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Critical Kinases Required for the Proliferation and Survival of Diffuse Large B-cell Lymphoma

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CRITICAL KINASES REQUIRED FOR THE PROLIFERATION AND SURVIVAL OF DIFFUSE LARGE B-CELL LYMPHOMA

By

Julie M. Matthews

A DISSERTATION

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CRITICAL KINASES REQUIRED FOR THE PROLIFERATION AND SURVIVAL OF DIFFUSE LARGE B-CELL LYMPHOMA

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Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL). Previously, distinct molecular subtypes: Germinal Center B-Cell like (GCB-like) and Activated B-Cell like (ABC-like) DLBCL were identified by large-scale microarray analysis. In the current study, we set out to determine whether there are distinct profiles for kinase activity/expression within or across DLBCL subtypes. Herein we demonstrate the existence of broad intracellular activation/upregulation of components of the mitogen-activated protein kinase (MAPK) signaling pathway. We specifically identified the MAPK components germinal center kinase (GCK) and c-jun N-terminal kinase 1 (JNK1) as being critical to the proliferation and survival of DLBCL cell lines and primary tumors. Immunohistochemical staining of GCK and p-JNK demonstrated strong positive GCK staining in 88% and 53% of primary DLBCLs, respectively. The expression of GCK and p-JNK did not correlate with the expression patterns of ABC-like or GCB-like markers, demonstrating that this expression/activation is not DLBCL subtype specific. Examination of GCK, p-JNK1 and JNK1 levels in DLBCL cell lines by Western blotting demonstrated
robust expression in most cell lines, irrespective of the subtype. Knockdown of GCK and JNK1 by small interfering RNAs (siRNAs) demonstrated the important role of these kinases in DLBCL cell proliferation and survival. A specific inhibitor for GCK, HG-6-64-1, was produced and tested. Treatment with the GCK inhibitor HG-6-64-1 or the JNK inhibitor 9L, led to cell cycle arrest, the induction of apoptosis in DLBCL cell lines and apoptosis in primary tumors. Overall our results demonstrate that JNK1 activation through GCK contributes to the proliferation and survival of DLBCLs, identifying a previously unrecognized mechanism of DLBCL survival and two new potential DLBCL therapeutic agents.
ACKNOWLEDGEMENTS

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List of Abbreviations

ABC-like - activated B-cell like
ABL1/2 - V-abl Abelson murine leukemia viral oncogene homolog 1/2
AID- activation-induced (cytidine) deaminase
BCL2 - B-cell lymphoma 2
BCL6 - B-cell lymphoma 6
CLL - chronic lymphocytic leukemia
c-raf - RAF proto-oncogene serine/threonine-protein kinase
C-REL - c-rel proto-oncogene protein
CSK - c-src tyrosine kinase
DLBCL - diffuse large B-cell lymphoma
DMSO - dimethyl sulfoxide
ERK - extracellular-signal-regulated kinase
G1 – gap 1
GCB-like - germinal center B-cell like
GCK – germinal center kinase
IKK - IκB kinase complex
IL- interleukin
IPI - international prognostic index
JNK – c-jun N-terminal kinase
LCMS/MS - liquid chromatography-mass spectrometry/mass
LYN - V-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MAPK – mitogen-activated protein kinase
MCL - mantle cell lymphoma
MCP-1 - monocyte chemotactic protein-1
MEKK1 – MAP3K1
MK2 - mitogen-activated protein kinase activated protein kinase 2
MSK-1 - mitogen- and stress-activated protein kinase-1
MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MZL - marginal zone lymphoma
NF-κB - nuclear factor-κB
NHL - non-Hodgkin lymphoma
p38 - p38 MAP Kinase
PAX5 - paired box protein 5
PBL – peripheral blood lymphocyte
PBS – phosphate buffered saline
PIM1 - proto-oncogene serine/threonine-protein kinase 1
RhoH/TTF - ras homolog gene family, member H/translocation three four
S - synthesis
SAPK - mammalian stress-activated protein kinase
siRNA - small interfering RNA
STAT3 – signal transducer and activator of transcription 3
TAK1 - transforming growth factor β–activated kinase 1
TP53 - tumor protein 53
TRAF6 - tumor necrosis factor receptor associated factor 6
ZAK - sterile alpha motif and leucine zipper containing kinase AZK
INTRODUCTION

I. Diffuse Large B-Cell Lymphoma

*The Pathogenesis and Treatment of Diffuse Large B-cell Lymphoma*

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease which is considered, by many, to be a collection of diseases rather than one specific entity. The mechanisms of DLBCL oncogenesis vary widely, as do the clinical responses to treatment amongst DLBCL patients.

As with most cancers, DLBCL pathogenesis occurs as a multistep process. Frequently, the biological programs that operate within normal B-cells go awry and these alterations cause healthy lymphocytes at various stages of maturation to undergo genomic amplifications, deletions, mutations, translocations, and alterations in transcription leading to the emergence of a malignant clone (Table 1.1).¹

The most common type of genetic lesion in DLBCL is somatic hypermutation.²⁻⁴ The highly prolific and mutagenic nature of the germinal center environment is necessary to create B-cell receptors which are highly specific to their antigen. However, this same mutagenic process can affect a wide variety of genes.⁵⁻⁸
Table 1.1: Known and predicted mechanisms of pathogenesis due to common genetic and molecular lesions identified in DLBCL. Some lesions are subtype specific, occurring in only ABC-like or GCB-like DLBCL, while others occur in all subtypes of DLBCL.⁹

The process of somatic hypermutation in B-cells introduces mutations into the immunoglobulin variable (V) domains, thereby generating antibodies that have high affinity for a given antigen. Recent work has demonstrated that this same process leads to mutagenesis in non-immunoglobulin genes including c-MYC, BCL6, and others; a process which occurs due to activation-induced (cytidine)
deaminase (AID) expression in these cells. Thus, it is not surprising that 50% of DLBCLs exhibit somatic hypermutations in several genes such as B-cell lymphoma 6 (BCL6), c-MYC, proto-oncogene serine/threonine-protein kinase (PIM1), paired box protein 5 (PAX5), and ras homolog gene family, member H/translocation three four (RhoH/TTF). Beyond somatic hypermutation, there are a number of mechanisms of pathogenesis which occur across the spectrum of DLBCL tumors, most notably BCL6 translocations, occurring in 30-40% of DLBCLs and mutations of tumor protein 53 (TP53), which occur in approximately 20% of DLBCLs (Figure 1.1).

Figure 1.1: The frequency of occurrence of the most common genetic lesions in DLBCL. A compilation of data from numerous studies on DLBCL.

BCL6 protein is normally upregulated in germinal center B-cells to repress apoptosis and promote proliferation. This contributes to the high proliferative rate and genetic instability at the time of B-cell affinity maturation, predisposing to malignant transformation and thus contributing to oncogenecity. Similarly,
mutations in TP53 are well documented to lead to drug resistance, metastasis and transformation in a variety of cancers.\textsuperscript{16} While these, and other mechanisms of pathogenesis, are characteristic of DLBCL tumors as a whole, there are alterations which occur only in certain subsets of DLBCL.

**Classification of the subsets of DLBCL**

Understanding the origin of DLBCL subsets requires knowledge of the process of B-cell maturation (Figure 1.2). Hematopoietic stem cells in the bone marrow give rise to all the cells in the immune system. B-cells originate in the bone marrow and they must express BCR’s or they will apoptose. Once a B-cell precursor leaves the bone marrow, it travels through the peripheral circulation to the lymph node. B-cells encounter antigens, migrate to lymph nodes and initiate the germinal center reaction which promotes selection of cells expressing BCRs with the highest affinity to the initiating pathogens. During this process the immunoglobulin undergoes somatic hypermutations and class switching to create the best binding affinity to the antigen. Those cells with high binding affinity for antigen proliferate inside the lymph node and differentiate to either memory cells or plasma cells.\textsuperscript{15}

Due to the phenomenon that DLBCL patients with the same diagnosis have remarkably different responses to therapy, a collaborative gene profiling study was established to determine the molecular heterogeneity that exists between and within a variety of lymphomas. The major finding of this study was
that DLBCL could be divided into two distinct subtypes, namely Activated B-Cell like (ABC-like) and Germinal Center B-Cell like (GCB-like) DLBCL. It is hypothesized that GCB-like DLBCLs originate from early germinal center B-cells, whereas ABC-like DLBCLs arise from late germinal center or post germinal center B-cells.

Figure 1.2: *The process of B-cell maturation*. This process begins in the bone marrow and ends when B-cells exit the lymphoid organs as memory B-cells or plasma cells. It is the highly mutagenic process in between, specifically in the germinal center, where DLBCL tumors arise.

These DLBCL subtypes vary in underlying pathogenetic mechanisms, response to treatment and prognosis. GCB-like DLBCLs have gene expression profiles closely mirroring that of normal germinal center lymphocytes and
frequently harbor translocations of B-cell lymphoma 2 (BCL-2), amplifications in the c-rel proto-oncogene protein (C-REL) locus, intraclonal heterogeneity of immunoglobulin V (IgV) and/or upregulation of components of the interleukin (IL)-4 signaling pathway. In contrast, ABC-like DLBCLs have a gene expression pattern characteristic of peripheral blood B-cells that have been activated in vitro. These tumors frequently demonstrate constitutive activation of the IκB kinase complex (IKK), increased expression of nuclear factor-κB (NF-κB) and its target genes, mutations in genes regulating the NF-κB pathway and constitutive B-cell receptor signaling. Recently, constitutively active signal transducer and activator of transcription 3 (STAT3) has also been reported in the ABC-like DLBCL subtype.

DLBCL subtype profiling allowed identification of tumors with distinct prognoses. Prior to DLBCL subtype separation, the international prognostic index (IPI) was used to classify patients into high and low clinical risk based on a variety of factors such as age, stage of disease, serum lactate dehydrogenase level, performance status and number of extranodal sites. Using the IPI to separate 38 patients by risk factors, a low clinical risk group exhibited 62.5% survival, and high clinical risk group 21.4% survival. In contrast, subdivision by ABC-like and GCB-like diagnosis allowed a much better predictive algorithm, with 76% of GCB-like patients alive at the 5 year point, versus 16% of ABC-like DLBCL patients (Figure 1.3).
Figure 1.3. **GCB-like DLBCL patients have a distinct survival advantage over ABC-like DLBCL patients.** Kaplan Meier plots of A) all DLBCL patients, subdivided into GCB-like and ABC-like subtypes, B) IPI high and low clinical risk patients and C) low risk clinical patients, as identified by the IPI, subdivided by DLBCL subtype.17

**Current DLBCL Therapeutic Strategies**

Irrespective of ABC-like or GCB-like classification all DLBCL patients are treated with combination chemotherapy consisting of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP). Rituximab is a B-cell specific monoclonal antibody, targeting the B-lymphocyte antigen CD20, and its addition to the CHOP regimen has dramatically increased patient survival in the last ten years.26

However, only 50-60% of patients respond to the current standard treatment for DLBCL, as demonstrated by the current 5 year survival rates.26 The need for novel, targeted and increasingly efficacious therapeutics is clear.
II. Kinase Targeting For Cancer Therapeutics

Kinases in Cancer

Kinases, by definition, phosphorylate tyrosines, threonines and serines. There are 90 known tyrosine kinases and 388 serine-threonine kinases, and up to 50% of intracellular proteins are subject to phosphorylation. Therefore, kinases initiate broad intracellular signaling and aberrations in kinase activation and regulation can lead to a wide variety of human diseases. Comparative genomic hybridization has allowed mapping of chromosomal areas that are amplified in tumors and 164 of the 518 known human protein kinases map to these locations. Discoveries in cancer research dating back 25 years involve protein kinases, specifically the knowledge that protein kinases can transform cells by inappropriately phosphorylating signaling proteins. In fact, viral oncogene products are often protein kinases. This makes kinases attractive drug targets and the developing drug market reflects that, with releases such as imatinib mesylate (Gleevec), which inhibits the tyrosine kinase bcr-abl, gefitinib (Iressa) which slows tumor growth by inhibiting intracellular tyrosine kinases that affect the epidermal growth factor receptor (EGFR), and erlotinib (Tarceva) which is a specific EGFR tyrosine kinase inhibitor.

Chemical kinase inhibition is historically problematic due to the fact that a wide variety of kinases contain similar catalytic domains. Thus, kinase inhibitors marketed as “specific” often turn out to have off-target multi-specificity functions. Selectivity is a key issue in the development of any drug, and kinase-inhibitors
are no exception. Many chemical kinase inhibitors turn out to be non-selective for their kinase of interest. Gleevac, for example, is relatively non-specific which led to delays in its clinical use. However, eventually it was realized that its non-selective properties were well-tolerated in humans and may be beneficial in terms of clinical response. Correspondingly, H89, marketed as a “selective and potent PKA inhibitor” was later reported to have higher specificity for at least three other kinases.34

Although kinases are attractive drug targets in cancer, the need for mass-profiling of kinase specificity is critical to the development of safer, more efficacious cancer therapeutics.

**Targeted Analysis of Differential Kinase Activation and Kinase Inhibitor Specificity**

We have undertaken the task of profiling kinase activity in DLBCL cell lines to further delineate potential mechanisms of DLBCL pathogenesis. A comprehensive and highly specific kinase array has recently been developed which allows broad surveying of active kinases in protein or tissue lysates. In addition to identifying kinases which are differentially activated between model systems, this method of kinase profiling allows for the testing of specificity of kinase inhibitors. The basis of the ActivX kinase activity array is an adenosine triphosphate (ATP)-mimetic labeled probe that consists of a recognition element which provides specificity for protein kinases and a reactive group for covalent linkage of the probe to the target kinase (Figure 1.4). The probe was designed to
be compatible with conserved lysine residues in protein kinases and targets lysine residues within the kinase active site (Figure 1.5). When the ATP-mimetic probe binds an open ATP-pocket on a given kinase its’ terminal acyl phosphate reactive group is brought into proximity with the conserved lysines. In this position, the probe and the kinase bind irreversibly and release the ATP molecule which is inherent to the probe. The ActivX chemical proteomics platform thereby enables quantitative assessment of ATP binding site affinity/occupancy across greater than 80% of kinases present in cell or tissue lysates by using a combination of nucleotide acyl-phosphate probes and targeted liquid chromatography-mass spectrometry/mass (LCMS/MS). This technology allows for the expression/activity of the majority of kinases present in cell lines or primary tissues to be simultaneously analyzed alongside the exact effect of a given kinase inhibitor on those samples. The raw data from the array consists of peak heights from the mass spectrometer, which is an indication of the relative abundance of each kinase in possession of an open ATP-binding pocket.

The ability to identify greater than 80% of kinases with this method is a vast improvement over recent screens which identified less than 30% of the predicted kinome. ActivX also has an adenosine diphosphate (ADP)-pocket binding probe that assists with the detection of additional active kinases.
Figure 1.4: *Structure and mechanism of kinase probe binding.* A) Biotin-labeled probes contain ATP or ADP binding groups and an acyl phosphate reactive group. B) When the probe binds a kinase, its acyl phosphate interacts with conserved lysine residues in the kinases’ active site. The reaction releases ATP or ADP and forms an amide bond between the kinase and the biotin tag.\(^{35}\)

Figure 1.5: *Basis of the ActivX Probe Labeling Technology.* Sequences surrounding the conserved lysine residues in A1) The ATP binding loop, A2) The catalytic aspartate in all protein kinases, A3) The catalytic
aspartate in serine/threonine kinases. In A1-3 the size of the letter corresponds to the abundance of the residue across the spectrum of kinases represented. B) The crystal structure of CDK2 showing the proximity of the ActivX probe to the conserved lysine residues. C) The distance between terminal nitrogens and the terminal phosphate groups of interest (in angstroms).^{35}

**Targeting Kinases in the MAPK Cascade**

The mitogen-activated protein kinase (MAPK) family consists of kinases which phosphorylate serines and threonines. The functions of this kinase family vary widely, from promoting survival to initiating cellular death. MAPK family members can also induce cellular proliferation, transformation and differentiation. Aberrations in MAPK signaling have been linked to a variety of cancers and neurodegenerative diseases.^{37}

A variety of signals, such as oxidative stress or cytokine stimulation can activate MAPK signaling. Cytokine induction of MAPKs begins at the level of an extracellular receptor. The activating signal is then transferred to either a MAPK kinase kinase kinase (MAP4K) or a MAPK kinase kinase (MAP3K), and then on to MAPK kinases (MAP2Ks) and MAPKs (Figure 1.6). Herein we focus specifically on MAP4K2, also termed germinal center kinase (GCK) and MAPK8, which is also referred to as c-jun N-terminal kinase 1 (JNK1).
Figure 1.6: A diagram of MAPK signaling. Typical MAPK signaling can be initiated by stimuli from the extracellular environment, or internal stress. The cascade then flows through MAP4Ks to MAP3Ks, MAP2Ks and MAPKs, like JNK1, which phosphorylate transcription factors. Kinases critical to this dissertation are noted alongside their relevant category.

GCK was discovered in 1993 by a group that was isolating cDNA clones for genes differentially expressed between populations of B-cells. GCK was named because of its preferential expression in the germinal center region of the lymphoid follicle. However, is not exclusively expressed in germinal center B-cells. GCK mRNA was found in several other tissues, including the heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. Since that time, GCK has been shown to activate components of the mammalian stress-activated protein kinase (SAPK) pathway, and the MAPK pathway.

In terms of regulation of its kinase activity, although GCK can be phosphorylated, this phosphorylation does not contribute to its active state.
Instead, GCK is subject to constitutive degradation. Its over-expression is the only way to “increase” its kinase activity, as over-expressed GCK is stabilized by dimerizing, or by binding scaffold proteins such as the tumor necrosis factor receptor associated factor 6 (TRAF6). In its stabilized state, GCK is then able to interact with and activate MAPK signaling components.\textsuperscript{40}

GCK-/- mice are viable, which is a positive indicator for the implementation of GCK interruption as a cancer therapeutic strategy. The macrophages from GCK-/- mice have a reduced capacity to secrete ILs such as IL-6, IL-10 and monocyte chemotactic protein-1 (MCP-1). This secretory capacity is restored with GCK reintroduction.\textsuperscript{41}

GCK is traditionally activated in response to proinflammatory cytokines, environmental stress and apoptotic agents through a variety of membrane bound receptors.\textsuperscript{42} GCK preferentially activates JNKs.\textsuperscript{41} JNK1 will be focused on herein, due to its described roles in the proliferation and survival of various cancers. JNK1 activation generally occurs in response to pro-inflammatory signaling through MAP3Ks, such as MAP3K1, which is also referred to as MEKK1.\textsuperscript{43-45} These MAP3Ks activate MAP2Ks such as MAP2K4 which directly activates JNK1.\textsuperscript{46,47}

JNK family proteins were once described to solely induce apoptosis.\textsuperscript{48} It was historically suggested the JNK pathway promotes apoptosis in non-lymphoid cells, and may have the opposite roles in cells derived from the lymphoid compartment.\textsuperscript{49} However, recent reports have emerged showing the duplicitous
role of different JNK family proteins as both inhibitors and inducers of oncogenesis,\textsuperscript{50-55} even in select non-lymphoid cells.\textsuperscript{56} For example, JNK has also been shown to induce apoptosis in neuronal cells,\textsuperscript{57} but in sharp contrast, JNK promotes the cell cycle in some primary cells and cell lines.\textsuperscript{56} More recently, constitutively active JNK was discovered in mantle cell lymphoma (MCL), and inhibition of p-JNK led to growth arrest in MCL cell lines.\textsuperscript{55} JNKs role in the cell cycle has not been precisely elucidated; however, there are some clues as to its mechanism of action. For example, it is known that the kinase activity of JNK is inhibited by p21.\textsuperscript{58} In primary T-lymphocytes and the human cervical cancer cell line, HeLa, JNK is associated with p21 during (Gap1/Synthesis) G1/S arrest and at that time JNK is unphosphorylated. As cells enter S-phase, p21 dissociates from JNK and JNK becomes phosphorylated.\textsuperscript{56} This phosphorylation precedes the cells exiting S-phase. JNKs’ role in activating the transcription factor c-jun is most likely responsible for this observation, since fibroblasts that are c-jun (-/-) do not efficiently activate the transcription factor E2F.\textsuperscript{59} Activation of E2F is the critical step in the exit from S-phase, and it is possible that c-jun activation by JNK is necessary to allow progression through the cell cycle.

Despite the numerous aberrations and mechanisms of pathogenesis that have been elucidated in the development of DLBCL tumors, no components of the MAPK pathway have been previously identified as being crucial to DLBCL origination or progression. The studies within this dissertation were prompted by our determination that several components of the MAPK pathway have increased
kinase activity in all of our DLBCL cell lines when compared to a pool of B-cells extracted from healthy human tonsils.

The hypothesis of this application is that the MAPK signaling proteins GCK, and JNK1 are upregulated and activated in DLBCL versus healthy human B-cells, and this induction is related to DLBCL pathogenesis. To examine this hypothesis, we aimed specifically to determine the variable components of MAPK activation in DLBCL by identifying all critical signaling components leading to the broad MAPK activation. After identifying GCK and JNK1 as the critical activator and effector, respectively, we rigorously confirmed the activation of those signaling components. We next set out to describe the consequences of MAPK activation in DLBCL by determining the effects of that activation on proliferation, cell cycle and apoptosis, and by dissecting the critical components of MAPK signaling using kinase inhibitors with varying specificity. The final step in this study was to design small molecules inhibiting this pathway and evaluate their activity on DLBCL cell lines and primary tumors. The experiments herein were structured based on the desire to elucidate the importance of JNK1 activation through GCK, as it relates to DLBCL and to determine whether GCK and JNK1 are viable molecular therapeutic targets.
CHAPTER 2
METHODS

I. Reagents

HG-6-64-1 and the HG-6-64-1 variants NG1-NG25 were kindly provided by Nathanael S. Gray from the Dana Farber Cancer Institute (Boston, MA).
JNK1 (FL), phospho-JNK1 (G-7), GC Kinase (N-19), and GAPDH (0411) were from Santa Cruz Biotechnology (Santa Cruz, CA). p38α (5F11) and phospho-p-
38α (28B10) were from Cell Signaling (Boston, MA). siRNAs were Human MAP3K1, MAPK8, MAP4K2 and scrambled ON-TARGET plus SMARTpool
(Dharmacon, Lafayette, CO), MAP4K2 Silencer® select (Ambion, Austin, TX), and MAPK8 Stealth RNAi (Invitrogen, Carlsbad, CA).

II. DLBCL cell lines

The DLBCL cell lines SU-DHL-6, SU-DHL-8, VAL, G452, OCI-LY-3, OCI-
LY-8, OCI-LY-10, OCI-LY-19 and RIVA were grown in Iscoves Modification of Dulbecco Medium (Mediatech Inc., Manassas, VA) supplemented with 20%
human plasma, 2 nM glutamine (Gibco/BRL, Grand Island, NY) and penicillin/streptomycin (Gibco/BRL).
III. Primary Tissues and Tumors

B-cells were enriched from primary tissues and human tonsils that were obtained during routine tonsillectomies, or scheduled biopsies. Fresh tissues were rinsed in phosphate buffered saline (PBS), cut into small sections and ground in a metal sieve. The suspension was passed through several layers of sterile mesh to remove clumps. Informed consent was obtained in accordance with the Declaration of Helsinki and Institutional Review Board approval was obtained from the University of Miami for inclusion of anonymized data in this study.

IV. B-cell Isolation

B-cells were purified using the B-cell Isolation Kit II and an AUTOMACS sorter (Miltenyi Biotec, Auburn, CA) according to the manufacturers’ instructions.

V. KiNativ Analysis

For the analysis of various DLBCL cell lines, cells were grown in identical media and reseeded every two days at 300,000 cells/ml. A total of $2\times10^8$ cells were pelleted and flash frozen. For control, B-cells were isolated from human tonsils as described in “B-cell Isolation.” After sorting, cells were pelleted and flash frozen.
For KiNativ profiling, cell pellets were resuspended and sonicated in lysis buffer (20 mM Hepes, 150 mM NaCl, 0.1% Triton X-100, and 20 mM MnCl₂) using a tip sonicator. Lysate (0.5 ml of 5 mg/ml concentration) was labeled with 20uM biotin labeled ATP or ADP-mimetic probe. To measure drug inhibition, drugs were incubated with lysates for 5 minutes before probe addition. Labeled probes were eluted with a 50% CH₃CN/H₂O solution containing 0.1% TFA and analyzed by LCMS/MS using a Finnigan LCQ Deca XP ion trap mass spectrometer.

VI. Immunohistochemistry

Normal and neoplastic formalin fixed, paraffin-embedded human hematolymphoid tissues were obtained after Institutional Review Board approval from the Department of Pathology, Stanford University Medical Center, Stanford, CA. The 2008 World Health Organization standards were used to classify hematolymphoid neoplasias.

IHC was performed by the sectioning of human lymphoma samples at a thickness of 0.4 µM. Sectioned samples were then deparaffinized in xylene followed by hydration using graduated alcohols. Primary antibodies directed at Phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology, Millipore, MA), and GCK (W-24; Santa Cruz Biotechnology, Santa Cruz, CA), were used. Samples were pretreated with 20 mM EDTA/50 mM TRIS, pH 9.0 and both
antibodies were diluted 1:4. The Vector VIP staining kit (VECTOR Laboratories, Burlingame, CA) was used to develop slides.

The TMA scoring was as follows: A score of 0 represents no staining, a score of 2 indicates weak staining with 5% - 20% of cells staining positive and a score of 3 is assigned for samples with strong staining, in which greater than 20% of cells were positive. A TMA score of 1 means the data was uninterpretable. Reasons for a TMA score of 1 include high background staining, and the loss of sample tissue. To optimize staining, normal tonsil sections were paraffin-embedded and tested. The cut-off of for a positive staining score, wherein greater than 20% of lymphoma cells stain positive, was established using these tonsil sections. No consideration was given to differences in normal and neoplastic tissue with regard to the establishment of this cut-off, it reflects simply the need for a non-ambiguous threshold in order to score the TMAs.

VII. Whole-cell extract preparation and Western blotting

Whole-cell extracts were prepared by lysing 5 x 10^6 cells for 15 minutes on ice in NP-40 lysis buffer (50 mM HEPES pH7.5, 150 mM NaCl, 1% NP-40) with PhosSTOP phosphatase inhibitor cocktail tablets and complete mini EDTA-free protease inhibitor tablets (Roche Diagnostics, Indianapolis, IN). Cellular lysates were assayed for protein concentration using Coomassie Protein Assay Reagent (Pierce, Rockford, IL) in 96 well-plates using a Biorad Benchmark Microplate Reader (Biorad Laboratories Inc., Hercules, CA). Western blotting was performed as electrophoresis of whole cell lysates, using 10% polyacrylamide
gels, with subsequent transfer to nitrocellulose membranes (Biorad Laboratories, Inc.) Immunoblotting for total proteins was performed with the indicated antibodies in 5% milk. All phospho-antibody primary antibody incubation was performed in 5% Bovine Serum Albumin (BSA). Immunocruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc.) was added for 5 minutes to visualize protein levels with light sensitive-film (Phenix Research, Candler, NC). Immunoblots were quantified using ImageJ software (National Institute of Health, Bethesda, MD).

VIII. Cell line transfection

Cells were transfected with an Amaxa Electroporator (Lonza Biotechnology, Walkersville, MD) using 2 µg of Dharmacon siRNAs and incubated at 37°C for 48 hours or 5 µg of Ambion/Invitrogen siRNAs and incubated at 37°C for 72 hours. VAL and DOHH2 cells were transfected with Amaxa solution V (Lonza Biotechnology) and program X-001. SU-DHL-6 cells were transfected with Amaxa solution C (Lonza Biotechnology) and program P-027.

IX. Proliferation and apoptosis studies

For proliferation studies, 3x10^5 cells/mL were plated at the onset of each experiment. At specified time intervals, 20 µl samples were assayed in triplicate
for each experimental condition using the CellTiter 96® Aqueous Non-
Radioactive Cell Proliferation Assay (Promega, Madison, WI), which is a 3-(4,5-
dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-
tetrazolium (MTS) reagent, and an ELx800 Universal Microplate Reader (Bio-
TEK Instruments). MTS readings were compared to the readings at the time of
transfection.

For apoptosis studies, 10^5 cells/mL were collected at the specified time
periods, washed with 1x PBS, and stained with propidium iodide (PI) (Invitrogen)
and YO-PRO (Invitrogen) as per the manufacturer's instructions. Analysis was
performed on a Becton Dickinson LSR1 analyzer (Becton Dickinson, Franklin
Lakes, NJ).

X. Cell Cycle Analysis

2 x 10^6 cells were washed, fixed in 70% ethanol on ice for 15 minutes, and
permeablized overnight at -20°C. For analysis, cells were rinsed in PBS and
incubated at room temperature with RNase I and PI for 20 minutes. Cell cycle
profiles were then assessed by flow cytometry on the LSR1 (Becton Dickinson)
and analyzed using the accompanying software from Becton Dickinson.
XI. Kinase Assays

The p38 MAP Kinase Assay Kit (non-radioactive) and SAPK/JNK Kinase Assay Kit (non-radioactive) were from Cell Signaling. Briefly, 200 µg of total protein were subject to immunoprecipitation with ATF-2, or c-jun immobilized beads or with rabbit IGG (Santa Cruz Biotechnology) and rProtein G Agarose beads (Invitrogen). Immunoprecipitates were then rinsed 2x in lysis buffer, 2x in kinase assay buffer and incubated in kinase assay buffer and ATP for 30 minutes at 30°C. Readout for the kinase assay was an immunoblot of p-ATF-2 or p-c-jun.

XII. Statistics

The intensity signal for each kinase detected in the KiNativ assay was compared to the same kinase in a pool of B-cells derived from human reactive tonsils using the students’ t-test with Bonferoni correction.

Error bars on all graphs represent the standard error across multiple experiments, unless otherwise stated.
CHAPTER 3

Results

I. The MAPK pathway is broadly activated in DLBCL

In order to supplement the broad mRNA expression data available to DLBCL researchers, we set out to prolife functional kinase expression and activity within the entity of DLBCL. Specifically, we intended to address whether there are different kinase activity profiles within the DLBCL subtypes, or whether there is activation of previously unidentified pathways across DLBCL subtypes. We selected the KiNativ Platform from ActivX Biosciences because it is the most comprehensive kinase array currently available.

For KiNativ analysis, control B-cells were isolated, as described in the materials in methods chapter, from human reactive tonsils collected from patients undergoing routine tonsillectomy. Isolated B-cells from two patients were flash frozen and pooled for analysis. The GCB-like DLBCL cell lines SU-DHL-6, VAL, and OCI-LY-19 and the ABC-like cell lines SU-DHL-8, G452, OCI-LY-3, OCI-LY-8, OCI-LY-10, and RIVA were grown in identical media supplemented with human plasma from a pool of three plasma donors.

The KiNativ platform detected a total of 153 kinases which were active/expressed in at least some of the analyzed samples. Of those, several kinases exhibited significantly higher or lower activity/expression in DLBCL cell lines compared to the pool of normal B-cells. Kinases with at least 2-fold
higher/lower expression in DLBCL relative to normal B-cells were selected, mapped to gene symbols and annotated by comparison to curated gene sets corresponding to canonical molecular pathways (Broad Institute Molecular Signatures Database, mSigDB). Hypergeometric testing showed a statistically significant enrichment ($p = 9.30 \times 10^{-11}$) in components of the MAPK signaling pathway that were differentially active/expressed in DLBCL cell lines compared to the normal B-cells.

Of the 153 kinases detected in the ActivX array, we identified 18 proteins which were either classical MAPKs or that are kinases that are either effectors or targets of the MAPK signaling pathway and which demonstrated a two-fold or greater increase in activation/expression in DLBCL cell lines compared to normal B-cells. Six kinases within the MAPK family demonstrated lower activity/expression than in normal B-cells. A heat map comprised of all of the kinases identified as potentially involved in MAPK signaling is depicted in Figure 3.1.

In order to determine if the activation of MAPK family proteins is a phenomenon of in vitro growing cells, or whether this activation is specific to DLBCL we examined the results of previous studies which analyzed GCK kinase expression/activity in non-DLBCL cell lines and peripheral blood mononuclear cells (PBMCs) using the same methodology. The LCMS/MS signal for GCK in PBMCs was compared to the T-cell leukemia cell line, Jurkat and the human promyelocytic leukemia cell line, HL60. In comparison to the primary PBMCs, HL60 cells showed a slight upregulation of GCK and Jurkat showed significant
downregulation (Figure 3.2). The bi-directional enrichment of GCK in other cell lines suggests that the upregulations we detected in the MAPK signaling pathway are not an innate property of \textit{in vitro} growing cells.

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{\textbf{The MAPK family of proteins is upregulated in DLBCL.} The MAPK protein activity/expression profile is shown in 9 DLBCL cell lines. Each row represents a separate kinase identified and quantified by LCMS/MS and each column a separate DLBCL cellular lysate. The displayed matrix represents the relative log base 2 ratio of LCMS/MS detected activity/expression of the kinases from each experimental DLBCL cell line relative to normal B-lymphocytes, as depicted by the corresponding color scale.}
\end{figure}
Figure 3.2. **MAPK upregulation is not a phenomenon of in vitro growing cells.** Cell lines were profiled simultaneously using the KiNativ platform, and the activity/expression of each cell line was normalized to PBMCs.

To narrow the selection of initial targets, we performed an extensive literature review of the MAPK signaling cascade. We searched for the most proximally activated kinase targets, and analyzed the activation status of their downstream components. The synchronization of data in the literature with ActivX findings revealed that one of the activated MAPK pathways begins with GCK. GCK was upregulated 3.9 fold over tonsillar B-cells according to the ActivX data. GCK is known to activate MEKK1, which showed 3.2 fold upregulation in the same data set. MEKK1 is capable of activating MKK4 or MKK6. Both of these kinases can activate all isoforms of p38 and MKK4 can activate the JNK family of kinases, though p38 MAP Kinase (p38) is preferentially activated by MKK3 and MKK7. MKK4 demonstrated a 62.8 fold induction in expression/activity over tonsillar B-cells, and the JNK family (JNK1, JNK2 and JNK3), which was assayed simultaneously, was upregulated 3.5 fold. The accessibility of the p38α active site was downregulated in DLBCL cell lines, though its downstream targets mitogen- and stress-activated protein kinase-1 (MSK1) and IKKβ demonstrated activation.
The ActivX KiNativ platform has been validated by the company by comparison of their technology to a range of kinase arrays and to Western blotting. However, to independently determine the authenticity of their results, we performed p38 and JNK immunoprecipitation-based non-radioactive kinase assays.

p38α binds the ATP-mimetic ActivX probe in two locations. One site is the ATP-binding pocket, and is referred to as the active site. Probe binding to the active site is an indicator of p38α being in the active state. The second site, referred to as the mitogen-activated protein kinase activated protein kinase 2 (MK2) site, is accessible to the ActivX probe only when the kinase is inactive, due to a conformational change of this particular kinase upon activation. Thus, the best indicator of p38α activity is the ratio of the probe’s active site binding to inactive site binding. This ratio was calculated and graphed in Figure 3.3.A.

ATF-2 is a well described target of p38. Thus, lysates from purified tonsillar B-cells and from the DLBCL cell lines OCI-LY-8, OCI-LY-19, G452 and VAL were incubated overnight with beads bound to immobilized ATF-2, and a subsequent immunoprecipitation-based non-radioactive kinase assay was performed, as described in the materials and methods section. Consistent with KiNativ data, tonsillar B-cells exhibited significantly higher levels of p-ATF-2 in the classical immunoprecipitation based kinase assay, when compared to DLBCL cell lines (Figure 3.3.B).
Unlike p38α, the JNK family kinases have only one probe binding site. Thus, the raw KiNativ readout is a good estimate of JNK family activity/expression, and is graphed in Figure 3.3.C.

To assess JNK family activity and relate it to the KiNativ data, a classical immunoprecipitation based kinase assay was performed on tonsillar B-cells and on the cell lines SU-DHL-6, OCI-LY-19 and VAL using substrate c-jun and IGG as control. The results of this kinase assay, as depicted in Figure 3.3.D, are consistent with KiNativ data in that the JNK immunoprecipitated from DLBCL cell lines was able to phosphorylate a greater quantity of the substrate c-jun, when compared to the assayed immunoprecipitates from tonsillar B-cells.

![Figure 3.3. Validation of ActivX's KiNativ Platform by Non-Radioactive Kinase Assays. A) The ratio of the ATP-binding site (active) to the MK2 binding site (inactive) on p38α, as measured by the KiNativ Platform in B-cells derived from human reactive tonsils and in DLBCL cell lines, is used to calculate relative p38α kinase activity. B) Immunoblotting of substrate p-ATF-2 after immunoprecipitating p38 from the whole cell lysates followed by incubation with substrate ATF-2 and ATP. C) LCMS/MS signal for JNK1/2/3 after pulldown with the ActivX ATP-mimetic probe in B-cells.](image-url)
purified from human tonsils, and in DLBCL cell lines. D) Immunoblotting of substrate p-c-jun after JNK pulldown, using either c-jun conjugated beads or rabbit IGG as control, and subsequent non-radioactive kinase assay.

To further validate the results of the KiNativ kinase array, and to determine whether GCK and p-JNK were present in primary DLBCL tumors, we performed immunohistochemical staining for GCK and p-JNK. These experiments were critical in order to firmly establish that the upregulation of GCK and p-JNK is not a phenomenon of in vitro growing DLBCL cell lines. We stained for GCK in 33 primary DLBCL tumors. Twenty-nine out of 33 tumors, 87.8%, exhibited strong positive staining for GCK. This corresponds well to the ActivX data in which 8 out of 9 DLBCL cell lines, 88.9%, were GCK positive. GCK is a cytoplasmic protein which associates with scaffolding proteins such as TRAF6, so we expected to find GCK localized to the cytoplasm and co-localized to areas of the cell membrane, which it was. The levels of p-JNK were assessed in 32 primary DLBCL tumors. JNK, when activated, phosphorylates transcription factors. Thus, p-JNK was anticipated in the nucleus of positively stained cells. We confirmed nuclear p-JNK staining in 17 of 32, or 53.1%, of the primary DLBCL tumors tested. Similarly, in the tested DLBCL cell lines 6 out of 9, or 66%, had upregulation of JNK family activity/expression in comparison to tonsillar B-cells. Representative staining for GCK and p-JNK is shown in Figure 3.4.A. ActivX data also revealed downregulation of p38α in DLBCL cell lines compared to primary tonsillar B-cells. To confirm p38α active site downregulation, we performed IHC staining for p-p38α. As anticipated, p-p38α staining was negative in the majority, 12 of 78, or 15.4% of tested samples.
Figure 3.4. *GCK and p-JNK are expressed in primary DLBCL tumors.* Immunohistochemical staining for p-JNK in a primary DLBCL tumor.

Finally, we implemented Western blotting for total GCK protein, total JNK1, and p-JNK1 in order to compare basal levels of these proteins to the ActivX kinase array readouts. Heat maps corresponding to the GCK ATP-binding probe, the GCK ADP-binding probe and the JNK family ADP binding probe are shown in Figure 3.5.A. The ActivX ATP-binding probe did not efficiently target the JNK active site, as is the case with some kinases. Thus, only the ADP-binding probe produced relevant data. Figure 3.5.B depicts unmanipulated DLBCL cells which were lysed and immunoblotted for GCK, JNK1 and p-JNK1. The kinase activity of GCK is regulated at the protein level, because GCK is primarily active when dimerized, thus p-GCK was not assessed by phosphorylation specific Western blotting. GCK phosphorylation has been shown to be irrelevant to the kinase activity of this particular kinase. As expected, Western blotting confirmed activation of p-JNK in all cell lines and robust expression of GCK in most cell lines. Notably, G452 minimally expresses GCK. This corresponds well to the MAPK heat map, which shows low GCK expression/activity in the cell line G452. Similarly, SU-DHL-8 and RIVA have a low signal for JNK family activity in both the heat map of ActivX data, and in the Western blot for p-JNK1.
II. The Role of the GCK-JNK pathway in DLBCL

Having determined that GCK and its downstream effector, p-JNK1, were upregulated in DLBCL cell lines and primary tumors, we wanted to examine their potential role in DLBCL biology. To this end, we tested the effect of GCK and JNK knockdown using siRNAs on the proliferation and survival of DLBCL cell lines.

Transfection of VAL cells with siRNA directed against GCK and JNK1 revealed that these proteins have cross reactive responses, even when specifically targeted (Figure 3.6). The siRNA directed at GCK was effective in knocking down GCK in VAL cells, and led to a small reduction in total JNK1. JNK1 siRNA also resulted in a partial knockdown of GCK, suggesting a feedback loop.
Figure 3.6. **GCK and JNK1 exhibit cross-reactive reductions in total protein.** VAL cells were transfected with JNK1, GCK, or control siRNA (Dharmacon). Cells were collected for immunoblotting 48 hours after transfection. The depicted blot is representative of results from three independent experiments.

We next performed siRNA knockdown of GCK and JNK1 in SU-DHL-6 cells. GCK knockdown led to a reduction in total JNK1 protein, as seen in VAL cells. To examine the effects of GCK interruption *in vitro* in cultured cells, cells were harvested 48 hours after transfection and subject to cell cycle analysis which revealed a minor induction in cell death (Figure 3.7.A). A dramatic effect was seen with JNK1 knockdown in SU-DHL-6 cells. JNK1 siRNA reduced total GCK protein, and killed the cells, resulting in a complete reduction of GAPDH. The JNK1 siRNA mediated cell death is apparent in the cell cycle analysis figure, where the population of cells shifts to the sub-G1 zone (Figure 3.7.B).

To extend these findings to another cell line, we analyzed the effect of GCK, JNK1 and MEKK1 siRNAs on proliferation and death in the DLBCL cell line DOHH2. The degree of MEKK1 knockdown has not been assessed. However, in DOHH2 cells it is apparent that siRNA directed against MEKK1 decreases total GCK, though the effects on MEKK1 itself were not quantified. JNK1 siRNA decreases both JNK1 and GCK (Figure 3.8.A).
Figure 3.7. Knockdown of GCK and JNK1 leads to G1 arrest and kills DLBCL cells. SU-DHL-6 cells were transfected with 2 µg of A) GCK siRNA, B) JNK1 siRNA or scrambled Dharmacon siRNA as control. Cell cycle was analyzed at 48 hours. The filled traces depict control transfected cells and the unfilled traces depict cells transfected with the indicated specific siRNA. This experiment is representative of three independent knockdowns.

The MTS assay was then used to test for the induction of death or potential anti-proliferative effects of these siRNAs. For this assay, MTS is combined with phenazine methosulfate (PMS) and these reagents are added to cells to simultaneously assess cell proliferation and viability. The reduction of MTS to a colored formazan product occurs only in living cells. Therefore, cells are seeded and treated with MTS reagent on day zero of a given experiment, and again at select time points. If the colorimetric reading from the MTS reaction
is less than it was on day zero, it is clear that some cells have died. If the MTS readout increased after seeding, despite treatment, cells are clearly proliferating.

Interestingly, the siRNAs directed against GCK led to a decrease in MTS response, indicating that this transfection lowered the number of viable cells in culture (Figure 3.8.B). MEKK1 and JNK1 siRNA transfected cells had a reduced MTS response compared to control transfected cells, but did exhibit some proliferation post-transfection. Proliferation relative to control was calculated by dividing the increase in MTS readout from 0 to 48 hours in each sample by the increase in MTS readout in the dimethyl sulfoxide (DMSO) treated, control, sample over the same time period. GCK siRNA resulted in a negative value because in addition to proliferative arrest, some cells in the GCK siRNA treated sample died. Overall, the siRNAs against GCK, JNK1 and MEKK1 demonstrate crosstalk between members of this pathway. Knockdown of any single pathway member dramatically reduced the MTS response at 48 hours, with the most dramatic response seen in GCK knockdown.

Alternate siRNAs were tested in VAL cells to ensure the specificity of response. GCK siRNA was obtained from Invitrogen and JNK1 siRNA from Ambion. The resulting knockdowns of both GCK (Figure 3.9.A) and JNK1 (Figure 3.9.B) with alternate siRNAs led to a small, but statistically significant, decrease in proliferation as detected by MTS assay at 72 hours post transfection (Figure 3.9.C).
Figure 3.8. *GCK and JNK knockdowns in DOHH2 cells.* A) DOHH2 cells were transfected with 2μg of the indicated Dharmacon Smartpool siRNAs. At 48 hours, cells were collected and immunoblotted with the indicated antibodies and B) subject to MTS assay. Knockdown and MTS experiments were performed two times.

Figure 3.9. *Ambion siRNAs are less efficient in knockdown but have directionally similar proliferation results compared to Dharmacon Smartpool siRNAs.* A) SU-DHL-6 cells were transfected with 5μg of GCK siRNA or control siRNA. Cells were collected, lysed and immunoblotted with the indicated antibodies at 72 hours. B) Cells were transfected with JNK1 siRNA or control. C) Transfected cells were subject to MTS assay at time zero and 72 hours after transfection. Proliferation was normalized to control transfected cells.
In summary, GCK, MEKK1 and JNK1 all appear to be critical contributors to proliferation and survival in DLBCL as demonstrated by specific siRNA knockdowns.

III. Chemical Interruption of MAPK Signaling

In order to chemically manipulate critical components of the MAPK pathway that we discovered to be activated in DLBCL cell lines, we began a collaboration with Nathanael S. Gray from the Dana Farber Cancer Institute at Harvard Medical School. Dr. Grays’ laboratory specializes in small molecule inhibitors with selectivity towards desired kinases. The prototype inhibitor, HG-6-64-1, was designed for initial experiments targeting GCK. This compound inhibits GCK, but not the related proteins GCKR or HPK1.

To determine the specificity of HG-6-64-1, HL60 cells were treated with a range of concentrations, up to 10 µM, and inhibition of the entire spectrum of detectable kinases was analyzed by LCMS/MS. The IC₅₀ with respect to all detectable targets was calculated and is depicted in Figure 3.10. c-src tyrosine kinase (CSK), V-yes-1 Yamaguchi sarcoma viral related oncogene homolog (LYN) and transforming growth factor β-activated kinase 1 (TAK1) were also potently inhibited by HG-6-64-1. The potential effects of inhibition of these targets were partially examined by multi-specificity kinase inhibitors (see Figures 3.15 and 3.16), and may be further examined at a later date. Our working
hypothesis, that GCK->MEKK1->MKK4->JNK1 signaling leads to proliferation and survival, led us to determine the effects of HG-6-64-1 on these endpoints.

To determine the appropriate concentration of HG-6-64-1 to use in our cell line studies, and to test the effects of this drug on the proliferation and survival of DLBCL cell lines, we treated cells with serial dilutions ranging from 500 nM to 326 pM.

Figure 3.11.A shows curves produced from MTS readings in the cell lines SU-DHL-6, OCI-LY-3, OCI-LY-10, OCI-LY-19, and DOHH2. Proliferation and survival were then assessed by comparing MTS assay readings from time zero to the 48 hour time point, relative to proliferation in control DMSO treated cells. SU-DHL-6 was most sensitive to proliferative arrest and death as measured by MTS assay. OCI-LY-3 was the least sensitive cell line. However, all cell lines showed robust decreases in MTS response using concentrations of HG-6-64-1 which are physiologically relevant. The concentration 400 nM was selected during this assay as a concentration which fully inhibited proliferation in all cell lines tested, and this concentration was used for all subsequent studies.
**Figure 3.10. HG-6-64-1 Potential Targets.** The kinase inhibitor HG-6-64-1 was profiled by ActivX Biosciences to determine the kinases inhibited by 5 minutes of HG-6-64-1 treatment. The IC\textsubscript{50} for each kinase, in HL60 cells, is listed on the right in µM units.

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<th>Kinase</th>
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<tr>
<td>p38α</td>
<td>10</td>
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JNK1 is a downstream target of GCK. Therefore, we examined whether inhibition of GCK by HG-6-64-1 leads to a downstream reduction in JNK kinase activity. ActivX profiling clearly demonstrated that none of the JNK family proteins are direct targets of HG-6-64-1. However, HG-6-64-1 inhibits GCK and GCK may activate JNK family proteins through MEKK1 and MKK4. To confirm whether chemical inhibition of GCK decreases JNK family member kinase activity in DLBCL cell lines, SUD-HL-6 and VAL cells were treated with DMSO or HG-6-64-1 for 8 hours. Cells were then lysed and subject to the JNK immunoprecipitation-based kinase assay, as described. SU-DHL-6 cells exhibited a 59% decrease in JNK family kinase activity after 8 hours of HG-6-64-1 treatment. Similarly, VAL cells exhibited a 35% decrease over the same time period.
period (Figure 3.11.B). These results confirm that JNK family proteins are downstream targets of GCK in DLBCL.

Figure 3.11. HG-6-64-1 inhibits JNK kinase activity, decreases proliferation and induces cell death. A) DLBCL cell lines were treated with 500 nM, 250 nM, 125 nM, 63 nM, 21 nM, 10 nM, 5 nM, 2.6 nM, 1.3 nM, 651 pM, and 326 pM HG-6-64-1. In OCI-LY-3 the pM concentrations were substituted with the intermediate concentrations of 188 nM and 94 nM. MTS readings were taken just prior to, and 48 hours after drug treatment. Cellular proliferation was calculated as the change in MTS reading for each experimental condition, divided by the increase in DMSO treated cells. Percent live cell values below zero indicate cellular death, due to the presence of fewer live cells assayed at the conclusion of the experiment compared to time zero. B) SU-DHL-6 and VAL cells were treated with 400 nM HG-6-64-1 or DMSO for 8 hours. 200 µg of cell lysate was used in each experimental condition, as quantified by Coomasie Protein Assay. Lysates were then subjected to a non-radioactive kinase assay for JNK activity, as described previously. Densitometry readings are listed beneath each immunoblot as a proportion of control. Quantification was performed using ImageJ software.

To determine whether the decrease in MTS response after HG-6-64-1 treatment was due to cell cycle arrest, cells were subject to cell cycle analysis after 24 hours of treatment with 400 nM HG-6-64-1. As expected, most cell lines arrested with treatment (Figure 3.12). The cell lines VAL, SU-DHL-6 and OCI-LY-19 exhibited increases of 19%, 25% and 34% of cells in G0/G1 with corresponding decreases in S phase and G2.
Figure 3.12. HG-6-64-1 inhibits proliferation in DLBCL cell lines. Asynchronously growing DLBCL cell lines were subject to media change followed by treatment with 400nM HG-6-64-1 or the vehicle, DMSO. Twenty-four hours after treatment cells were ethanol permabilized and saturated with PI in PBS. Cell cycle was analyzed by flow cytometry. Solid purple histograms represent control, DMSO treated, cells and unfilled green histograms represent HG-6-64-1 treated cells. Histograms are representative of results from three independent experiments.

In order to assess the specificity of HG-6-64-1, and to determine that it is acting through GCK as opposed to another kinase, we treated G452, the cell line which expresses low levels of GCK, with 400 nM HG-6-64-1 for 24 hours. G452 did not arrest after treatment with HG-6-64-1, as demonstrated by cell cycle analysis (Figure 3.13.A) and MTS assay (Figure 3.13.B). These results demonstrate the specificity of the response to HG-6-64-1, both by exhibiting that it is not a generally toxic compound, and that it inhibits the cell cycle and induces death only in GCK expressing cells.
HG-6-64-1 does not inhibit proliferation in cells which do not express GCK. A) G452 cells were treated with 400 nM HG-6-64-1 or DMSO for 24 hours. B) Cells were assayed for proliferation and death using MTS reagent at time zero and 24 hours after treatment with HG-6-64-1 or DMSO.

These results demonstrate that HG-6-64-1 arrests DLBCL cells in the G1 phase of the cell cycle. However, the changes in MTS readout seen in Figure 3.11.B suggest that cells are dying in addition to experiencing proliferative arrest. To quantify this death, cells were treated with HG-6-64-1 in 4 fold serial dilution from 1.280 µM to 1.25 nM for 72 hours and then stained with the DNA-intercalators YO-PRO and PI. YO-PRO can cross the cell membrane in early apoptosis whereas cells only become permeable to PI once larger holes in the membrane are created later in the death progression. Therefore, early apoptotic cells are YO-PRO positive whereas dead cells are stained with both dyes. Cells were then analyzed by flow cytometry to determine the percentage of live, apoptotic, and dead cells. Slightly over 40% of OCI-LY-19 cells were killed at 72 hours by 80 nM HG-6-64-1 (Figure 3.14.A). However, increasing the concentration of HG-6-64-1 beyond 80 nM did not result in any further increase in killing. The concentration needed to induce death appears to be lower than the concentration required for complete proliferative arrest in some cell lines.
Therefore, the concentration selected by proliferative arrest studies, 400 nM, appeared sufficient to test all cell lines.

We tested additional cell lines for the induction of death by treatment with 400 nM HG-6-64-1 for 72 hours. Cells were then assayed using YO-PRO and PI staining (Figure 3.14.B). HG-6-64-1 induced cell death of 48%, 37%, 41% and 58% in DOHH2, OCI-LY-10, OCI-LY-19 and SU-DHL-6 cells, respectively. The combination of these results shows that HG-6-64-1 is efficient in proliferative arrest and in the induction of death in DLBCL cell lines.

![Figure 3.14. HG-6-64-1 induces death in DLBCL cell lines. A) OCI-LY-19 cells were treated with DMSO or 1.25 nM, 5 nM, 20 nM, 80 nM, 320 nM or 1.280 µM HG-6-64-1 and induction of death was quantified by flow cytometry at 72 hours. B) The DLBCL cell lines DOHH2, OCI-LY-10, OCI-LY-19 and SU-DHL-6 were treated with 400 nM HG-6-64-1 for 72 hours.]

IV. Inhibitor-based kinase target profiling

HG-6-64-1 was designed as a prototype GCK inhibitor. It was profiled at ActivX revealing its specificity for GCK, CSK, LYN and TAK1 kinases at low concentrations and various other kinases as the concentration of HG-6-64-1 increased. To determine whether HG-6-64-1 was the best inhibitor for our
purposes, and to derive information on the variations in inhibition achieved with other small molecule inhibitors, our collaborators created 25 structural variants of HG-6-64-1. Each HG-6-64-1 variant was profiled at ActivX to determine its specificity for the detectable range of kinases. Each engineered structural variant exhibited a unique specificity profile.

In order to determine the efficacy of each HG-6-64-1 variant we designed a single concentration screen. We selected a treatment condition of 400 nM for this screen, due to the efficacy of HG-6-64-1 in inducing proliferative arrest and death at that concentration in a number of DLBCL cell lines. SU-DHL-6 and VAL cells were assayed for proliferative arrest at 400 nM treatment for 48 hours with HG-6-64-1 and the 25 structural variants of HG-6-64-1 variants named NG1, NG2, etc., to NG25. The 25 NG variants tested had a similar effect on the cell lines SU-DHL-6 and VAL in terms of proliferative arrest. Five of the NG variants, specifically NG14, NG15, NG17, NG19 and NG25 were able to induce proliferative arrest comparable to HG-6-64-1 (Figure 3.15). The HG-6-64-1 variant NG3 was inefficient in arresting cells.
Figure 3.15. *Comparison of the potential to inhibit proliferation by HG-6-64-1 versus engineered structural variants*. SU-DHL-6 and VAL cells were seeded at 300,000 cells/ml and treated with 400nM HG-6-64-1 or NG1-25. Cells were counted on a Beckman Coulter Particle Counter in triplicate for each treatment condition at the beginning of the experiment and at 48 hours after treatment. The proliferation response in each cell line was normalized to HG-6-64-1. Red arrows indicate proliferative arrest comparable to HG-6-64-1. Green arrows indicate no apparent effect on proliferation.

To address the question of the specificity of each NG variants, HUH7 cells were profiled after treatment. All detectable kinases were then tested for their degree of inhibition. We created a table comparing the inhibition of the primary HG-6-64-1 targets, GCK, CSK, TAK1 and LYN as well as any kinase which was potently inhibited by at least 5 out of 6 of the effective inhibitors. The specificity profile for NG3, which does not effectively inhibit DLBCL cell line proliferation, is listed as a control (Figure 3.16). The degree of inhibition of each kinase for the NG variants was calculated from data in HUH7 cells, and for HG-6-64-1 it was
calculated in HL60 cells. Therefore, the precise levels of inhibition of each kinase may be slightly different in each of these lines, and in DLBCL cells. The p38α MK2 site was potently inhibited by the 5 effective NG variants. Therefore, the effect of each inhibitor on the p38α active site is also shown.

<table>
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<tr>
<th></th>
<th>HG-6-64-1</th>
<th>NG14</th>
<th>NG15</th>
<th>NG17</th>
<th>NG19</th>
<th>NG25</th>
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<tr>
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<td>88%</td>
<td>91%</td>
<td>25%</td>
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<td>90%</td>
<td>96%</td>
<td>98%</td>
<td>92%</td>
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<td>92%</td>
<td>98%</td>
<td>80%</td>
<td>99%</td>
<td>31%</td>
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<td>27%</td>
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<td>6.8%</td>
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<td>97%</td>
<td>85%</td>
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<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
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<td>N/D</td>
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<tr>
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<td>75%</td>
<td>85%</td>
<td>31%</td>
<td>-42%</td>
<td>96.1%</td>
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Figure 3.16. Kinase specificity profiles of HG-6-64-1 “NG” variants. The ActivX specificity profiles of HG-6-64-1 and each potent NG variant are shown as percent inhibition of each kinase compared to the same kinase in untreated cells HL60 cells for HG-6-64-1 and in HUH7 cells for the NG variants.

To summarize the specificity data derived from the NG variant study - GCK and Sterile alpha motif and leucine zipper containing kinase AZK (ZAK) were targeted by at least 5/6 of the potent inhibitors and p38α and V-abl Abelson murine leukemia viral oncogene homolog 1/2 (ABL1/2) were targeted by all 6. ZAK and ABL1/2 were excluded from further analysis because they were potently inhibited by NG3, but NG3 did not arrest proliferation in either SUD-HL-6 or VAL cells. The p38α MK2 site was inhibited, but this is the inactive site. Analysis of the p38α active site revealed 4/6 of the effective inhibitors do not inhibit binding to p38α’s ATP-pocket. The HG-6-64-1 targets CSK and TAK1 were each
inhibited by 3/6 inhibitors. LYN is not expressed in HUH7 cells, so we did not receive data on its inhibition.

Interestingly, NG17 does not inhibit GCK, but it uniquely inhibits RAF proto-oncogene serine/threonine-protein kinase (c-raf) (data not shown). None of the other inhibitors target c-raf, so it was not pursued herein. However, possible roles of c-raf in DLBCL should be examined in future studies. The compilation of this specificity data led to GCK remaining our principal kinase of interest. JNK1 also remains of interest as a possible downstream/crosstalk effector. However, JNK1 is not recognizably a direct target of any of these drugs.

To examine the effects of HG-6-64-1 on GCK, p-p38α, p38α, JNK1 and p-JNK1 in our cell lines, SU-DHL-6 cells were treated with 400nM HG-6-64-1 for 1, 2, 4, and 8 hours. As expected, p-p38α was reduced within one hour with a time dependent subsequent decrease in total p38α protein and p-JNK1 (Figure 3.17.A). The cell lines VAL, OCI-LY-3 and OCI-LY-10 showed similar reduction in p-p38α when treated with HG-6-64-1 for 2 hours (Figure 3.17.B). p-JNK1 was reduced in OCI-LY-10 and VAL cells, but increased in OCI-LY-3 cells after 2 hour treatment with HG-6-64-1.
Figure 3.17. **HG-6-64-1 inhibits the phosphorylation of JNK1 and p38α in DLBCL cell lines.** A) Asynchronously growing SU-DHL-6 cells were treated with 400 nM HG-6-64-1 for 0, 1, 2, 4 or 8 hours. Lysates were subject to immunoblotting using the indicated antibodies. B) DLBCL cell lines were treated with 400 nM HG-6-64-1 or DMSO for 2 hours.

HG-6-64-1 and the NG variants are the first GCK inhibitors that we are aware of. However, p38α and JNK1 are susceptible to chemical inhibition by a variety of well described small molecule inhibitors. In order to directly assess the potential anti-proliferative and pro-death effects of direct JNK and p38 inhibition we tested the effects of the JNK inhibitors 9L, 2G-10 and Kin188 alongside the p38 inhibitors TAK-751 and RWJ-67657 by MTS assay. The JNK inhibitors 9L, 2G-10 and Kin188 led to proliferative arrest, similar to HG-6-64-1. The p38 inhibitors TAK-751 and RWJ-67657 required very high, µM, concentrations to create similar effects. The JNK inhibitors displayed EC50s in the nM range, with proliferation assays yielding similar results to HG-6-64-1 (Figure 3.18, left).

However, neither p38 inhibitor resulted in proliferative arrest until µM concentrations were reached (Figure 3.18, right). Drugs that do not demonstrate efficacy until µM concentrations are generally considered to act non-specifically; therefore, these p38 inhibitors were excluded from further analysis. The
combination of multi-drug efficacy despite lack of inhibition of p38α’s active site and the lack of ability of p38 inhibitors alone to create the proliferative arrest seen with GCK and JNK inhibitors led us to believe that p38 is not a viable target for DLBCL therapeutics.

Figure 3.18. **JNK inhibitors, but not p38 inhibitors, induce proliferative arrest in DLBCL cell lines.** SU-DHL-6 cells were treated with either DMSO, the JNK inhibitors 9L, 2G-10 or Kin188 or the p38 inhibitors TAK-751 or RWJ-67657 at concentrations from 10 nM to 30 µM. Cellular viability and proliferation were assessed using the MTS assay at time zero and 48 hours after treatment.

Cells treated with the JNK inhibitors 2G-10, 9L and Kin188 exhibit dose dependent decreases in MTS response. This may be due to proliferative arrest or death. To differentiate between proliferative arrest and induction of apoptosis,
cells were simultaneously assayed for MTS response and for death using YO-PRO and PI staining by flow cytometry (Figure 3.19). The decrease in MTS response occurs at lower concentrations of JNK inhibition than the induction of death. Thus, JNK inhibition leads to both death and proliferative arrest in DLBCL cells.

![Graph showing proliferation and death of cells treated with JNK inhibitors Kin-188, 2G-10, and 9L.]

**Figure 3.19. The JNK inhibitor 9L arrests proliferation and induces apoptosis of DLBCL cells.** SU-DHL-6 cells were treated with 4 fold serial dilutions of each JNK inhibitor from 1.280 µM to 1.25 nM and assayed for proliferative arrest by MTS assay at 48 hours and for death using YO-PRO/PI by flow cytometry at 72 hours. Proliferation was calculated as a percent of growth in control, DMSO treated, cells.

**V. MAPK inhibition and the survival of primary tissues**

It is critical that MAPK inhibition lead to the death of primary B-cells if it is to be an efficacious cancer treatment. In order to analyze primary tissues it was necessary to isolate the B-cells from within each tissue. This was achieved by magnetic labeling to remove all the non-B cell components from the
immunological tissue of interest. In whole blood samples, Ficoll density centrifugation was also used to remove red blood cells. To test the purity of B-cells after magnetic isolation, cells were stained with the B-cell specific marker, CD19. Figure 3.20 shows a primary tonsil before and after B-cell isolation. The primary tonsil contains mostly CD19 negative cells with a small population of CD19 positive B-cells. After isolation, all cells are CD19 positive.

Figure 3.20. **Purity of Primary B-cells.** Fresh human reactive tonsils were separated by negative selection into B-cell and non-B-cells compartments and analyzed by flow cytometry for CD-19 staining. The red, filled, histogram depicts the unsorted tonsil and the green, unfilled, histogram depicts the purified B-cells.

To determine the effect of HG-6-64-1 and 9L on primary tissues, we used a combination of YO-PRO and PI staining to assess the viability of B-cells isolated from healthy primary tissue and from lymphoma tumor samples. Viability was assessed after 48 hours of treatment with 400 nM HG-6-64-1, 400 nM 9L or DMSO. Figure 3.21 depicts a DMSO treated (left) and HG-6-64-1 treated (right) DLBCL tumor. After 48 hours, some B-cells remain alive in the DMSO treated sample. These cells can be seen in the lower left quadrant. The lower right quadrant contains YO-PRO positive early apoptotic cells and the upper right
quadrant houses cells which are completely dead and therefore stain positive for both YO-PRO and PI. All subsequent primary tissue studies were performed via this method.

![DMSO treated vs HG-6-64-1 treated cells](image.png)

**Figure 3.21. HG-6-64-1 kills primary DLBCL tumors.** Isolated B-cells from a primary DLBCL tumor were treated with DMSO (left) and HG-6-64-1 (right) for 48 hours.

It is also of interest to know whether HG-6-64-1 and 9L are effectively killing only B-cells, or all cells of lymphoid and non-lymphoid compartments. To test this, five healthy human tonsils were separated into their B-cell and non-B-cell components by magnetic labeling and treated with 400 nM HG-6-64-1, 9L or DMSO. The unsorted tonsil was also treated. There was no significant difference between the killing of unsorted tonsillar cells, the non B-cell compartment or isolated B-cells. Rather, all components of the tonsil were equally killed by HG-6-64-1 and 9L, demonstrating the broad specificity of these compounds for cells of lymphoid origin (Figure 3.22). This data suggests that HG-6-64-1 and 9L may eventually be tested for use in T-cell tumors and other cancers arising from cells in the immune compartment.
Figure 3.22. Tonsillar tissues are efficiently killed with HG-6-64-1 and 9L. Primary tonsillar tissue was sorted, as described and treated with DMSO, 400 nM HG-6-64-1 or 9L. Live cell percentage was derived as a proportion of control, DMSO, treated cells at 48 hours. Cell viability was assessed by flow cytometry.

The observation that these drugs can kill healthy B-cells is not a major drawback, since normal lymphoid cells are also killed by chemotherapy and anti-CD20 antibodies, that target both normal and malignant B cells.

We next examined the ability of HG-6-64-1 and 9L to target malignant B-cells in primary tumor samples, B-cells were isolated from primary tissues, treated with either drug at 400 nM concentration, or with an equivalent amount of DMSO, and assayed for death 48 hours after treatment (Figure 3.23). B-cells isolated from primary tumors from lymph node biopsies are shown in Figure 3.23 alongside 6 similarly assayed reactive, non-cancerous, lymph nodes. B-cells from one DLBCL, one chronic lymphocytic leukemia (CLL) and one marginal zone lymphoma (MZL) were efficiently killed by HG-6-64-1 and 9L, but a solid Mantle Cell Lymphoma (MCL) tumor was not.
Figure 3.23. *Cancerous lymph nodes are efficiently killed by HG-6-64-1 and 9L. Lymph nodes were processed, isolated, analyzed and graphed as described in Figure 3.22.*

Circulating B-cells in the blood, peripheral blood lymphocytes (PBLs), have distinct characteristics from B-cells located in solid lymph node tissue. To test the efficacy of these drugs on circulating B-cells, we obtained PBLs from three healthy volunteers. The effect of 400 nM HG-6-64-1 or 9L is depicted alongside B-cells isolated from two blood-borne tumors. HG-6-64-1 killed healthy B-cells from the peripheral blood of 3 volunteers, while 9L was not toxic to those cells. A blood-borne CLL was killed by both drugs, whereas a blood-borne MCL was resistant.
Figure 3.24. A blood-borne CLL is susceptible to killing by HG-6-64-1 and 9L. Freshly collected blood was subject to Ficoll density centrifugation, then processed, isolated, analyzed and graphed as described in Figure 3.22.

The efficacy of these drugs in destroying CLL cells is a positive indicator for their potential clinical implementation also in CLL. Most interesting is the ability of 9L to kill CLL tumor cells, but not healthy PBLs. This suggests that CLL treatment with 9L may leave healthy circulating blood cells intact, which would be beneficial to the patient population receiving such treatment. Overall, these results demonstrate that both HG-6-64-1 and 9L may be useful treatment in multiple B-cell cancers including DLBCL, but not MCL.
CHAPTER 4

Discussion

In this study we demonstrate for the first time that the MAPK pathway has an important role in DLBCL, specifically in the induction of proliferation and inhibition of apoptosis of these tumors. In particular, we demonstrate that the kinases GCK and JNK1 are upregulated at the protein level, and constitutively activated in DLBCL, respectively. Furthermore, we have generated and characterized a small molecule GCK inhibitor and identified an existing small molecule inhibitor for JNK, both potentially applicable as novel therapeutic approaches for DLBCL.

GCK

GCK is a kinase in the MAPK family which is preferentially expressed in the germinal centers of lymphoid follicles.\textsuperscript{38} It can be activated by a variety of agonists such as lipid A, polyinosine-polycytidine [poly(IC)], interleukin-1 (IL-1), lipopolysaccharides (LPS) and CD40 ligand engagement. The activation of GCK occurs by its stabilization, through a TRAF6 dependent mechanism, leading to downstream activation of JNK through the signaling intermediaries MEKK1 and MKK4.\textsuperscript{40}
Herein we demonstrate that GCK kinase activity is elevated in the majority of DLBCL tumors irrespective of their gene expression subtype and that its knockdown leads to a decrease in DLBCL cell proliferation and an increase in cell line and tumor apoptosis. Based on our knowledge, this is the first time that GCK is implicated in tumor pathogenesis. In contrast, its downstream effector-JNK, was previously implicated in tumor pathogenesis (see Discussion - JNK).

One of the potential explanations for the prior failure to identify GCK in tumors is the absence of biochemical methodology to directly measure GCK kinase activity, as the phosphorylation status of GCK is irrelevant to its activation state. Our application of a novel methodology, the KiNativ platform, enabled identification of its role in DLBCL.

The mechanism of GCK activation within DLBCL is unknown. The subset of agonists that are known to activate GCK suggest a role in inflammation, and in immune response to pathogens. GCK’s potential role in inflammation may explain the reported associations of DLBCL with inflammatory disorders such as Sjögren syndrome, hepatitis C, rheumatoid arthritis and HHV-8-positive multicentric Castleman disease. However, none of the known activators of GCK are consistently or broadly upregulated in DLBCL. The possibility exists that GCK is upregulated by an unidentified secretory product in these cell lines and tumors. However, the cytokine arrays we performed on DLBCL cell line supernatants (data not shown) have not revealed verifiable targets for this activation. More extensive cytokine array experiments are in progress.
An alternate mechanism for the activation of GCK in DLBCL is somatic mutations, which may increase protein stability. DLBCL tumors frequently exhibit somatic hypermutations,\(^1\) which recently were shown to affect a wide array of proteins implicated in different signaling pathways.\(^5\) Therefore, the possibility that stabilizing mutations are present in GCK exists, but has not been investigated. Sequencing of GCK and its upstream activators is necessary in primary DLBCL tumors to confirm or deny this possibility, and should be addressed in future studies.

**JNK**

GCK, among other kinases, has been previously demonstrated to activate the JNK family kinases through signaling intermediaries.\(^37\) ActivX profiling confirmed a single previous study that suggested JNK is required for the survival of murine B-lymphoma cells.\(^76\) Similarities in the JNK1/2/3 binding site made it impossible to determine which isoform was specifically activated within DLBCL using LCMS/MS. However, the varying expression patterns and roles of JNK1/2/3 family members directed our focus herein. JNK1 is known to stabilize c-jun, which leads to the transcriptional activation of a variety of pro-proliferative effectors. In contrast, JNK2 suppresses proliferation.\(^70\) JNK3 was excluded from our analysis because it is only expressed in the brain and testis.\(^71\) We focused on JNK1 because we were interested in potential pro-survival and pro-proliferative effects.
In addition to affecting proliferation, JNK family members can exhibit pro-apoptotic or pro-survival effects, depending on the cellular context. Activated JNK1 can regulate Bcl-2 family members, including Bcl-2–associated X protein (BAX) and Bcl-2 antagonist of death (BAD), leading to the induction of apoptosis in some cells.\(^6^4\) In other cells, chemical inhibition of constitutively active JNK1 leads to induction of apoptosis through caspase-3.\(^6^5\)

We confirmed the relationship between GCK and JNK1 in DLBCL by two methodologies. First, specific siRNA knockdown of GCK and JNK1 led to an apparent feedback response whereby JNK1 knockdown reduced total GCK and GCK knockdown reduced total JNK1. The feedback between GCK and JNK1 is a novel finding of our study. The knockdowns of JNK1 and GCK also both induced G1 arrest and cell death. MEKK1 siRNA reduced total GCK protein, and all three knockdowns led to a decrease in proliferation and viability as measured by MTS assay. Second, our kinase assay demonstrated a reduced ability of JNK to phosphorylate the substrate c-jun after chemical inhibition of GCK, suggesting a role for GCK in the activation of JNK in DLBCL. Thus, the results of our study are most consistent with activated JNK1 being a pro-proliferative, pro-survival mechanism within DLBCLs.

Both JNKs 1 and 3 have been previously demonstrated to have oncogenic potential. JNK1 is highly activated in hepatocellular carcinoma (HCC). In HCCs increases in JNK1 kinase activity are associated with increased proliferation of tumor cells and increased tumor number.\(^5^0\) In prostate cancer, PTEN loss leads to subsequent AKT activation and increased JNK activity,\(^5^2\) and brain tumors are
frequently associated with JNK3 loss-of-function mutations.\textsuperscript{51} In this study, we have additionally demonstrated a role for JNK1 in the proliferation and survival of DLBCL. Our success in the implementation of GCK and JNK small molecule inhibitors in other B-cell lymphomas also suggests that JNK1 may have a role in multiple B-cell neoplasms.

It is clear from our study that GCK has a role in the activation of JNK1 in DLBCL. However, the possibility has not been excluded that there are other upstream activators of JNK1 within this lymphoma subtype. Other MAP4K’s are capable of leading to downstream phosphorylation of JNK1;\textsuperscript{37} we discovered activation of the MAP4K family member, HPK1 in this study. There are also recent publications implicating TAK1 in JNK1 activation,\textsuperscript{66,67} and the potential role of both of these kinases in JNK1 activation needs to be explored in future studies. AKT is activated in a subset of DLBCL and may also contribute to JNK activation.\textsuperscript{68} As with GCK, the possibility exists that there are undetected somatic hypermutations within the JNK family, or in its upstream effectors. However, demonstration that GCK inhibition leads to decreased activation of JNK and affects cell proliferation and survival suggests that this is one of the dominant pathways activating JNK in DLBCL.

\textit{p38}\textsubscript{α}

There is a great deal of literature on the pathogenic potential of the kinase p38\textsubscript{α}. This kinase is reported to control proliferation and apoptosis, frequently
through crosstalk with JNK1.\textsuperscript{57} Although p38α is downstream of GCK, we found it to be downregulated in DLBCL cell lines compared to tonsillar B-cells, and our IHC studies of primary DLBCL tissues revealed that p-p38α was not detected in the majority of samples. Our inability to detect constitutively active p38α is most likely attributable to the preferentially activation of JNK1 by MKK4\textsuperscript{37,57}, which we discovered to be upregulated in DLBCL. GCK activates MEKK family homologs, in this case MEKK1, and those MEKK family members activate MKKs. MKK3 specifically activates p38α\textsuperscript{57} and we did not detect upregulation of MKK3 in our DLBCL cell lines. The preferential activation of MKK4 in DLBCL, versus MKK3, may explain the absence of p-p38α in these tumors.

Despite its downregulation in DLBCL, p38α was of interest because it is a potential downstream target of GCK,\textsuperscript{37} and because it is a direct target of HG-6-64-1. However, HG-6-64-1 was no more effective in inhibiting the growth of the tested DLBCL cell lines than 4 inhibitors which had no effect on the p38α active site. Additionally, siRNA knockdown of p38α induced minor cell death, but did not lead to G1 arrest (data not shown). Eliminating p38α as a necessary element in the treatment of DLBCL via this pathway was critical, given the negative clinical side effects associated with p38 inhibition.\textsuperscript{69} Western blotting demonstrated that HG-6-64-1 does inhibit the phosphorylation of p38α in DLBCL cell lines, but the equally efficacious NG variant NG25 does not, thus the future implementation of NG25 over HG-6-64-1 remains a possibility.
**Therapeutic Promise**

In B-cell lymphomas, and other cancers of the immune system, death does not have to be specific to cancerous cells as long as it leaves critical organs intact. This is due to the ability of the immune system to reconstitute itself. We demonstrated herein that these small molecule inhibitors can destroy tumor tissue at least as effectively, if not more effectively, than healthy components of the immune system. 9L had the additional advantage of leaving a portion of the hematopoietic compartment, the healthy PBLs, intact.

The small molecule inhibitors generated and identified herein specifically target GCK and JNK and are effective in killing most primary B-cells, both healthy, and those in the cancers DLBCL, CLL and MZL. These drugs do not kill MCL tumors, or the low-GCK expressing DLBCL cell line, G452, which is a good indicator that their inhibitory effects are specific and not due to general toxicity. Ideally, any cancer drug would target only tumor cells, however in current cancer therapeutics this is not always achievable. Rituximab, for example, is the most robust treatment for DLBCL.\(^{26}\) Rituximab targets and destroys all B-cells which it encounters, regardless of their oncogenic potential.

The destruction of the immune compartment is clinically tolerable for cancer patients. However, the risk of novel drug therapies is that critical organs or tissues will be targeted, causing unwanted side effects. GCK has not been demonstrated to be necessary for normal cellular homeostasis in healthy tissues, which is a good indicator for its potential for clinical implementation. However,
the activity of JNK2 is required to control the proliferation of some cells.\textsuperscript{70} We have not yet determined the effect of GCK on JNK2, thus it is possible that GCK inhibition will lead to negative side effects in human subjects. Several JNK inhibitors have successfully completed Phase I trials,\textsuperscript{71} indicating promise for JNK inhibition as a therapeutic strategy. After successful completion of our ongoing xenograft studies, Phase I trials to determine human tolerance to GCK inhibition will be critical in establishing the viability of this therapeutic option.

A second positive indicator for clinical implementation is our demonstrated increased activity/expression of GCK and JNK1 in both the ABC-like and the GCB-like subtypes of DLBCL. A treatment option which is not sub-type specific will ease and broaden clinical implementation of these drugs, especially given that DLBCL patients are classified as having ABC-like or GCB-like DLBCL by IHC staining of their tumors by either the Hans model,\textsuperscript{72} or by other published models of ABC-like and GCB-like markers. However, compared to the gold standard gene expression profiling, these models harbor an inherent error rate that limit their daily implementation. Thus, it is currently much more practical to focus on the development of drugs that do not require a precise determination of DLBCL subtype.
Future Studies

Xenograft models for the drugs 9L, HG-6-64-1 and NG25 are in progress in the Lossos' laboratory. This is the critical next step towards determining the viability of these small molecule inhibitors as DLBCL therapeutics.

Additionally verifying the specificity of GCK is also critical. The ActivX platform provided considerable specific information about the compound HG-6-64-1. We were able to solidify the target GCK through comparison of MTS response to HG-6-64-1 structural variants with diverse specificity. However, to further ensure the specificity of the chemical inhibition of GCK we are currently producing mutants of GCK in which the gatekeeper residue for HG-6-64-1 binding, a methionine, is substituted for phenylalanine or threonine. Additional GCK mutants in production include the deletion of one glycine in the HG-6-64-1 binding area, and the mutation of the gatekeeper methionine to threonine alongside deletion of the same glycine. These later mutations will produce a sequence similar to that found in the ATP binding area of proto-oncogene serine/threonine-protein kinase (c-raf), which is not inhibited by HG-6-64-1. In future studies, we hope to rescue the anti-proliferative and pro-apoptotic effects of HG-6-64-1 treatment by transfecting DLBCL cell lines with these mutants, prior to HG-6-64-1 treatment, demonstrating the specificity of the response.

Finally, there are interesting targets in our kinase screen which have not been explored. Specifically, the upregulation of IKKβ, which provides a survival signal, through NF-kB activation is of interest due to the proposed role of NF-kB
in a variety of B-cell malignancies. We also discovered that one of our HG-6-64-1 inhibitors which was highly specific for c-raf potently inhibited the proliferation of two of our DLBCL cell lines. C-raf is a MAP3K that activates the extracellular-signal-regulated kinases (ERK) pathway, neither of which have been implicated in DLBCL pathogenesis, but which are implicated in regulation of the cell cycle.

**Summary**

The combination of a newly identified molecular pathway which is critical to the survival of DLBCL and the creation and identification of small molecule inhibitors as potential DLBCL therapeutics adds considerably to the base of knowledge surrounding DLBCL. These ongoing studies will verify the viability of the small molecule inhibitors HG-6-64-1 and 9L as new DLBCL drugs, which may eventually improve the outcome of DLBCL patients.
References


