalytic loop (magenta) and the activation loop (red). Amino acid residues that participate in hydrogen bond interactions are shown as sticks and are labeled.
Figure 2-3. Effects of the G6 derivatives on HEL and Ba/F3-EpoR-Jak2-V617F cell proliferation. A) HEL cells were treated with the indicated concentrations of each compound for 72 h and cell viability was determined by trypan blue exclusion. B) HEL cells were treated with a 10 µM concentration of each drug for the indicated periods of time and viability was determined by trypan blue exclusion. C) Ba/F3-EpoR-Jak2-V617F cells were treated with the indicated concentrations for 72 h and cell viability was determined by MTS assay. D) Ba/F3-EpoR-Jak2-V617F cells were treated with a 10 µM concentration for the indicated times and viability was determined by MTS assay. Shown are results from two independent experiments, each performed in triplicate.
Figure 2-4. Effects of the G6 derivatives on Jak2 in vitro kinase activity. A) Recombinant Jak2 protein was incubated in a kinase buffer with GST-STAT1 substrate in the presence of DMSO or 2.5 µM of each compound. The kinase reactions were analyzed by Western blot. Shown is one of two independent results. B) and C) Quantification of STAT1 and Jak2 phosphorylation, normalized to the positive control. n=2. *, p<0.05 vs. positive control (Jak2+STAT1+ATP).
Figure 2-5. Effects of the G6 derivatives on STAT5 phosphorylation in HEL cells. A) HEL cells were treated with a 25 μM concentration of each compound for 24 h and lysates were immunoprecipitated for STAT5 and Western blot was used to detect phospho-STAT5. Shown is one of four representative results. B) Quantification of STAT5 phosphorylation, measured as a percent of total and normalized to the DMSO control. n=4. *, p<0.05 vs. DMSO.
Figure 2-6. Induction of cell cycle arrest and apoptosis in HEL cells. A) Cells were treated with a 10 µM concentration of each compound for 24 h and analyzed by flow cytometry for DNA content. Shown are results from two experiments, each measured in triplicate. *, p<0.05. B) Western blot was performed on cells treated with a 25 µM concentration for 24 h to examine cyclin A, cyclin B1, and CDK 1 expression. β-actin was used as a loading control. Shown is one of two independent results. C) Cells were treated with a 10 µM concentration of each compound for 48 h and analyzed by flow cytometry for Annexin V and propidium iodide staining. Plotted are the percentages of cells in early apoptosis (Annexin V positive, propidium iodide negative). Shown are the results from two independent experiments, each performed in triplicate. *, p<0.05. D) Western blot analysis was performed on cells treated with a 25 µM concentration of each compound for 24 h to examine expression of PARP, Bid, Bim, and caspase 3. Shown is one of two representative results for each blot.
Figure 2-7. Effects of the G6 derivatives on the *ex vivo* clonogenic growth potential of Jak2-V617F-positive, murine bone marrow cells. Bone marrow cells were isolated from 6 month old Jak2-V617F transgenic mice and were incubated with media containing a 25 µM concentration of each drug for 0, 12, or 24 hours. Cells were then washed and plated in semi-solid media, and the number of BFU-E and CFU-GM were counted 6 days later. Panels A-D show the results for NB4, NB6, NB13, and NB15, respectively. Each condition was measured in duplicate. *, p<0.05 vs. 0 h.
CHAPTER 3
ANTI-TUMOR EFFECTS OF THE JAK2 SMALL MOLECULE INHIBITOR G6 IN GLIOBLASTOMA CELLS

Glioblastoma multiforme (GBM) is the most common and most aggressive form of primary brain tumor. The median survival is 14 months after optimal therapy, such as surgical resection, radiation therapy and chemotherapy. The most commonly used chemotherapeutic agent for GBM is temozolomide, which acts as a DNA alkylating agent. However, temozolomide resistance in a large number of GBM patients has prompted the development of alternate therapies (Friedman et al. 2000). Recently, many of the molecular mechanisms involved in GBM pathogenesis have been identified and these discoveries have led to the development of molecular targeted therapies. Pathways that have been targeted to date include VEGF, EGFR, PDGF, PI3K, Akt, and mTOR (Agarwal et al. 2011). Although many of these therapies have shown promising pre-clinical efficacy, the clinical outcomes have not been highly successful thus far (Ohka et al. 2012; Mao et al. 2012).

Recently, there has been increasing interest in the role of Jak/STAT signaling in GBM, and small molecule Jak/STAT inhibitors have been investigated for GBM treatment in vitro and in vivo. In 2007, constitutive phosphorylation of Jak2 was found in the GL15 glioblastoma cell line, and treatment with the tyrphostin AG490 was shown to induce cell cycle arrest in these cells (Sciaccaluga et al. 2007). More recently, several studies have demonstrated efficacy of AG490 and other more specific Jak2 inhibitors in cell culture and animal models (McFarland et al. 2011; Sai et al. 2012). In the current study, we sought to examine the effects of the Jak2 small molecule inhibitor G6 in glioblastoma cells. G6 was developed by our lab for the treatment of Jak2-dependent neoplasia, and we have demonstrated efficacy of this compound in numerous in vitro,
ex vivo, and in vivo models of Jak2-mediated disease (Kiss et al. 2009; Majumder et al. 2010; Kirabo et al. 2011a, 2011b, 2012). Here, we hypothesized that G6 treatment would reduce the tumorigenic potential of GBM cells that exhibit hyperactive Jak2 signaling. In order to test this, we examined the effects of G6 in the T98G cell line, which expresses high levels of phosphorylated Jak2 protein.

**Materials and Methods**

**Cell lines**

T98G glioblastoma cells and glioblastoma stem cell lines (L0, L1, L2, S2, S3 and S7) were kindly provided by Dr. Jeffrey Harrison and Dr. Brent Reynolds. T98G cells were maintained in MEM supplemented with 10% FBS, penicillin, streptomycin, and L-glutamine at 37 °C and 5% CO₂. GBM stem cells were maintained in DMEM:F12 supplemented with EGF, bFGF, penicillin and streptomycin.

**Cell Proliferation Assay**

T98G cells were plated in 96-well plates and treated with the indicated concentrations of G6 for 72 hours. Cell viability was determined using MTS colorimetric assay (Promega) according to the manufacturer’s protocol.

**Colony Formation Assay**

T98G cells were plated in 100 mm dishes at a density of 200 cells per dish and were cultured for 24 hours in the presence or absence of G6. The drug was then removed, and cells were cultured for 9 days. Cells were fixed in 90% methanol and stained with crystal violet, and the number of colonies was counted.

**Western Blotting**

Cells were lysed in RIPA buffer and protein concentration was determined using a Bradford assay (BioRad). Approximately 30 μg of soluble protein was separated by
SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked at room temperature for 1 hour with 5% milk in TBS-T and incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: phospho-Jak2 at 1:300 (Invitrogen), total Jak2 at 1:1000 (Millipore/Invitrogen), phospho-STAT3 at 1:300 (Santa Cruz), total STAT3 at 1:1000 (Santa Cruz), and β-actin at 1:500 (Cell Signaling). Secondary antibodies were applied for 1 hour at room temperature. Anti-rabbit and anti-mouse secondary antibodies were used at 1:4000 (GE Healthcare).

**Cell Migration and Invasion Assays**

For scratch assays, cells were plated on 4-well chamber slides, grown to confluency and treated with G6 for 24 hours. The drug was removed, a scratch was made and migration into the scratch area was monitored for up to 48 hours. For cell migration assays, T98G cells were plated in 24-well migration inserts (PET membrane with 8 μm pores - BD). Cells were plated in the top of the chamber in serum-free MEM and the bottom of the chamber was filled with serum-containing MEM. Cell migration was analyzed after 24 hours. Non-migrating cells were removed from the top of the chamber using a cotton swab, and migrated cells were stained with crystal violet and counted. For cell invasion assays, T98G cells were plated in 24-well inserts coated with matrigel (BD). The top chamber contained cells in serum-free MEM and the bottom chamber contained complete MEM. Cell invasion was analyzed after 48 hours.

**Apoptosis Assays**

For TUNEL staining, cells were plated on 4-well chamber slides and treated with G6 for 48 hours. TUNEL staining was performed as previously described (Baskin and Sayeski 2012).
Cell Cycle Analysis

T98G cells were treated with G6 at various concentrations for 48 hours and DNA content was analyzed using the CycleTEST PLUS™ DNA Reagent Kit according to the manufacturer’s protocol (BD Biosciences). Cells were analyzed using a FACSCalibur™ flow cytometer (BD Biosciences).

Lentiviral shRNA-mediated Jak2 Knockdown

HEK293T cells were transfected with Jak2 shRNA constructs (pool of Thermo catalog numbers RHS3979-9571807, RHS3979-9571808, RHS3979-9571809, RHS3979-9571810, and RHS3979-9571811) or a non-targeting shRNA construct (AddGene plasmid 1864) along with packaging and envelope vectors (psPAX2 and pMD2.G). Lipofectamine (Invitrogen) was used as the transfection reagent. Approximately 40 hours post-transfection, culture media containing virus was collected and supplemented with polybrene, sterile filtered, and applied to T98G cells. A second round of infection was performed 24 hours later. Infected cells were identified by puromycin selection and used for experiments.

Jak2 Immunostaining and Flow Cytometry

GBM stem cell lines (L0, L1, L2, S2, S3, and S7) were stained with Live/Dead Fixable Blue Stain (Invitrogen) followed by fixation in 1:1 methanol:acetic acid at -20°C. After fixation, cells were incubated with goat anti-pJak2 (Santa Cruz) and rabbit anti-Jak2 (Cell Signaling) primary antibodies in 2% BSA at 1:100 each. Cells were incubated with Alexa-Fluor 594 anti-goat and Alexa-Fluor 488 anti-rabbit antibodies (Invitrogen) at 1:400 each and analyzed by flow cytometry.
Quantitative RT-PCR

RNA was extracted using the RNeasy® mini kit (Qiagen) and 1 μg of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Expression of GFAP (Hs00909236_m1), Jak2 (Hs01078124_m1), and GAPDH (Hs02758991_g1) were analyzed using TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) in a StepOne™ real time PCR system.

Results

G6 Reduces Jak2 and STAT3 Phosphorylation in T98G Cells

Expression of active Jak2 and/or STAT3 has been observed in a number of glioblastoma cell lines and primary cells (Sciaccaluga et al. 2007; McFarland et al. 2011; Sai et al. 2012). Several studies have demonstrated the efficacy of Jak2 inhibitors in both *in vitro* and *in vivo* models of glioblastoma (McFarland et al. 2011; Sai et al. 2012). Here, we initially examined expression of Jak2 protein in three glioblastoma cell lines: A172, U87MG, and T98G. We found a high level of Jak2 phosphorylation in T98G cells by Western blot (Figure 3-1A). Based on the initial screening results, we selected the T98G cell line for further experiments in testing the hypothesis that Jak2 inhibitors are efficacious against Jak2-dependent GBM.

We first examined the effects of G6 treatment on Jak2 and STAT3 phosphorylation in this cell line. Cells were treated for 24 hours at the indicated concentrations and we found that G6 caused a dose-dependent decrease in Jak2 and STAT3 phosphorylation (Figure 3-1B). Phosphorylation levels were quantified by densitometry (Figure 3-1C and D). These results suggest that G6 effectively inhibits Jak2/STAT3 signaling in T98G cells. We also examined the effects of the Jak2 small molecule inhibitor AG490, which is commonly used as a model Jak2 inhibitor in cell-
based assays. We found that AG490 inhibited Jak2 and STAT3 phosphorylation, but only minimally at a concentration of 30uM and markedly at 100 uM (data not shown). Thus, in addition to being a more specific Jak2 inhibitor, G6 also has greater potency than AG490.

**G6 Inhibits Cell Proliferation and Colony Formation in T98G Cells**

We next wanted to determine if the G6-dependent inhibition of Jak2 phosphorylation in T98G cells would correlate with a reduction of cell viability and/or colony formation. For cell viability assays, T98G cells were treated with increasing concentrations of G6 for the indicated times and viability was determined by colorimetric MTS assay. We found that G6 caused both a dose- and time-dependent decrease in T98G cell viability (Figure 3-2A and B). For colony formation assays, cells were treated with the indicated concentrations of G6 for 24 hours and plated in 100 mm dishes. The number of colonies was counted after 6 days. We found that G6 caused a dose-dependent decrease in the ability of T98G cells to form colonies (Figure 3-2C). Collectively, these results indicate that G6 reduces both the cell viability and colony forming ability of T98G cells *in vitro.*

**G6 Treatment Reduces the Migratory and Invasive Potential of T98G Cells**

The ability to migrate into and invade surrounding tissue is an important clinical characteristic of glioblastoma cells. Here, we wanted to determine if G6 treatment would reduce the migratory and/or invasive potential of T98G cells. We first used a scratch assay to examine cell migration. Cells were grown to confluency and treated with G6 for 24 hours, at which point the drug was removed and a scratch was made. We found that G6 treatment markedly reduced migration of cells into the scratch area (Figure 3-3A). For a more quantitative examination of cell migration, we used a trans-well
migration/invasion assay. Cells were treated with G6 for 24 hours and then plated in 24-well chamber inserts. Inserts were either uncoated (for migration assays) or coated with matrigel (for invasion assays). The numbers of migrating and invading cells were counted. We found that G6 treatment reduced cell migration and invasion in a dose-dependent manner (Figure 3-3B, C, D, and E). These results indicate that G6 is effective at reducing the migratory and invasive potential of T98G glioblastoma cells.

**Lentiviral-mediated Knockdown of Jak2 Reduces Migratory and Invasive Potential of T98G Cells**

We next wanted to determine if the effects of G6 on migratory and invasive potential of T98G cells could be reproduced by specifically knocking down Jak2. The main goal of these experiments was to determine if the effects of G6 could be attributed directly to inhibition of Jak2 signaling, rather than to any off target effects of the drug. Here we used lentiviral-mediated shRNA to silence Jak2 in these cells. We found that Jak2 mRNA expression was reduced by approximately 80% in Jak2 knockdown cells, when compared to scrambled shRNA (Figure 3-4A). We also found that Jak2 protein expression was reduced by approximately 60% in these cells (Figure 3-4B and C). After puromycin selection, cells were plated in uncoated or matrigel-coated 24-well chamber inserts and cell migration and invasion were examined. The numbers of migrating and invading cells were counted. We found that Jak2 knockdown reduced the migratory and invasive potential of T98G cells (Figure 3-5A, B, C and D). These results indicate that the effects of G6 on T98G cell migration and invasion can also be observed through specific silencing of Jak2. Thus, it appears that Jak2 plays an important role in maintaining the migratory and invasive potential of these glioblastoma cells.
G6 Induces Caspase-Dependent Apoptosis and Mild Cell Cycle Arrest in T98G Cells

Next, we wanted to determine if the growth inhibitory properties of G6 in T98G cells could be attributed to induction of apoptosis and/or cell cycle arrest. We have previously shown that G6 treatment induces caspase-dependent apoptosis and cell cycle arrest in human erythroleukemia cells (Kirabo et al. 2011a). In the current study, we treated T98G cells with varying concentrations of G6 for 48 hours and examined the level of apoptosis by TUNEL staining. We found that G6 treatment caused a dose-dependent increase in the number of TUNEL-positive cells, which was statistically significant (Figure 3-6A and B). In order to examine the mechanism of apoptosis in these cells, we examined the level of caspase 3/7 activity after treatment with G6. We found that G6 caused an increase in the level of caspase 3/7 activity when compared with the DMSO control (Figure 3-6C). During apoptosis, full length caspase 3 is cleaved into an active form. The disappearance of full length caspase 3 typically correlates with an increase in caspase activity and thus indicates an increase in apoptotic signaling.

We examined the expression of caspase 3 by Western blot and found that G6 treatment caused a dose-dependent decrease in the expression of full-length caspase 3 (Figure 3-6D).

To determine if G6 induces cell cycle arrest in T98G cells, the cells were treated with varying concentrations of the drug for 48 hours and were analyzed by flow cytometry. We found that only the highest concentration of G6, 30 uM, caused a significant decrease in the percent of cells in G1 and a significant increase in G2 (Figure 3-7). However, the shift from G1 to G2 was minimal, so we concluded that G6 may induce a mild G2 arrest in T98G cells. Overall, these results indicate that G6 treatment
induces caspase-dependent apoptosis, and that apoptosis may be the primary mechanism by which the drug reduces cell viability in T98G cells.

**Jak2 Expression in Glioblastoma Stem Cell Lines**

In recent years, the role of stem-like cells in the development of GBM tumors has been investigated (Chen et al. 2012). It has been demonstrated that tumor initiating cells, or tumor stem cells, derived from primary GBM tumors have different phenotypic and genotypic characteristics from commonly used GBM cell lines. It has also been shown that the stem-like cells cultured in serum-free conditions give rise to tumors resembling the original tumor, but if cultured long-term in serum, these cells will eventually give rise to tumors with different morphological characteristics (Lee et al. 2006). There has been a recent drive to develop therapies specifically targeting these tumor stem-like cells, and several stem-cell maintenance signaling pathways have been targeted (Chen et al. 2012). The role of Jak2 in GBM stem cells has been controversial to date, with some reports suggesting that Jak2 is important in stem cell survival (Sai et al. 2012) while others suggest that Jak2 promotes glioma stem cell differentiation (Shu et al. 2011).

Here, we wanted to determine if Jak2 expression and activation were elevated in different glioblastoma stem-like cell lines. We first examined the expression and phosphorylation of Jak2 in six primary glioblastoma cell lines by Western blot. We found that there was no detectable Jak2 tyrosine phosphorylation, but there were varying levels of total Jak2 expression in the six cell lines (Figure 3-8A). One issue that arises when studying glioblastoma stem-like cell cultures is the heterogeneity of GBM tumors (Chen et al. 2012). Therefore, we wanted to determine if there might be a subset of cells within each stem-like cell line that express active and/or total Jak2 protein. Here, we
examined Jak2 phosphorylation and total Jak2 levels by flow cytometry. We found that a small percentage of cells in each cell line expressed total Jak2, but that hardly any of the cells expressed active Jak2 (Figure 3-8B and Table 3-1). Based on the relatively low levels of detectable Jak2 expression and phosphorylation, we were uncertain of a significant role for Jak2 in maintenance of these various cell lines.

Since Jak2 has been implicated in the differentiation of glioma cells (Shu et al. 2011), we next wanted to determine if differentiation of these stem-like cell lines would lead to an upregulation of Jak2. We cultured the cells for 72 hours either in serum-free stem cell media containing EGF and bFGF or in serum-containing media. We then examined Jak2 expression by quantitative RT-PCR. We found that expression of Jak2 was elevated by approximately 2-fold in the L0, L1, and L2 cell lines (Figure 3-9A). To confirm differentiation status of the cells, we examined expression of glial fibrillary acidic protein (GFAP). We found a significant elevation of GFAP in these three cell lines (Figure 3-9B). However, serum incubation had no effect on Jak2 or GFAP expression in the S2, S3 or S7 cell lines (data not shown).

In order to determine if Jak2 played a critical role in the serum-induced differentiation of GBM stem-like cells, we treated the L2 cell line with the Jak2 inhibitor G6 during differentiation experiments. Interestingly, we found that G6 treatment caused a further elevation of GFAP expression in the presence of serum, and that G6 treatment also caused a slight elevation of GFAP expression even in the absence of serum (Figure 3-9C and D). Based on these preliminary results, we believe that Jak2 may have an undefined role in stem cell property maintenance in the L2 cell line, and that inhibition of Jak2 may drive the cells toward a more differentiated state.
Discussion

Here, we have identified the Jak2 small molecule inhibitor G6 as a potential therapy for Jak2-dependent glioblastoma. We found that the T98G glioblastoma cell line expresses a high level of active Jak2 protein, and that treatment with G6 reduced Jak2/STAT3 phosphorylation in a dose-dependent manner. We also found that this correlated with a reduction in cell viability, colony formation, cell migration, and cell invasion. One mechanism by which G6 reduced cell viability in these cells was through induction of caspase-dependent apoptosis. Overall, these results indicate that G6 may have therapeutic potential in glioblastoma.

GBM is a highly aggressive and highly invasive form of cancer that is associated with a very poor prognosis even after optimal therapy. The primary chemotherapeutic agent used to treat glioblastoma is the DNA alkylating agent temozolomide. However, many tumors exhibit temozolomide resistance, and tumor heterogeneity makes treatment of GBM very challenging. A number of molecular mechanisms involved in GBM pathogenesis have been described to date, including VEGF, EGFR, PDGF, PI3K, Akt, and mTOR signaling pathways (Agarwal et al. 2011). Molecular targeted therapies have been investigated targeting each of these pathways, and there are a number of small molecule drugs that have been developed and tested in clinical trials. Unfortunately, the clinical efficacy of these drugs has been limited. For example, EGFR inhibitors such as gefitinib have not been able to improve overall or progression-free survival in phase I and II trials. The anti-VEGF antibody bevacizumab was able to reduce tumor size and increase progression-free survival, but didn’t improve overall survival in clinical trials (Mao et al. 2012).
This overall lack of clinical efficacy has prompted further examination of the molecular mechanisms driving GBM tumor initiation, progression, and drug resistance. One pathway that has come to light in recent years is the Jak/STAT signaling pathway. In the initial studies examining the role of Jak2 in glioblastoma, the tyrphostin compound AG490 was used to block Jak2 activation. These studies showed that inhibition of Jak2 activity could impact viability and invasive properties of GBM cells (Sciaccaluga et al. 2007; Senft et al. 2011). AG490 is a broad spectrum tyrosine kinase inhibitor, but further studies have demonstrated efficacy of more specific Jak2 inhibitors against glioblastoma growth (McFarland et al. 2011; Sai et al. 2012). Our results provide more evidence of a role for Jak2 signaling in glioblastoma cells and the potential application of Jak2 inhibitors for GBM therapy. Due to the heterogeneity of glioblastoma tumors, combination therapy with multiple molecular targeted drugs may be necessary in order to improve patient outcomes. We believe that Jak2 small molecule inhibitors, such as G6, may be a viable therapeutic option in cases where Jak2 signaling is elevated. However, the mechanisms of Jak2 hyper-activation in glioblastoma are still poorly understood and will require further investigation.

We have also investigated the role of Jak2 in glioblastoma stem-like cells. Cancer stem cells (CSCs), or tumor-initiating cells, are thought to be a driving factor in the development and progression of glioblastoma. Here, we examined the expression and phosphorylation of Jak2 in six primary glioblastoma stem-like cell lines. We found that Jak2 was expressed in a small fraction of the cell population, and that it was not highly phosphorylated. We found that upon serum-induced differentiation, Jak2 expression increased two-fold in several of these cell lines. In addition, we found that
treating the cells with the Jak2 inhibitor G6 led to an increase in GFAP expression, indicating a more differentiated state. This change in GFAP expression occurred whether the cells were cultured in basal medium or in serum-containing medium. This finding may implicate Jak2 as an important factor in stem cell maintenance, but these results are very preliminary and further studies must be conducted in order to fully characterize these cell lines.
Figure 3-1. G6 reduces Jak2 and STAT3 phosphorylation in T98G cells. A) Expression of phospho-Jak2 in three glioblastoma cell lines. γ2A+Jak2 cell lysates were used as a Jak2-positive control. B) T98G cells were treated with the indicated concentrations of G6 for 24 hours and examined for Jak2 and STAT3 phosphorylation by Western blot. C) and D) Quantification of Jak2 and STAT3 phosphorylation, respectively. n=3. *, p<0.05
Figure 3-2. G6 treatment reduces T98G cell viability and clonogenic potential. A) T98G cells were treated with increasing concentrations of G6 for 72 hours and viability was measured by MTS assay. B) T98G cells were treated with G6 for the indicated times and viability was measured by MTS assay. C) T98G cells were treated with G6 for 24 hours, washed and plated in 100 mm dishes. Colonies were grown for two weeks, stained with crystal violet and counted. *, p<0.05.
Figure 3-3. G6 reduces migratory and invasive potential of T98G cells. A) Cells were treated with G6 for 24 hours, washed and a scratch was made. Cell migration was monitored 24 and 48 hours after the scratch was made. B) Cells were treated with G6 for 24 hours, washed, and plated in un-coated 24-well inserts. Migration was examined by crystal violet stain after 24 hours. C) Cells were treated with G6 for 24 hours and plated in matrigel-coated 24-well inserts. Invasion was examined by crystal violet stain after 48 hours. D) Quantification of migrating cells. E) Quantification of invading cells. *, p<0.05
Figure 3-4. Lentiviral-mediated knockdown of Jak2 in T98G cells. A) Jak2 mRNA expression was examined by qRT-PCR. B) Jak2 protein expression was examined by Western blot. C) Quantification of Jak2 protein expression. *, p<0.05
Figure 3-5. Jak2 knockdown reduces the migratory and invasive potential of T98G cells. 
A) Cells were plated in 24-well inserts and migration was monitored after 24 hours. B) Quantification of migrating cells. C) Cells were plated in matrigel-coated 24-well inserts and invasion was monitored after 48 hours. D) Quantification of invading cells. *, p<0.05
Figure 3-6. G6 induces caspase-dependent apoptosis in T98G cells. A) Cells were treated with G6 for 48 hours and apoptosis was examined by TUNEL immunofluorescence. B) Quantification of the percentage of TUNEL-positive cells. C) Cells were treated for the indicated times with a 10uM concentration of G6 and Caspase 3/7 activity was measured by a luminescent assay. D) Cells were treated with G6 for 48 hours and Caspase 3 expression was measured by Western blot. *, p<0.05
Figure 3-7. G6 induces mild G2 arrest in T98G cells. Cells were treated with G6 for 48 hours and analyzed by flow cytometry. *, p<0.05
Figure 3-8. Expression and phosphorylation of Jak2 in glioblastoma stem cells. A) Cells were analyzed for Jak2 phosphorylation and expression by Western blot. B) Cells were analyzed for phospho-Jak2 and total Jak2 expression by intracellular FACS. Representative plots are shown. Q4 represents cells expressing only total Jak2, while Q2 represents cells expressing phospho-Jak2.
Table 3-1. Quantification of Jak2 flow cytometry staining in GBM stem cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>% of cells expressing phospho Jak2</th>
<th>% of cells expressing total Jak2</th>
</tr>
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<tbody>
<tr>
<td>L0</td>
<td>0.33 +/- 0.12</td>
<td>2.43 +/- 0.32</td>
</tr>
<tr>
<td>L1</td>
<td>0.07 +/- 0.12</td>
<td>1.80 +/- 1.65</td>
</tr>
<tr>
<td>L2</td>
<td>0.77 +/- 0.45</td>
<td>5.10 +/- 1.06</td>
</tr>
<tr>
<td>S2</td>
<td>0.87 +/- 0.23</td>
<td>6.87 +/- 1.80</td>
</tr>
<tr>
<td>S3</td>
<td>0.53 +/- 0.35</td>
<td>7.93 +/- 2.37</td>
</tr>
<tr>
<td>S7</td>
<td>0.60 +/- 0.17</td>
<td>4.73 +/- 2.11</td>
</tr>
</tbody>
</table>
Figure 3-9. Effects of differentiation on Jak2 expression and effects of G6 treatment on GFAP expression in GBM stem cells. A) Cells were cultured in serum-free or serum-containing media and Jak2 expression was examined by qRT-PCR. B) GFAP expression was examined by qRT-PCR. C) Cells were cultured in serum in the presence or absence of 1uM G6 and GFAP expression was examined by qRT-PCR. D) Cells were cultured in the absence of serum and in the presence or absence of 1uM G6, and GFAP expression was analyzed by qRT-PCR.
CHAPTER 4
SCREENING OF G6 DERIVATIVES FOR JAK2 INHIBITION

After the identification and characterization of the small molecule Jak2 inhibitor G6, our lab became interested in further optimizing the structure of this compound to enhance Jak2 inhibition. As mentioned in Chapter 3, we determined that the para-hydroxyl orientation of G6 derivatives lends greater Jak2 inhibitory potential than the meta-hydroxyl orientation. The results of that study emerged from screening of numerous G6 derivatives, and this chapter will describe the structures and *in vitro* efficacy of some of these other derivative compounds. Although the native structure of G6 is highly efficacious, it contains two free hydroxyl groups, which is not an optimal structure for achieving high bioavailability. In order to address this issue, here we have also examined the *in vitro* efficacy of G6 derivatives containing protected hydroxyl groups. The goals of this work are two-fold: 1) to maintain a level of Jak2 inhibition similar to that of the parent compound G6 and 2) to improve upon the bioavailability of the drug. The preliminary results of these studies will be described in this chapter.

**Materials and Methods**

**Compounds and Cell Lines**

Compounds were synthesized and kindly provided from the laboratory of Dr. Kirpal Bisht (University of South Florida, Tampa, FL). Human erythroleukemia (HEL) cells were purchased from the American Type Culture Collection. Ba/F3-EpoR-Jak2-V617F cells were created as described previously (Pradhan et al. 2010). Both cell lines were cultured in RPMI 1640 (Mediatech) supplemented with 10% FBS, penicillin, streptomycin, and L-glutamine at 37°C and 5% CO₂.
Cell Viability Assays

HEL cells or Ba/F3-EpoR-Jak2-V617F cells were plated in 96-well plates and treated with either 0.25% DMSO or varying concentrations of G6 and its derivatives for 72 hours. Cell viability was determined by trypan blue exclusion staining or by MTS (Promega) according to the manufacturer's protocol.

Results

Screening of NB1-NB34

The initial screening of the “NB” series of G6 derivatives included 34 compounds, named NB1-NB34. This screen, along with further experiments, identified NB15 as a potent Jak2 inhibitor with favorable in vitro, in silico, and ex vivo properties. Here, we report the results for the other compounds tested in this screen. All of the compounds in this screen have stilbenoid-derived structures, because we have previously reported that the stilbene core of G6 is critical for its ability to inhibit Jak2-mediated cell growth (Majumder et al. 2010). Of the initial 34 compounds, two (NB11 and NB20) were insoluble in DMSO at a 10 mM concentration and were therefore excluded from the study. The remaining compounds were tested for their ability to inhibit HEL cell growth. HEL cells were plated in 96-well plates and treated for 72 hours with the indicated concentrations of each drug. Cell viability was determined by direct cell counts using Trypan blue exclusion. The effects of these compounds on HEL cell viability are presented in Figure 4-1, Figure 4-2, and Figure 4-3. Table 4-1 shows the structures and relative HEL cell growth inhibition compared with the parent compound G6 at a 10 µM concentration. We found that a number of the compounds had equal or greater potency when compared with G6, including NB10, NB15, NB32 and NB34. However, some of
the structures completely lost Jak2 inhibitory potential, such as NB2, NB6, NB18 and NB33.

**Screening of NB35-NB43**

After this initial screening and the identification of NB15 as reported in Chapter 3, we began investigating other structural properties of G6 in order to improve its drug-like characteristics. Namely, as shown in Table 4-1, the structure of G6 contains two free phenolic groups and two amine groups. In *in vitro* liver microsome assays, we found that G6 is susceptible to metabolism by liver enzymes (unpublished observations). The presence of these amine and free hydroxyl groups may contribute to this instability. The structure of G6 is similar to that of resveratrol, a stilbenoid compound with free hydroxyl groups that is highly metabolized into a number of sulfate derivatives (Boocock et al. 2007; Cottart et al. 2010). In order to improve the stability of G6, we developed a set of G6 derivatives containing cyclization of the amine and hydroxyl groups. These compounds, known as NB35-NB43, maintain the stilbene core of G6 while protecting the hydroxyl groups from sulfation or other reactions by incorporating them into ring structures. Simultaneously, these compounds maintain the spatial positioning of the reactive groups in order to preserve Jak2 ATP-binding pocket interactions.

Here, we tested the ability of these G6 derivatives to inhibit cell growth in a Jak2-dependent cell line (Ba/F3-EpoR-Jak2-V617F). This murine pro B cell line has been retrovirally transduced with the Jak2-V617F mutant and demonstrates cytokine independent cell growth. In these studies, we utilized this cell line instead of the HEL cell line because it may serve as a better cell-based model of Jak2-mediated malignancy due to its complete dependence on the Jak2-V617F mutation for cytokine independent growth. Due to insolubility at a 10 mM concentration in DMSO, three of the
compounds were eliminated from the study (NB35, NB40, and NB43). The structures of the DMSO-soluble compounds are shown in Figure 4-4. For these compounds, cells were plated in 96-well plates and treated with the indicated concentrations of each drug for 72 hours. Cell viability was determined by MTS assay. The effects of these compounds on BaF3-Jak2-V617F cell viability are shown in Figure 4-5. We found that many of these derivatives maintain equal or greater potency when compared with G6, including NB36, NB37, NB41, and NB42. These compounds are being further investigated for their in vitro ADME-tox properties including metabolic stability, cytotoxicity, plasma stability, and aqueous solubility.

Discussion

Here, we have described the in vitro effects of a number of G6 derivative compounds against Jak2-mediated cell growth. These preliminary results may be of use for further development of stilbenoid Jak2 inhibitors for the treatment of Jak2-mediated pathologies. G6 is the first stilbenoid-derived Jak2 inhibitor that has been described to date, and it exhibits a high degree of efficacy against Jak2-dependent pathologic cell growth in vitro, ex vivo, and in vivo (Kiss et al. 2009; Majumder et al. 2010; Kirabo et al. 2011a, 2011b, 2012). Therefore, this compound has high potential for further clinical development. The first derivatives, known as NB1-NB34, were designed in order to examine the SAR properties of G6 in relation to the placement of amine and hydroxyl groups. This screening identified the potent Jak2 inhibitor NB15 as described in Chapter 3. From those studies we learned that the native para-hydroxyl orientation of G6, when paired with an overall minimized structure, lent high Jak2 inhibitory potential. Here, we also identified other structures that may be of further interest.
The second set of derivatives included NB35-NB43, and these compounds were designed to improve the metabolic stability of the parent compound. This screening has identified several potential candidates, which are being further investigated for their Jak2 inhibitory and metabolic properties. Specifically, four of the NB compounds that showed high potency against BaF3-Jak2-V617F cell growth are currently being examined for their solubility, metabolic stability, toxicity, and membrane permeability. Identifying a stilbene compound that has equal or greater potency when compared with G6, but which also has improved bioavailability, will be an important step in improving the current therapy for Jak2-mediated pathologies such as MPNs.
Table 4-1. Structures of the NB1-NB34 derivative compounds and their HEL cell growth inhibitory potentials (mean +/- SD).

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Structure</th>
<th>Mol. Wt</th>
<th>% of G6 HEL cell growth inhibition, 10 uM, 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6</td>
<td><img src="image1" alt="Structure" /></td>
<td>438.65</td>
<td>100 +/- 6</td>
</tr>
<tr>
<td>NB1</td>
<td><img src="image2" alt="Structure" /></td>
<td>382.54</td>
<td>85 +/- 5</td>
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<tr>
<td>NB2</td>
<td><img src="image3" alt="Structure" /></td>
<td>410.59</td>
<td>26 +/- 17</td>
</tr>
<tr>
<td>NB3</td>
<td><img src="image4" alt="Structure" /></td>
<td>410.59</td>
<td>61 +/- 6</td>
</tr>
<tr>
<td>NB4</td>
<td><img src="image5" alt="Structure" /></td>
<td>354.59</td>
<td>54 +/- 18</td>
</tr>
<tr>
<td>Compound Name</td>
<td>Structure</td>
<td>Mol. Wt</td>
<td>% of G6 HEL cell growth inhibition, 10 uM, 72 h</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------</td>
<td>---------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>NB5</td>
<td><img src="image" alt="NB5 Structure" /></td>
<td>382.54</td>
<td>74 +/- 5</td>
</tr>
<tr>
<td>NB6</td>
<td><img src="image" alt="NB6 Structure" /></td>
<td>326.43</td>
<td>35 +/- 19</td>
</tr>
<tr>
<td>NB7</td>
<td><img src="image" alt="NB7 Structure" /></td>
<td>406.56</td>
<td>50 +/- 2</td>
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<td>NB8</td>
<td><img src="image" alt="NB8 Structure" /></td>
<td>483.51</td>
<td>112 +/- 7</td>
</tr>
<tr>
<td>NB9</td>
<td><img src="image" alt="NB9 Structure" /></td>
<td>427.41</td>
<td>114 +/- 0</td>
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Table 4-1. Continued

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<tr>
<th>Compound Name</th>
<th>Structure</th>
<th>Mol. Wt</th>
<th>% of G6 HEL cell growth inhibition, 10 uM, 72 h</th>
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<tr>
<td>NB10</td>
<td><img src="image" alt="NB10 Structure" /></td>
<td>455.46</td>
<td>73 +/- 12</td>
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<tr>
<td>NB12</td>
<td><img src="image" alt="NB12 Structure" /></td>
<td>479.48</td>
<td>62 +/- 6</td>
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<td>60 +/- 10</td>
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<td>NB14</td>
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<td>35 +/- 3</td>
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<td>NB15</td>
<td><img src="image" alt="NB15 Structure" /></td>
<td>326.43</td>
<td>99 +/- 5</td>
</tr>
<tr>
<td>Compound Name</td>
<td>Structure</td>
<td>Mol. Wt</td>
<td>% of G6 HEL cell growth inhibition, 10 μM, 72 h</td>
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<td>NB16</td>
<td><img src="image1" alt="Structure" /></td>
<td>434.61</td>
<td>85 +/- 5</td>
</tr>
<tr>
<td>NB17</td>
<td><img src="image2" alt="Structure" /></td>
<td>406.56</td>
<td>68 +/- 15</td>
</tr>
<tr>
<td>NB18</td>
<td><img src="image3" alt="Structure" /></td>
<td>410.51</td>
<td>26 +/- 8</td>
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<tr>
<td>NB19</td>
<td><img src="image4" alt="Structure" /></td>
<td>438.56</td>
<td>39 +/- 5</td>
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<tr>
<td>NB21</td>
<td><img src="image5" alt="Structure" /></td>
<td>378.51</td>
<td>97 +/- 9</td>
</tr>
<tr>
<td>NB23</td>
<td><img src="image6" alt="Structure" /></td>
<td>382.54</td>
<td>74 +/- 6</td>
</tr>
<tr>
<td>Compound Name</td>
<td>Structure</td>
<td>Mol. Wt</td>
<td>% of G6 HEL cell growth inhibition, 10 uM, 72 h</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------</td>
<td>---------</td>
<td>-----------------------------------------------</td>
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<td>NB24</td>
<td><img src="image" alt="Structure NB24" /></td>
<td>434.61</td>
<td>89 +/- 3</td>
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<tr>
<td>NB25</td>
<td><img src="image" alt="Structure NB25" /></td>
<td>462.67</td>
<td>81 +/- 18</td>
</tr>
<tr>
<td>NB26</td>
<td><img src="image" alt="Structure NB26" /></td>
<td>466.61</td>
<td>48 +/- 5</td>
</tr>
<tr>
<td>NB27</td>
<td><img src="image" alt="Structure NB27" /></td>
<td>434.61</td>
<td>73 +/- 11</td>
</tr>
<tr>
<td>NB28</td>
<td><img src="image" alt="Structure NB28" /></td>
<td>406.56</td>
<td>73 +/- 3</td>
</tr>
<tr>
<td>NB29</td>
<td><img src="image" alt="Structure NB29" /></td>
<td>353.497</td>
<td>74 +/- 5</td>
</tr>
<tr>
<td>NB30</td>
<td><img src="image" alt="Structure NB30" /></td>
<td>268.35</td>
<td>65 +/- 0</td>
</tr>
<tr>
<td>Compound Name</td>
<td>Structure</td>
<td>Mol. Wt</td>
<td>% of G6 HEL cell growth inhibition, 10 uM, 72 h</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------</td>
<td>---------</td>
<td>---------------------------------------------</td>
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<td><img src="image2" alt="Chemical Structure" /></td>
<td>212.24</td>
<td>106 +/- 4</td>
</tr>
<tr>
<td>NB33</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>240.29</td>
<td>35 +/- 3</td>
</tr>
<tr>
<td>NB34</td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td>212.24</td>
<td>106 +/- 6</td>
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Figure 4-1. Effects of G6 derivatives NB1-NB10 on HEL cell viability. Cells were treated with each concentration of drug for 72 hours and cell viability was determined by cell counting with trypan blue exclusion. G6 cell growth inhibition is shown in black as a reference.
Figure 4-2. Effects of G6 derivatives NB12-NB24 on HEL cell viability. Cells were treated with each concentration of drug for 72 hours and cell viability was determined by cell counting with trypan blue exclusion. G6 cell growth inhibition is shown in black as a reference.
Figure 4-3. Effects of G6 derivatives NB25-NB34 on HEL cell viability. Cells were treated with each concentration of drug for 72 hours and cell viability was determined by cell counting with trypan blue exclusion. G6 cell growth inhibition is shown in black as a reference.
Figure 4.4. Structures and molecular weights of the G6 derivative compounds NB36-NB42.
Figure 4-5. Effects of G6 derivatives NB36-NB42 on BaF3-Jak2-V617F cell viability. Cells were treated with each concentration of drug for 72 hours and cell viability was determined by MTS assay. G6 cell growth inhibition is shown in black as a reference.
CHAPTER 5
EXPRESSION AND PURIFICATION OF JAK2 PROTEIN IN E. COLI

The Jak2 kinase domain, JH1, is the main target of most small molecule drugs designed to inhibit Jak2 signaling. The kinase domain contains a number of critical residues that are important for the phospho-transferase activity of the protein. Some of these residues include Glu930 and Leu932 in the hinge region, Arg980 in the catalytic loop, and Asp994 in the activation loop (Lucet et al. 2006). Through in silico molecular docking studies, we have shown that the Jak2 inhibitory potential of G6 may be due to its interactions with each of these residues through hydrogen bonding (Majumder et al. 2010). In order to further validate these interactions, we sought to express and purify the Jak2 kinase domain using an E. coli system and subsequently co-crystallize the protein in a complex with G6. This chapter will describe the experimental methods and results of this study.

Materials and Methods

Cloning of Jak2-JH1 for Expression in E. coli

Human Jak2 cDNA encoding amino acids 835 to 1132 was cloned from pcDNA3-Jak2 to pQE-T7-2 using Not I and Xho I restriction digestion and ligation using T4 DNA ligase at 16°C overnight.

Expression of Jak2-JH1 in E. coli

Jak2-JH1 cDNA cloned into the pQE-T7-2 vector was used to transform chemically-competent JM-109-DE3 cells. Protein expression was induced by addition of various concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG) to E. coli cultures after an OD$_{600}$ of 0.6 was reached. After addition of IPTG, cultures were grown for 1 to 4 hours and cells were collected by centrifugation (4000g for 20 minutes at 4°C).
Isolation of Jak2 Protein from Inclusion Bodies

Cells were resuspended in a lysis buffer containing 100 mM Tris pH 8, 300 mM NaCl, 20 mM imidazole and 0.1 mg/mL lysozyme. Cells were sonicated on ice and centrifuged at 12,000g for 15 minutes at 4°C. Cell pellet was resuspended in fresh lysis buffer in order to remove remaining soluble content, and centrifuged at 12,000g for 15 minutes at 4°C. The remaining pellet, containing Jak2 inclusion bodies, was suspended in a denaturing buffer containing 100 mM Tris, 300 mM NaCl, 20 mM imidazole, and 8 M urea. After complete resuspension with vortexing, sample was centrifuged at 12,000g for 15 minutes at 4°C. The denatured protein supernatant was used for further analysis.

On-column Refolding of Jak2 Protein

Jak2 protein, containing a C-terminal 10x His tag, was purified using a Ni-NTA column. Approximately 15 ml of denatured protein was applied to a capped gravity flow column containing 2 ml of Ni-NTA agarose beads (Qiagen). The column was pre-equilibrated with denaturing buffer. The denatured protein/agarose bead slurry was incubated for 1 hour with shaking at 4°C. The column was then un-capped and the flow through was collected. A refolding buffer containing 100 mM Tris, 300 mM NaCl, 20 mM imidazole, 50 mM L-arg, 50-mM L-glu, and 1 mM DTT was applied to the column. The column was capped and incubated with shaking for 1 hour at 4°C. The flow through was then collected and the column was washed with 2x column volume of wash buffer (100 mM Tris/300 mM NaCl/20 mM imidazole). Protein was then eluted in 1mL fractions with an elution buffer gradient from 100 to 400 mM imidazole and 5% glycerol.

Anion-Exchange Purification of Jak2 Protein

Refolded Jak2 protein was dialyzed using Slide-A-Lyzer Mini Dialysis devices with a 10K molecular weight cutoff (Thermo) to a final buffer of 20 mM Tris pH 8.5, 100
mM NaCl, 5 mM DTT, and 10% glycerol. Protein was applied to a pre-equilibrated HiTrapQ anion exchange column (Qiagen) at a flow rate of 0.5 mL/min. The column was then washed with 5x column volume of the starting buffer. Protein was then eluted in 100 uL fractions with 20 mM Tris pH 8.5, 600 mM NaCl, 5 mM DTT, 100 uM G6, and 10% glycerol.

**SDS-PAGE and Western Blotting**

Proteins were analyzed by SDS-PAGE followed by either Coomassie staining or Western blotting. For blotting, an anti-His tag primary antibody (Santa Cruz) was used at 1:500 and an anti-mouse secondary antibody (GE) was used at 1:4000.

**Crystallization Trials**

Jak2 protein/G6 eluates were used to set up 96-well plate sitting drop vapor diffusion experiments. The screening conditions used in these experiments included the Hampton Crystal Screen 1 and 2 (Hampton Research) and two customized PEG/Ion screens.

**Results**

**Cloning and Expression of Jak2 in *E. coli***

We used the pQE-T72 cloning vector (Qiagen) in order to express Jak2-JH1 protein in *E. coli*. This vector contains a C-terminal 10x His tag for protein purification on a Ni-NTA column. Using the Not I and Xho I restriction sites, we successfully cloned in amino acids 835 – 1132 of the Jak2 kinase domain into the vector. For protein expression, we used JM109-DE3 cells, which contain the T7 RNA polymerase gene required for protein expression using the pQE-T72 vector. JM109-DE3 cells were transformed with the hJak2-pQE-T72 vector and selected by Kanamycin resistance. Protein expression was induced in small-scale cultures (3 mL) using 0.1 mM, 0.5 mM,
or 1.0 mM IPTG for an induction period of 2 or 4 hours. Cells pellets were resuspended in SDS loading buffer and Jak2 protein expression was examined by Western blot. We found that expression of Jak2 could be readily detected using an antibody against the His tag (Figure 5-1A). A band was detected at approximately 30 kDa, which was the expected molecular weight. We also found that the expression of Jak2 was consistent across all induction conditions that were tested (Figure 5-1A). We therefore chose to use an induction period of 4 hours with an IPTG concentration of 0.5 mM. We continued to use small cultures (10 mL) and lysed cells using a Tris buffer containing lysozyme in an attempt to obtain soluble Jak2 protein. Unfortunately, we found that all of the Jak2 protein was found in the cell pellet rather than in the soluble fraction (Figure 5-1B). This was true for a variety of other lysis conditions tested, so we moved forward with isolation of Jak2 protein from inclusion bodies.

Isolation of Jak2 from Inclusion Bodies and On-Column Protein Refolding

We initially attempted to isolate Jak2 protein from inclusion bodies using the CellLytic IB Inclusion Body Solubilization Reagent (Sigma) followed by dialysis for protein refolding. However, during dialysis, there was extensive protein precipitation and we were unable to obtain soluble refolded protein. We then switched to an on-column refolding method based on Qiagen on-column refolding protocols. Here, we lysed cells and solubilized the pellet in denaturing buffer. The denatured protein was applied to a column containing Ni-NTA agarose beads. The protein was then refolded on the column using a refolding buffer and eluted using an imidazole gradient. We found that pure, soluble refolded protein was obtained in the elution fractions (Figure 5-2). After optimizing this protocol, we moved forward with large scale protein expression and purification.
Purification and Concentration of Refolded Jak2 Protein

Protein expression was scaled up to maximize yield, and the on-column refolding procedure was performed as before. The protein obtained from on-column refolding was typically eluted at a maximum concentration of approximately 0.15 mg/mL. In order to concentrate the protein to a concentration suitable for co-crystallization studies, we used anion exchange chromatography. Refolded elution fractions (totaling 8-10 mLs) were first dialyzed to remove imidazole using Slide-A-Lyzer Mini-Dialysis devices from Thermo. After dialysis, protein was applied to a HiTrap Q anion exchange column and eluted in 100 uL fractions. After optimization, we found that the most suitable elution buffer was 20 mM Tris pH 8.5 containing 600 mM NaCl, 5 mM DTT, 100 uM G6, and 10% glycerol (Figure 5-3). We found that the protein precipitated out of solution when the concentration reached higher than approximately 2 mg/mL, even in the presence of various stabilizing additives. Therefore, the eluates ranging from 1 to 2 mg/mL concentration were collected and immediately used in crystallization trials.

Jak2-G6 Co-Crystallization Trials

The elution buffer for the anion exchange concentration experiments contained 100 uM G6 and was therefore directly ready for co-crystallization studies. However, the concentration of protein was not optimal for crystallization. We were unable to obtain soluble protein of a concentration higher than 2 mg/mL. Nevertheless, we set up various crystallization trials using the most concentrated protein samples obtained. Trials were conducted using a robotic 96-well plate system. Screening conditions included Hampton Screens 1 and 2 (Hampton Research) and two custom PEG/Ion screens (PEG 4000 and PEG 3350) which were established based on Jak2 crystallization conditions
reported in the literature (Lucet et al. 2006; Williams et al. 2009). Unfortunately, none of the conditions tested yielded protein crystals.

**Discussion**

Here, we have described our attempt to express, purify, and co-crystallize the Jak2-JH1 domain in complex with the small molecule Jak2 inhibitor G6. In these studies, we used an *E. coli* protein expression system and attempted to isolate and refold Jak2 protein from inclusion bodies. Much work has been done to improve the success of *E. coli*-based systems for the expression and purification of eukaryotic proteins (Sahdev et al. 2008). One benefit of using this system, particularly if the protein of interest is found in inclusion bodies, is that the protein will already be highly pure and concentrated after inclusion body solubilization. However, there are many challenges associated with refolding eukaryotic proteins after inclusion body purification. In many cases, the protein of interest will not properly refold to its native state, so many refolding conditions may need to be tested. In our case, we used an on-column refolding procedure that yielded soluble Jak2 protein. However, in our later experiments, we were unable to utilize this protein to obtain crystals.

Other groups have crystallized portions of the Jak2 kinase domain in the presence or absence of inhibitors (Lucet et al. 2006; Williams et al. 2009). In these studies, however, a baculovirus/Sf9 protein expression system was used to express Jak2 protein. In our case, we instead began our experiments with the *E. coli* system in an attempt to maximize protein yield, as expression in a baculovirus/Sf9 system had produced low yields of protein in other experiments from our laboratory (unpublished observations). However, based on the results we obtained in the current study, we believe the baculovirus/Sf9 system will be the best option for further Jak2 protein
purification studies. Characterizing the Jak2-binding properties of the small molecule inhibitor G6 would be important in verifying the compound’s interactions within the Jak2 ATP-binding pocket. We have so far observed these interactions in silico using molecular docking programs (Majumder et al. 2010), but verification of these results by obtaining structural data could provide important novel information. For example, because G6 is the first stilbene-derived Jak2 small molecule inhibitor, obtaining structural data could help us to determine what specific features distinguish G6 from other Jak2 small molecule inhibitors. These results could also assist in the development and optimization of more potent, specific, or metabolically stable stilbene-derived compounds for the purpose of Jak2 inhibition.
Figure 5-1. Expression of Jak2 protein in *E. coli*. A) Protein expression was induced by addition of IPTG to culture media for the indicated times. Cells were lysed in SDS loading buffer and analyzed by Western blot for Jak2 expression using an anti-His tag antibody. B) Protein expression was induced by addition of 0.5 mM IPTG for 4 hours. Cells were lysed with lysozyme. Supernatant (s) and pellet (p) fractions were analyzed by Western blot.
Figure 5-2. On-column refolding of Jak2 protein. Fractions were collected from on-column refolding experiments and analyzed by Coomassie staining.
Figure 5-3. Jak2 protein concentration on anion exchange column. Jak2 protein of an approximate 0.15 mg/mL concentration was applied to the anion exchange column and eluted in 100 uL fractions. Samples were analyzed by Coomassie stain to determine final protein concentration.
The serum and glucocorticoid inducible kinase 1 (SGK1) is a ubiquitously expressed serine/threonine kinase that regulates a wide variety of ion channels and transporters. SGK1 plays a key role in renal sodium retention and potassium excretion by regulating ENaC, ROMK, and Na⁺-K⁺-ATPase activity (Lang et al. 2006). The expression of SGK1 is controlled by a variety of stimuli, including numerous hormones and cytokines (Lang et al. 2006). Among its many functions, SGK1 is known to have anti-apoptotic effects through regulation of transcription factors such as FOXO3A (Brunet et al. 2001). SGK1 expression is elevated during wound healing and in several fibrotic diseases, including diabetic nephropathy and cardiac fibrosis (Lang et al. 2006). Additionally, SGK1 has been shown to promote cell survival in breast cancer cells, and contributes to glucocorticoid-induced chemotherapy resistance in breast cancer (Zhang et al. 2005; Wu et al. 2004).

Angiotensin II (Ang II) is the principle signaling molecule in the renin-angiotensin system (RAS). It acts through the Ang II Type 1 (AT₁) receptor to facilitate cell growth and pro-survival effects in a variety of cell types (Mehta and Griendling 2007). It has been shown that Ang II induces SGK1 expression in murine fibroblasts, causing increased expression of CTGF (Hussain et al. 2008). This pathway is believed to contribute to the pro-fibrotic effects of Ang II.

It has also been demonstrated that Ang II has a protective effect against apoptosis in fibroblast-like synoviocytes (Pattacini et al. 2007). Additionally, Ang II has
been shown to play a role in a number of cancers due to its proliferative effects via the AT₁ receptor (Haznedaroglu and Beyazit 2010; Uemura et al. 2011; Zhao et al. 2010b). However, whether Ang II can directly act through SGK1 in order to promote cell survival is unknown.

In the current study, we investigated the role of SGK1 in Ang II-mediated cell survival. Here, we hypothesized that Ang II would protect fibroblast-derived cells from serum starvation-induced apoptosis by upregulating and/or activating SGK1. To investigate this, we examined SGK1 mRNA expression, phosphorylation, downstream signaling, and apoptosis in a fibroblast-derived cell line in response to Ang II. We found that Ang II increased SGK1 mRNA and protein levels and increased the phosphorylation levels of SGK1 protein at Thr256 and Ser422. The Ang II-mediated increase in SGK1 mRNA levels required intracellular calcium mobilization and tyrosine kinase activation while the phosphorylation of SGK1 at Ser422 was mTOR-dependent. The consequence of Ang II-mediated SGK1 expression/activation was phosphorylation of FOXO3A and a subsequent increase in cell survival. As such, these results are significant in that they demonstrate a novel role of SGK1 in Ang II-mediated cell survival.

**Materials and Methods**

**Cell Lines**

The fibrosarcoma-derived cells stably expressing the AT₁ receptor have been previously described (Sandberg et al. 2004). Cells were cultured in DMEM supplemented with 10% FBS, penicillin, streptomycin, Zeocin, G418 and L-glutamine at 37°C and 5% CO₂. For serum starvation, cells were cultured in DMEM supplemented with 0.5% BSA (wt/vol), penicillin, streptomycin, and L-glutamine at 37°C and 5% CO₂.
**Ang II, Inhibitor Compounds, and Cell Treatments**

Ang II (Sigma) was stored in dd H2O at −20°C at a stock concentration of 10 µM. BAPTA-AM (Enzo Life Sciences) and PD98059 (Calbiochem) were stored at −20°C in DMSO at a stock concentration of 5 mM. Verapamil (Enzo Life Sciences), Genistein (Sigma), and Go6983 (Tocris) were stored at −20°C in DMSO at a stock concentration of 10 mM. Rapamycin (Sigma) was stored at −20°C in ethanol at a stock concentration of 500 µM.

For mRNA and protein analyses, cells were serum starved overnight and were then treated with 100 nM concentration of Ang II for the indicated periods of time. In drug pre-treatment experiments, cells were serum starved overnight and pre-treated with the following inhibitors prior to Ang II stimulation: BAPTA-AM (50 µM, 10 min), verapamil (10 µM, 5 min), PD98059 (50 µM, 1 h), Go6983 (10 µM, 1 h), genistein (100 µM, 1 h), or rapamycin (0.75–25 nM, 1 h). After appropriate inhibitor pre-treatment, the cells were treated with 100 nM Ang II and processed as described in each figure.

**Quantitative RT-PCR**

For quantitative RT-PCR, RNA was extracted using an RNeasy® mini kit (Qiagen) according to the manufacturer’s protocol. 2 µg of each RNA sample was reverse transcribed into cDNA in a final reaction volume of 20 µL using a high capacity cDNA reverse transcription kit (Applied Biosystems). SGK1 mRNA levels were determined using the TaqMan® gene expression assay Hs00178612_m1 (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase expression was determined as an internal loading control using Hs02758991_g1 (Applied Biosystems). Real time PCR was performed with TaqMan® universal PCR Master Mix (Applied Biosystems) in
a final reaction volume of 20 µL in a StepOne™ real time PCR system according to the manufacturer’s protocol.

**Western Blotting**

Cells were lysed in radioimmunoprecipitation assay buffer on ice and protein concentration was determined using a Bradford assay (Bio-Rad). Approximately 50 µg of soluble protein was separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked using 5% milk/TBS-T for 1 h. The following primary antibodies were obtained from Santa Cruz and were used at a 1:500 dilution: pSGK1 (Ser422), pSGK1 (Thr256), pFOXO3A (Thr32), and FOXO3A. The β-actin primary antibody (Cell Signaling Technology) and the total SGK1 antibody (Sigma) were also used at a 1:500 dilution. An HRP-conjugated anti-rabbit secondary antibody (GE Healthcare) was used at 1:4000. Bands were visualized using the enhanced chemiluminescence system (Western Lightning® Ultra; PerkinElmer Life Sciences).

**Transfection with siRNA**

Cells were transfected using DharmaFECT4 transfection reagent (Dharmacon) and a 25 nM concentration of SmartPool siRNA targeting SGK1 or a non-targeting SmartPool siRNA (Dharmacon). Cells were plated in a 6-well plate and incubated with transfection reagents for 6 h in antibiotic-free media. Cells were then allowed to recover overnight in complete media prior to experiments.

**TUNEL Staining**

After siRNA transfection and recovery, cells were transferred to 8-well chamber slides for TUNEL assay. Cells were subsequently serum starved for 48 h in the presence or absence of 100 nM Ang II. Cells were fixed in 1% paraformaldehyde at
room temperature for 10 min, washed 2× with PBS, and post-fixed with ethanol:acetic acid (2:1) for 5 min at −20°C. TUNEL staining was performed using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer's protocol. Images were obtained using an Olympus fluorescent microscope at 20× magnification.

Statistics

Statistical evaluation was performed using Student's t-test. Conditions were considered statistically significant when p < 0.05.

Results

Ang II Increases SGK1 mRNA and Protein Levels in a Calcium- and Tyrosine Kinase-Dependent Manner

Aldosterone is known to increase SGK1 expression within kidney epithelial cells leading to increased Na⁺ retention (Lang et al. 2006). Ang II has been shown to similarly increase SGK1 expression in fibroblasts, but the cellular outcome is increased expression of the pro-fibrotic marker CTGF, rather than increased Na⁺ reabsorption (Hussain et al. 2008). These results indicate that SGK1 mediates distinct cellular outcomes in response to different stimuli. In the case of Ang II-mediated SGK1 activation in fibroblasts, the specific signaling pathways that mediate this effect and the overall impact on cell fate have not previously been determined. Therefore, in order to provide some fundamental understanding of these critical events, we employed a fibrosarcoma-derived cell line that stably expresses the AT₁ receptor and examined Ang II/SGK1 signaling within this system.

The creation and characterization of these cells has been previously published (Sandberg et al. 2004). In short, they robustly express the AT₁ receptor, but lack AT₂
receptor expression. In addition to being highly responsive to Ang II, they readily express SGK1. We first wanted to determine if Ang II treatment can increase SGK1 mRNA levels in these cells as it does in cultured fibroblasts. For this, the cells were treated with Ang II for 2 h and SGK1 mRNA levels were examined by quantitative RT-PCR. We found that 2 h of Ang II treatment increased SGK1 mRNA levels by 2-fold (Figure 6-1A). To determine if SGK1 protein levels were also increased, cells were treated with Ang II for 0, 8, 16, and 24 h and SGK1 protein levels were analyzed by Western blot. We found that SGK1 protein levels were significantly increased at 16 and 24 h with a peak increase of ~ 2-fold observed at the 24 h time point (Figure 6-1B and C). Overall, these results indicate that Ang II increases SGK1 mRNA and protein levels in these cells. Furthermore, the results are virtually identical to those observed in human and mouse fibroblasts (Hussain et al. 2008).

SGK1 mRNA expression is regulated by a variety of signaling pathways including PKC, MAPK, and calcium (Lang et al. 2006). In order to examine the mechanism(s) by which Ang II increases SGK1 mRNA levels, we took a pharmacological approach whereby cells were pre-treated with various inhibitors prior to Ang II stimulation. Specifically, cells were pretreated with the indicated inhibitors and then treated with Ang II for 0 or 2 h. SGK1 mRNA levels were then examined by quantitative RT-PCR. We found that pretreatment with the intracellular calcium chelator BAPTA-AM, completely blocked the ability of Ang II to increase SGK1 mRNA levels, while the L-type calcium channel blocker verapamil was without effect (Figure 6-2A). We also found that the ability of Ang II to increase SGK1 mRNA levels was insensitive to the MEK inhibitor PD98059, was minimally sensitive to the PKC inhibitor Go6983, and was very sensitive
to the tyrosine kinase inhibitor genistein (Figure 6-2B). When taken together, these results indicate that the ability of Ang II to increase SGK1 mRNA levels requires intracellular calcium mobilization and tyrosine kinase activation.

**Ang II Causes an Increase in SGK1 Phosphorylation at Thr256 and Causes an mTOR-Dependent Increase in SGK1 Phosphorylation at Ser422**

In addition to increased mRNA and protein levels, SGK1 enzymatic activity can be augmented via post translational modification of the protein. Specifically, activation of SGK1 requires phosphorylation at Ser422 in the hydrophobic motif and at Thr256 within the activation loop (Lang et al. 2006). Currently, little is known about the direct effects of Ang II on the phosphorylation state of SGK1. Therefore, to determine if Ang II treatment has an impact on the phosphorylation of SGK1 at these critical sites, cells were treated with Ang II for 0, 15, 30, 45, and 60 min and SGK1 phosphorylation was examined by Western blot using phospho-specific antibodies. Figure 6-3A shows a representative phospho-Ser422 SGK1 blot and Figure 6-3B shows densitometric quantification of blots. The data indicate that Ang II treatment significantly increased the phosphorylation of Ser422 with peak phosphorylation occurring at the 30 min time point. With respect to Thr256 phosphorylation, Figure 6-3C shows a representative phospho-Thr256 SGK1 blot and Figure 6-3D shows quantification of all blots. We similarly observed a significant increase in Thr256 phosphorylation with peak phosphorylation occurring at the 15-30 min time points. Thus, these results indicate that in addition to increasing SGK1 mRNA and protein levels, Ang II treatment activates SGK1 protein by increasing its phosphorylation at Ser422 and Thr256.

It is well established that SGK1 Thr256 phosphorylation is PDK1-dependent, so we did not examine this mechanism in our cell model (Kobayashi and Cohen 1999).
However, it has previously been shown by various groups that either mTOR complex 1 (mTORC1) or mTOR complex 2 (mTORC2) can directly phosphorylate SGK1 at Ser422 (Garcia-Martinez and Alessi 2008; Hong et al. 2008; Lu et al. 2010). In order to determine if the Ang II-mediated phosphorylation of SGK1 at Ser422 is mTORC1- or mTORC2-dependent, cells were pre-treated with rapamycin prior to Ang II stimulation and phosphorylation of Ser422 was again examined by phospho-specific Western blot analysis. Figure 6-4A shows that pretreatment for 1 h, with increasing concentrations of rapamycin, completely blocked the ability of Ang II to increase SGK1 Ser422 phosphorylation after 30 min of treatment, the time that normally induces maximal phosphorylation at Ser422 (Figure 6-3). Additionally, a 1 h pretreatment with 25 nM rapamycin completely blocked Ser422 phosphorylation over a wide range of Ang II treatment points (Figure 6-4B). The concentrations of rapamycin and the duration of treatment used in these experiments are below the threshold of those shown to inhibit mTORC2 activity in vitro (Jacinto et al. 2004; Sarbassov et al. 2006; Barilli et al. 2008). Therefore, the ability of rapamycin to block Ang II-mediated phosphorylation at Ser422 indicates that this signaling process is mTORC1-dependent.

Ang II Protects Cells from Serum Starvation-Induced Apoptosis in an SGK1-Dependent Manner

The AT1 receptor typically mediates pro-growth and pro-cell survival signaling (Mehta and Griendling 2007). Furthermore, SGK1 itself has been directly implicated in cell survival (Brunet et al. 2001). Given the ability of Ang II to activate SGK1 in this system, we wanted to determine if Ang II mediates cell survival, and if so, whether this effect is SGK1-dependent. To determine this, we elected to use an SGK1 siRNA knockdown approach in cells that would be serum starved in the presence or absence
of Ang II and then examined for survivability. To demonstrate the feasibility of this approach, cells were either left untransfected or transfected with non-targeting siRNA or SGK1 targeted siRNA. Two days later, the levels of SGK1 mRNA were determined via quantitative RT-PCR. We found that while transfection of the non-targeting siRNA was largely without effect, transfection of the SGK1 siRNA reduced SGK1 mRNA levels by ~80% (Figure 6-5A). To determine if this decrease in SGK1 mRNA translated to reduced levels of SGK1 protein, whole cell protein lysates were examined for SGK1 protein levels via Western blot analysis. Figure 6-5B shows a representative Western blot and Figure 6-5C shows the quantification of all blots. We found that transfection of SGK1 siRNA decreased SGK1 protein levels by ~80% when compared to non-targeting transfected controls. When taken together, the data in Figure 6-5 demonstrate that siRNA targeting can effectively reduce SGK1 mRNA and protein levels in these cells.

We next wanted to determine the role of SGK1 in Ang II-mediated cell survival. Specifically, cells were transfected as indicated and then serum starved in the presence or absence of 100 nM Ang II for 48 h, at which time cell survival was determined via TUNEL staining. TUNEL detects apoptotic cells and its presence correlates inversely with cell survival. We found that in untransfected cells, serum starvation caused a significant increase in the percentage of TUNEL-positive cells and Ang II significantly prevented this increase (Figure 6-6A). This result indicates that Ang II is capable of protecting these cells from serum starvation-induced apoptosis. When cells were transfected with the non-targeting control siRNA, a similar pattern was observed whereby serum starvation increased TUNEL staining and Ang II reversed this effect (Figure 6-6B). However, cells transfected with SGK1-targeting siRNA had a high level of
apoptosis under serum-starved conditions and Ang II-treatment was unable to reverse this effect (Figure 6-6C). To quantify these results, the percentages of TUNEL-positive cells were determined and plotted as a function of treatment condition (Figure 6-6D). The data indicate that when compared to serum-containing conditions, serum starvation significantly increased the percentage of TUNEL positive cells in untransfected, non-targeting transfected, and SGK1 siRNA transfected cells. Furthermore, Ang II was able to improve cell survivability (i.e., decrease apoptosis) in the untransfected and non-targeting transfected cells, but not in the SGK1 siRNA transfected cells, thereby indicating a critical role of SGK1 in Ang II protection against serum starvation-induced apoptosis.

**Ang II Induces FOXO3A Phosphorylation in an SGK1-Dependent Manner**

One mechanism by which SGK1 regulates cell survival is through the phosphorylation of FOXO3A (Brunet et al. 2001). This transcription factor activates transcription of pro-apoptotic genes, and its phosphorylation has an inhibitory effect by excluding it from the nucleus. Therefore, we wanted to determine if Ang II regulates cell survival by inducing SGK1-dependent phosphorylation of FOXO3A. To determine this, untransfected cells were treated with Ang II for 0, 15, 30, 45, and 60 min and FOXO3A phosphorylation at Thr32 was examined by Western blot. We found that Ang II caused an increase in FOXO3A phosphorylation after 15 min and this persisted through the 60 min time point (Figure 6-7A). To determine if this effect was SGK1-dependent, cells were transfected with either non-targeting or SGK1-targeting siRNA and then treated with Ang II. We found that in cells transfected with non-targeting siRNA, the results closely mirrored those seen in the untransfected cells as phosphorylation of FOXO3A at Thr32 was significantly increased after 15 min of Ang II treatment (Figure 6-7B).
However, in cells transfected with SGK1-targeting siRNA, there was no increase in FOXO3A phosphorylation at Thr32 after Ang II treatment (Figure 6-7C). All blots were quantified and the relative phosphorylation levels of FOXO3A were plotted as a function of Ang II treatment time (Figure 6-7D). The results indicate that Ang II induces FOXO3A phosphorylation in an SGK1-dependent manner. Thus, FOXO3A phosphorylation may be a potential mechanism by which Ang II and SGK1 mediate survival in these cells.

**Discussion**

Ang II signaling via the AT₁ receptor has numerous biological effects, one of which is cell survival. Some mechanisms by which Ang II/AT₁ signaling can regulate cell survival include activation of NFκB, p38/MAPK, and PDK1-AKT signaling pathways (Mehta and Griendling 2007). The current study reveals that SGK1 plays a critical role in Ang II-mediated cell survival in fibroblast-derived cells. Figure 8 represents a working model of the proposed signaling pathways involved in this Ang II-mediated cell survival. Ang II, acting through the AT₁ receptor, leads to increased levels of SGK1 via calcium- and tyrosine kinase-dependent mechanisms (Figure 6-2). Additionally, Ang II causes phosphorylation of SGK1 at Ser422 in an mTOR-dependent manner (Figure 6-3 and Figure 6-4). Activated SGK1 then phosphorylates FOXO3A on Thr32 (Figure 6-7). The combined effect of increased SGK1 mRNA/protein levels and its concomitant activation, leads to an overall increase in cell survival (Figure 6-6).

These results may have a number of physiological and/or pathophysiological implications as SGK1 and Ang II are involved in many of the same cell signaling events and clinical pathologies. For example, SGK1 and Ang II are both involved in the progression of numerous fibrotic diseases including renal, hepatic and cardiac fibrosis (Lang et al. 2006; Vallon et al. 2006; Lang et al. 2000; Periera et al. 2009; Ruiz-Ortega...
et al. 2006; Billet et al. 2008). Additionally, it has been shown that Ang II contributes to hepatic myofibroblast survival during liver fibrosis (Oakley et al. 2009). It is therefore possible that some of the pro-survival effects of Ang II in hepatic myofibroblasts may be mediated by SGK1 signaling. Ang II and SGK1 have also been independently found to promote cell survival in breast cancer cells, and both SGK1 and the AT₁ receptor are involved in multiple myeloma (Wu et al. 2004; Haznedaroglu and Beyazit 2010; Zhao et al. 2010b; Fagerli et al. 2011). Given these overlapping roles in such a wide range of pathologies, it is possible that Ang II and SGK1 signaling contribute concurrently in a number of these cases.

Many of the studies examining Ang II-mediated SGK1 expression and function have focused on the actions of the RAS, and therefore have concentrated more on aldosterone release and subsequent SGK1 upregulation. However, the current study focuses on aldosterone-independent effects of Ang II on SGK1 expression and signaling. We have shown that Ang II impacts both the phosphorylation of SGK1 at activating sites and the expression of SGK1 mRNA/protein, leading to an overall increase in cell survival. Therefore, these results add to the body of literature on Ang II signaling and demonstrate that the Ang II/AT₁ signaling axis can influence cell survival through multiple, independent pathways.

Interestingly, we found that phosphorylation of SGK1 at Ser422 in response to Ang II was dependent on mTORC1 activity. In order to exclude the possibility of mTORC2 inhibition contributing to this effect, we treated with rapamycin at concentrations and durations that are not shown to inhibit mTORC2 in cell culture models (Jacinto et al. 2004; Sarbassov et al. 2006; Barilli et al. 2008). Various reports in
the literature have shown that Ser422 of SGK1 can be phosphorylated by mTORC2, and fewer groups have shown that mTORC1 is capable of phosphorylating this residue (Garcia-Martinez and Alessi 2008; Hong et al. 2008; Lu et al. 2010). However, no reports have focused specifically on Ser422 phosphorylation in response to Ang II. Therefore, the results found here indicate that the involvement of mTORC1 and/or mTORC2 may vary depending on the initial stimulus. It was recently found that Ang II activates mTOR via the AT₁ receptor, leading to tubulointerstitial fibrosis in a rat model, which further supports the proposed model of AngII/AT₁R/mTOR-mediated activation of SGK1 (Whaley-Connell et al. 2011).

In this study, we employed a fibrosarcoma cell line expressing the AT₁ receptor and lacking the AT₂ receptor (Sandberg et al. 2004). This was beneficial, as it allowed for isolation of Ang II signaling via the AT₁ receptor alone, and thus did not require additional confirmation with the use of AT₁ or AT₂ receptor blockers. Also, we used serum starvation as the model of apoptosis induction. This strategy was favorable as it allowed for examination of the effects of Ang II signaling on apoptosis in the absence of other growth factors or stimuli. Nevertheless, it would be interesting in future studies to investigate the role of these AngII/AT₁R/SGK1 signaling events in cell survival in other models, such as drug-induced apoptosis. Additionally, it would be interesting to investigate the physiological role of this signaling axis in disease models.
Figure 6-1. Increase in SGK1 expression in response to Ang II. A) Cells were serum starved overnight and treated with Ang II for 0 or 2h and SGK1 mRNA was quantified by qRT-PCR. n=6. *, p<0.05 vs. 0 h control. B) Cells were serum starved overnight and treated with Ang II for 0, 8, 16, or 24 h and SGK1 protein levels were analyzed by Western blot. C) SGK1 protein levels were quantified using densitometry and normalized to β-actin. n=3. *, p<0.05 vs. 0 h control.
Figure 6-2. Ang II increases SGK1 mRNA levels via mobilization of intracellular calcium and tyrosine kinase activation. A) Cells were serum starved overnight and pre-treated with BAPTA-AM (50 µM, 10 min) or verapamil (10 µM, 5 min) prior to treatment with Ang II for 0 or 2 h. SGK1 mRNA was quantified via qRT-PCR. n=3. *, p<0.05 vs. 0 h control. B) Cells were serum starved overnight and pre-treated with PD98059 (50 µM, 1 h), Go6983 (10 µM, 1 h), or genistein (100 µM, 1 h) prior to treatment with Ang II for 0 or 2 h. SGK1 mRNA was quantified using qRT-PCR. n=3. *, p<0.05 vs. 0 h control.
Figure 6-3. Ang II increases the phosphorylation of SGK1 at Ser422 and Thr256. Cells were serum starved overnight and then treated with Ang II for the indicated times and SGK1 phosphorylation at Ser422 (A and B) and Thr256 (C and D) was examined by Western blot. Phosphorylation was quantified using densitometry and normalized to total SGK1 protein levels. n=3. *, p<0.05 vs. 0 min control.
Figure 6-4. The Ang II-mediated phosphorylation of SGK1 at Ser422 is mTOR-dependent. A) Cells were serum starved overnight and then pre-treated with rapamycin (0 – 25 nM, 1 h) prior to treatment with Ang II for 0 or 30 min. n=3. B) Cells were serum starved overnight and then pre-treated with rapamycin (25 nM, 1 h) prior to treatment with Ang II for 0-60 min. n=3.
Figure 6-5. Confirmation of SGK1 knockdown. A) Cells were left untransfected or transfected with non-targeting or SGK1-targeting siRNA and SGK1 mRNA levels were then quantified by qRT-PCR. n=4. *, p<0.05 vs. untransfected and non-targeting siRNA. B) Cells were transfected with non-targeting or SGK1-targeting siRNA and SGK1 protein levels were examined by Western blot. C) SGK1 protein levels were quantified using densitometry and normalized to β-actin. n=2. *, p<0.05 vs. non-targeting siRNA.
Figure 6-6. Ang II protects cells from starvation-induced apoptosis and this is SGK1-dependent. Cells were left untransfected (A), transfected with non-targeting siRNA (B), or transfected with SGK1-targeting siRNA (C) and then serum starved for 48 h in the presence or absence of 100 nM Ang II. Cells were analyzed for apoptosis by TUNEL assay. TUNEL-positive cells are green and the DAPI nuclear counter stain is blue. D) The percentage of TUNEL-positive cells was quantified and plotted as a function of condition. n=4. *, p<0.05 vs. serum containing groups. #, p<0.05 vs. untransfected Ang II-treated group.
Figure 6-7. Ang II induces FOXO3A phosphorylation in an SGK1-dependent manner. Cells were left untransfected (A), transfected with non-targeting siRNA (B), or transfected with SGK1-targeting siRNA (C) and then serum starved overnight prior to Ang II treatment for 0-60 min. FOXO3A phosphorylation at Thr32 was examined by Western blot. D) FOXO3A phosphorylation was quantified using densitometry and normalized to total FOXO3A protein. n=3 for untransfected and n=2 for non-targeting and SGK1-targeting siRNA.
Figure 6-8. Proposed signaling pathway for the role of SGK1 in Ang II-mediated cell survival. Ang II acts via the AT₁ receptor to 1) increase SGK1 mRNA and SGK1 protein levels via calcium and tyrosine kinase-dependent mechanisms and 2) increase SGK1 and FOXO3A phosphorylation levels via an mTORC1-dependent mechanism. The combined effect of increased SGK1 mRNA levels, increased SGK1 protein levels, and activation of existing pools of SGK1 protein via post translational phosphorylation, is an increase in overall cell survival.
CHAPTER 7
CONCLUSIONS AND PERSPECTIVES

Characterization of Jak2 Inhibitors: Discoveries and Challenges

In the following sections, we will discuss some of the information that has been gained from our studies of Jak2 small molecule inhibitors. We will also discuss some of the challenges that are still faced in the field of molecular targeted cancer therapies and potential avenues of research that may be able to address these challenges.

Stilbene Compounds for Molecular Targeted Therapy

In Chapters 2 through 4, we have described novel SAR properties and novel applications for the Jak2 small molecule inhibitor G6. In the first set of studies, we found that the para-hydroxyl orientation of G6 is important for its ability to bind Jak2 \textit{in silico} and to inhibit Jak2 activity \textit{in vitro} and \textit{ex vivo}. G6 is a novel stilbene-derived Jak2 inhibitor with a structure similar to that of the compound resveratrol. Resveratrol, found in red grapes and other natural sources, has been shown to have various beneficial physiological effects, including anti-oxidant properties. Interestingly, resveratrol and some of its bioactive derivatives have actually been investigated for the treatment of hematological malignancies (Kelkel et al. 2010).

Even more interestingly, the para-hydroxyl derivatives of resveratrol were shown to have greater anti-leukemic activity, but less overall cell toxicity (Kelkel et al. 2010). These features may make para-hydroxyl stilbene compounds suitable scaffolds for developing specific, molecular-targeted chemotherapeutic agents. We believe that the results of our SAR studies support this notion. Specifically, we observed that the para-hydroxyl stilbene compound NB15 had the highest Jak2 inhibitory potential as measured by a reduction in Jak2 activity \textit{in vitro}, which was accompanied by a potent
inhibition of cell growth. However, the *meta*-hydroxyl compound NB4 had no effect on Jak2 activity, but still caused significant reductions in cell viability and induced apoptosis and cell cycle arrest. Thus, we believe the rearrangement of the active groups in this compound may have reduced its specificity for Jak2 and therefore increased its overall toxicity. Further studies to confirm this hypothesis could be conducted by testing the toxicity of these compounds in non-Jak2 dependent cell lines or in standard toxicity assays. In addition, obtaining structural information through co-crystallization with the Jak2 kinase domain may be an important step in fully understanding the basis for the differences in activity we observe between *para*- and *meta*-hydroxyl compounds. This structural information is not only useful in terms of Jak2 inhibition, but may be applicable in the development of other stilbene compounds targeting various signaling pathways.

Reseveratrol and its derivatives, for example, have been shown to interact with various signaling proteins involved in disease pathogenesis. These include cyclooxygenases, tyrosine kinases and serine/threonine kinases (Pirola and Frojdo 2008). In several *in vitro* and *in vivo* studies, resveratrol was shown to inhibit COX-1 activity and reduce tumor growth in mouse skin tumor models (Pirola and Frojdo 2008). Some of these studies identified key structural requirements for COX inhibition and led to the development of more specific stilbene-derived COX-1 inhibitors (Szewczuk et al. 2004; Handler et al. 2007). Such results validate our rationale for further development of specific, molecular-targeted small molecules derived from a stilbene core structure. Based on our observations, we particularly believe that a *para*-hydroxyl stilbene scaffold may be the optimal starting point for such studies.
Targeting Jak2 in Hematological Malignancies

The phenotypically diverse MPN diseases, including PV, ET, and PMF, have been shown to arise from a clonal Jak2 mutation that occurs within the pseudo-kinase domain of the protein (JH2 domain). This domain serves an auto-inhibitory role over the kinase domain, and the Jak2-V617F mutation relieves this auto-inhibition, leading to constitutive kinase activation (Zhao et al. 2010a; Gnanasambandan et al. 2010). However, the majority of Jak2 inhibitors that have been developed to date target the Jak2 kinase domain, and the ATP-binding pocket in particular. The structure of the ATP-binding pocket is highly homologous from one family of tyrosine kinases to another, so there is often much concern regarding the specificity of drugs that target this particular region.

From a clinical standpoint, many of the toxicities associated with Jak inhibitor therapy are limited to the hematopoietic system (anemia, thrombocytopenia, etc) (Majumder and Sayeski 2010). This would suggest that the effects of these drugs may be limited to tissues in which Jak2 is known to play an important role. However, in some cases, therapeutic efficacy of Jak2 inhibitors in MPN patients has been observed in the absence of the Jak2-V617F mutation (Verstovsek et al. 2008a). Such results call to question the real specificity of the drug for Jak2 inhibition, as opposed to non-specific myelosuppression. In addition, all of the current Jak2 inhibitors that have been tested clinically fail to correct the mutant Jak2 allele burden in patients, and thus are not curative in nature (Majumder and Sayeski 2010).

All of these concerns raise the question of whether targeting the Jak2 ATP-binding pocket is the best strategy for treating Jak2-mediated diseases. This is a complicated issue, but there are some structural features of the Jak2 kinase domain
that may justify using it as a target. In particular, Jak2 has a couple of unique features that may lend some degree of specificity for ATP-competitive drugs. First, the Jak2 ATP-binding pocket has a unique, somewhat closed structure when compared with other tyrosine kinases. This allows for drug-protein interactions that may not be achieved in other, more “open” kinase domains (Lucet et al. 2006). In addition, Jak2 has a Jak insertion loop that is not conserved in other tyrosine kinases outside of the Jak family (Lucet et al. 2006). We believe that these structural features of Jak2 make it a suitable target for small molecule kinase inhibitors. Specifically, we believe that the unique stilbene-derived structure of G6 may lend greater specificity for Jak2 versus other tyrosine kinases, in part due to this more closed ATP-binding pocket structure. We have demonstrated in vitro that G6 has no activity against c-Src or Tyk2, and that it does not inhibit pathologic cell growth that is Jak3- or c-Myc-dependent (Kiss et al. 2009). The unique structure of the Jak2 ATP-binding pocket may be a possible explanation for this selectivity.

While we have a rationale for targeting the Jak2 ATP-binding pocket, there is still a need for other, more selective drugs that can specifically target Jak2-V617F mutant clones rather than wild-type Jak2 protein. It should be noted that G6 is capable of reducing the Jak2-V617F allele burden within the bone marrow of transgenic MPN mice (Kirabo et al. 2011b). However, the basis for this selectivity over wild-type clones is currently unknown. In many cases, current drugs inhibit wild-type and mutant Jak2 protein activity equally, which may explain some of the myelosuppressive side effects of drugs in clinical trials. The ultimate goal, then, would be to specifically eliminate Jak2 mutant clones without inhibiting the wild-type protein in healthy cells. A major limitation
that has been faced so far has been the lack of any structural information on the Jak2 pseudo-kinase (JH2) domain. Until recently, there was no crystal structure for this portion of the protein, so creating a drug to specifically target this region was not possible through computational screening. However, a recent report described the crystallization of both the wild-type and V617F mutant Jak2 JH2 domains (Bandaranayake et al. 2012). This report identified some of the key structural differences between the wild-type and mutant JH2. This structural characterization of the pseudo-kinase domain opens the door for the development of novel allosteric Jak2 inhibitors targeting the mutant Jak2-V617F protein. Such compounds may be able to improve the overall efficacy and tolerance of Jak2 inhibitor therapy in a clinical setting.

**Targeting Jak2 in Solid Tumors**

In Chapter 4, we described the effects of the Jak2 small molecule inhibitor G6 in the T98G glioblastoma cell line. We found that inhibition of Jak2 in these cells was able to reduce their tumorigenicity as measured by reductions in cell viability, colony formation, migration, and invasion. These results contribute to the field of cancer biology and add to the body of evidence suggesting that Jak2 plays an important role not only in hematological malignancies, but also in solid tumors such as glioblastoma.

To date, Jak2 is believed to play a role in a variety of solid tumors through mechanisms that are independent of any activating Jak2 mutations such as V617F. These tumor types include non-small cell lung carcinoma (Looyenga et al. 2012), colorectal cancer (Seavey et al. 2012), breast cancer (Berishaj et al. 2007), prostate cancer (Liao and Nevalainen 2011), and glioblastoma (GBM) (Sciaccaluga et al. 2007). Although these malignancies occur in the absence of activating Jak2 mutations, hyperactive Jak2 signaling has been observed and Jak2 inhibitors have been effective
against pathologic cell and tumor growth. The mechanisms of Jak2 activation in solid tumors are not clear, but some argue that Jak2 is important because multiple upstream signaling cascades converge into the Jak/STAT signaling pathway. Additionally, Jak2 single nucleotide polymorphisms (SNPs) have recently been identified and associated with prostate cancer risk (Kwon et al. 2012).

In our study, we focused on the role of Jak2 in GBM. This type of primary brain tumor is highly aggressive and is associated with very dismal survival outcomes. Recent studies have identified a potential role of Jak2/STAT3 signaling in the pathogenesis of GBM. In various GBM cell lines, different groups have observed constitutive Jak/STAT signaling and have shown that these cell lines are sensitive to Jak2 inhibition. We examined a group of GBM cell lines and identified one (T98G) that expressed high levels of phosphorylated Jak2 protein. We subsequently showed that treatment with the Jak2 small molecule inhibitor G6 reduced the tumorigenicity of these cells. Our results support the notion that Jak2 inhibitor therapy may be beneficial in glioblastoma patients.

However, there is still much uncertainty regarding a widespread role of Jak2 in GBM. This type of cancer is highly heterogeneous and can involve a number of different cell types within the brain. The list of activating mutations associated with glioblastoma has continued to grow, and numerous molecular targeted therapies have been tested at both the pre-clinical and the clinical level. Unfortunately, these have largely been unsuccessful at increasing overall survival in glioma patients (Ohka et al. 2012). The heterogeneity of glioma tumors greatly increases the complexity and difficulty of developing therapies. Additionally, it contributes to drug resistance and tumor relapse in patients. In recent years, many groups have proposed and validated the concept of
tumor initiating cells, or cancer stem cells (CSCs), which are believed to drive glioma tumor growth and drug resistance (Bonavia et al. 2011). These tumor stem-like cells have been shown to have distinct genotypic and phenotypic signatures when compared with standard glioma cell lines (Lee et al. 2006).

Both the heterogeneity of glioma tumors and the discovery of CSCs have called to question the use of standard, serum-cultured glioma cell lines for the purpose of drug development. Many believe that targeting the stem-like cell niche may be the best way to treat glioma, and much effort has been made to develop such therapies. The role of Jak2 in glioblastoma CSCs has not been fully characterized, but some evidence suggests that inhibition of Jak2 in these cells can reduce tumor growth (Sai et al. 2012).

In our study, we sought to investigate the role of Jak2 in six different primary glioblastoma stem-like cell lines. We found very low levels of Jak2 activation, and we found that Jak2 expression was increased upon differentiation of the cells toward an astrocyte phenotype. Interestingly, treatment with the Jak2 inhibitor G6 caused an increase in GFAP expression in the presence or absence of serum. This result was intriguing, because it implies a role for Jak2 in maintenance of a stem-like state in these cells, in spite of the relatively low levels of Jak2 phosphorylation we observed. However, these results are very preliminary and a further characterization of the cell lines would be necessary in order to verify them. Theoretically, inducing differentiation through Jak2 inhibition could cause GBM stem-like cells to become less malignant or more chemosensitive. Testing such a hypothesis could demonstrate a novel application for Jak2 inhibitor therapy. Additionally, a widespread screening of multiple cell lines could help to
determine how common Jak2 activation is among glioma stem-like cells. Such studies could help to clarify whether Jak2 is truly a viable target in the treatment of GBM.

Future studies could also investigate the role of Jak2 in drug-resistant glioma cells. It would be interesting to determine if there is any change in Jak2 expression or activation once a cell acquires drug resistance, and to examine whether Jak2 inhibition may be able to sensitize these resistant clones to other chemotherapeutic agents. In addition, based on the genetic characterization of the various GBM subtypes and the corresponding molecular targeted therapies, Jak2 inhibition could be combined with other therapeutic agents in order to improve patient outcomes. Overall, there are still relatively few studies that have specifically examined Jak2 signaling in glioblastoma. So far, we believe that there is evidence that Jak2 signaling may contribute significantly in this malignancy, and that G6 may therefore be a candidate for glioblastoma therapy.

**Novel Discoveries in Cell Survival Signaling**

In this final section, we will discuss some of the implications of our cell survival study and will outline some of the potential future studies related to this work.

**Angiotensin II and SGK1: Mechanisms of Disease Pathogenesis**

In Chapter 6, we described our study examining the role of SGK1 in Ang II-mediated fibroblast cell survival. We found that Ang II protected cells from apoptosis in an SGK1-dependent manner, potentially through mTOR and FOXO3A signaling mechanisms. These results highlight a possible mechanism involved in Ang II-mediated pathologies such as fibrosis and cancer.

Ang II, as the primary signaling molecule of the RAS, has potent effects on blood pressure and is known to contribute to a plethora of cardiovascular pathologies. Many of the physiological and pathological actions of Ang II are mediated through the AT1R.
One signaling molecule downstream of Ang II/aldosterone signaling is SGK1, which regulates renal sodium retention and fluid homeostasis. Interestingly, both Ang II and SGK1 have independently been associated with a variety of fibrotic pathologies in various tissues, including the kidney (Ruiz-Ortega et al. 2006; Lang et al. 2006). In addition, both have been implicated in the growth promotion and survival of cancer cells (Zhao et al. 2010b; Uemura et al. 2011; Wu et al. 2004; Lang et al. 2010).

Few studies to date have examined the direct, aldosterone-independent effects of Ang II on the expression and activation of SGK1. We found that in fibroblast-derived cells, Ang II was capable of both upregulating and activating SGK1. One consequence of this signaling mechanism was increased cell survival under serum starvation conditions. There is also evidence that Ang II mediates some of its pro-fibrotic effects through SGK1 signaling in fibroblasts, namely through the upregulation of CTGF (Hussain et al. 2008). We believe that a combination of these effects, with Ang II acting locally through an SGK1 signaling axis, may contribute to a number of tissue-specific fibrotic diseases. Thus, we believe that regulation of SGK1 via Ang II signaling is not only important in terms of the systemic RAS, but also in terms of local tissue RAS. More _in vitro_ and _in vivo_ studies need to be conducted in order to fully characterize the signaling pathways involved in Ang II-mediated SGK1 activation and the physiological or pathological consequences.

Some limitations of our study include the use of only one cell line, which was derived from a fibrosarcoma. Future studies could examine the effects of Ang II/AT1R signaling on SGK1 expression and activity in other cell lines, including primary fibroblast cells. In addition, it would be interesting to examine the physiological impact of SGK1
inhibition or deletion in models of Ang II-mediated fibrosis. In fact, a recent report showed that SGK1 knockout mice were protected from Ang II-induced cardiac inflammation and fibrosis (Yang et al. 2012). In this report, wild type mice receiving Ang II infusion developed cardiac hypertrophy and fibrosis, while their SGK1 knockout littermates were largely unaffected. To further demonstrate the aldosterone-independent effects of SGK1 under Ang II infusion, similar studies could be carried out in the presence of a mineralocorticoid receptor antagonist such as spironolactone. Such studies could demonstrate whether the beneficial effects of inhibiting SGK1 and/or aldosterone signaling in the presence of Ang II infusion would be similar, additive, or synergistic.

We are also interested in a possible role for this Ang II/AT1R/SGK1 survival signaling pathway in other cell types, and possibly in cancers in which Ang II and/or SGK1 are known to promote cell growth. For example, it has been demonstrated that RAS components are sometimes upregulated in breast cancer (Vinson et al. 2012). In addition, AT1R blockers have been shown to reduce the proliferative effects of Ang II in MCF-7 breast cancer cells (Du et al. 2012). Also in MCF-7 cells, other groups have shown that SGK1 is activated via mTOR-dependent mechanisms (Hall et al. 2012). As we demonstrated in fibroblast culture, Ang II treatment led to an mTOR-dependent increase in SGK1 phosphorylation. Thus, it would be interesting to examine the proliferative effects of Ang II in breast cancer cells, such as the MCF-7 cell line, and to determine if these effects involve mTOR/SGK1 signaling. Identification of these signaling mechanisms in breast cancer, or other solid tumors, may contribute to the development and application of novel therapeutic strategies.
Another pathophysiological event that may involve this Ang II/AT1R/SGK1 signaling axis is the process of restenosis. After angioplasty, restenosis often occurs due to the proliferation and migration of vascular smooth muscle cells. It has been shown that the use of the mTOR inhibitor rapamycin in stents helps to prevent this process (Sousa et al. 2001). It is known that Ang II promotes proliferation of vascular smooth muscle cells and plays a role in neointimal formation after vascular injury (Laporte and Escher 1992). Additionally, it was recently demonstrated that administration of the insulin sensitizer rosiglitazone can prevent Ang II-mediated vascular smooth muscle cell proliferation via mTOR-dependent mechanisms (Kim et al. 2012). Given the ability of Ang II to activate and up-regulate SGK1 through mTOR signaling, we believe this pathway may be involved in the process of restenosis and may be of further interest. Future studies to investigate a role for SGK1 could involve examination of SGK1 expression and activation before and during restenosis in the presence or absence of SGK1, mTOR, and/or Ang II blockade.

This Ang II/SGK1 cell survival signaling axis may also play a role in normal physiological events, such as embryonic development. It has been reported that Ang II acting through the AT1R promotes ureteric bud branching in the developing kidney, and that pharmacological inhibition of AT1R signaling induces apoptosis in ureteric bud tips (Yosypiv et al. 2008). It has also been shown that SGK1 is expressed in ureteric buds and in other parts of the kidney during embryonic development (Huber et al. 2001). The physiological role of SGK1 during kidney development is not known. Given its anti-apoptotic role in other tissues, it could be proposed that SGK1 regulates apoptosis in this developmental stage. Studies utilizing SGK1- and/or AT1R-knockout mice could
help to further understand any potential role these play during embryonic development, particularly within the kidney. In summary, the collective data presented in this dissertation have 1) defined novel Jak2 small molecule inhibitors for cancer therapy and 2) identified a novel mechanism of cell survival.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Rebekah Baskin was born and raised in the town of Alma, Georgia. During her high school studies, she became interested in science and was inspired to enter the field of chemistry. In 2004, she began her undergraduate studies at Georgia Southern University in Statesboro, GA, where she majored in chemistry. As an undergraduate, she worked in the laboratory of Dr. Laura D. Frost in the Chemistry Department, studying bile salt-phospholipid interactions using fluorescence spectroscopy techniques. Working in a biochemistry-focused laboratory and studying molecular disease mechanisms through her coursework inspired Rebekah to pursue a career in biomedical research. In May of 2008 she obtained her Bachelor of Science in chemistry and in August of 2008 she began her graduate studies at the University of Florida in the Interdisciplinary Program in Biomedical Sciences. There, she began working as a graduate assistant in the laboratory of Dr. Peter P. Sayeski, where she studied the role of Jak2 in cancer and the characterization of Jak2 inhibitors. In December 2012, she obtained her Ph.D. in biomedical sciences with a concentration in physiology and pharmacology.