Pharmacologic Approaches to Targeting Malignant Activation of Cap-Dependent Protein Translation in Aggressive B-Cell Lymphomas

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PHARMACOLOGICAL APPROACHES TO TARGETING MALIGNANT ACTIVATION OF CAP-DEPENDENT PROTEIN TRANSLATION IN AGGRESSIVE B-CELL LYMPHOMAS

By

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A DISSERTATION

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PHARMACOLOGICAL APPROACHES TO TARGETING MALIGNANT
ACTIVATION OF CAP-DEPENDENT PROTEIN TRANSLATION IN
AGGRESSIVE B-CELL LYMPHOMAS

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Despite decades of research identifying molecular drivers of cancer, targeted therapies fail to provide long-term survival benefits for cancer patients much of the time, primarily due to tumor heterogeneity and redundancies of signaling pathways. In the most common lymphoma, diffuse large B-cell lymphoma (DLBCL), malignant activation of B-cell receptor (BCR) signaling drives cancer cell growth. Expression of the PIM kinases downstream of this signaling is a common feature of the Activated B-cell like (ABC) subtype of DLBCL, and a target of interest for therapy. We found the sensitivity of ABC DLBCL to pan-PIM kinase inhibition by the clinical compound PIM447 to be dependent on mutational activation of BCR signaling. Cell lines with mutations downstream that directly activated NF-κB signaling through the CARD11, BCL10, MALT1 protein complex where dependent on PIM for activation of protein translation, while cell lines lacking those downstream mutations had multiple avenues for activation of translation and thus were resistant to PIM447 treatment. Importantly, all cell lines were sensitive to direct inhibition of translation through the RNA helicase eIF4A1, regardless of mutational status, highlighting the
advantage of directly targeting translation in cancer to avoid these resistance mechanisms.

Because there is currently an unmet need for eIF4A1 inhibitors in the clinic, we performed a novel screen for inhibitors of eIF4A1’s ATPase activity. We discovered the natural compound elatol, which in a 2:1 stoichiometry interacts with two key lysines in eIF4A1 to prevent ATP hydrolysis. Elatol shows antitumor activity in a wide range of cancer cell lines, but as our studies with PIM in DLBCL suggested, lymphoma is a particularly sensitive cancer type. Elatol induces apoptosis in DLBCL cells by inhibiting cap-dependent protein expression of key cancer drivers including c-MYC, Cyclin D3 and MCL1. Surprisingly, elatol treatment induces expression of stress response factor ATF4, but this is not important for elatol’s antitumor activity, which are driven by its action against eIF4A1. Elatol is tolerated at high doses in vivo, and significantly delayed tumor progression in an ABC DLBCL xenograft model. Together these studies emphasize the inhibition of translation as a therapeutic option in DLBCL and provide a pipeline for the development of eIF4A inhibitors.
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# TABLE OF CONTENTS

**LIST OF FIGURES**................................................................................................. v
**LIST OF TABLES** ................................................................................................. vii
**ABBREVIATIONS** ............................................................................................... viii
**CHAPTERS**

1 Introduction ........................................................................................................... 1
2 Materials and Methods .......................................................................................... 20
3 Control of Translational Activation by PIM Kinase in Activated B-Cell Diffuse Large B-Cell Lymphoma Confers Sensitivity to Inhibition by PIM447 ......................................................................................... 31
4 Targeted Screen Identifies Elatol as a Novel eIF4A Inhibitor with Potent Antitumor Activity .................................................................................................................. 47
5 Discussion and Future Directions ......................................................................... 74
Appendices .............................................................................................................. 83
References .............................................................................................................. 86
LIST OF FIGURES

Figure 1.1 Germinal center B-cell development and lymphomagenesis………3
Figure 1.2 Cap-dependent translation initiation………………………………..8
Figure 1.3 Deregulation of translation initiation in cancer…………………12
Figure 1.4 The Integrated Stress Response……………………………………14
Figure 3.1 Pan-PIM inhibition in NHL with PIM447…………………………33
Figure 3.2 PIM kinase expression in DLBCL cell lines………………………34
Figure 3.3 Differential sensitivity of ABC DLBCL to BCR signaling inhibitors.35
Figure 3.4 PIM447 treatment results in mTOR deactivation and PIM stabilization
in sensitive cell lines…………………………………………………………37
Figure 3.5 PIM447 shuts off translation activation by PIM2 in independent
system……………………………………………………………………………40
Figure 3.6 PIM447 treatment inhibits global translation in sensitive cell
lines………………………………………………………………………………41
Figure 3.7 Somatic mutations in PIM1 in DLBCL……………………………43
Figure 3.8 Recurrent PIM1 mutants preserve kinase function, but don’t overcome
PIM447…………………………………………………………………………44
Figure 3.9 BCR mutational activation as a biomarker for targeted therapy….46
Figure 4.1 Elatol is a specific inhibitor of eIF4A1 ATPase activity…………..48
Figure 4.2 Elatol shows broad antitumor activity……………………………50
Figure 4.3 Elatol is toxic to DLBCL cells………………………………………51
Figure 4.4 Elatol interacts with two key lysines in eIF4A1 in a 2:1
stoichiometry……………………………………………………………………….52
Figure 4.5 NHL cell line sensitivity to elatol and silvestrol..........................53

Figure 4.6 Elatol inhibits global and cap dependent translation.................55
Figure 4.7 Elatol reduces expression of translationally regulated oncogene..56
Figure 4.8 Elatol does not induce eIF4A2 transcriptional response.........57
Figure 4.9 Elatol affects mRNA expression at high concentrations, after effects on translation.................................................................59
Figure 4.10 Elatol causes immediate upregulation of ATF4...................61
Figure 4.11 Elatol induction of ATF4 is not mediated by PERK or a UPR.....63
Figure 4.12 Upregulation of ATF4 following elatol is dependent on eIF2α phosphorylation.................................................................64
Figure 4.13 eIF2α phosphorylation and ATF4 are not important for elatol’s potency against cancer cells.........................................................65
Figure 4.14 Elatol’s activity is dependent on eIF4A1...............................67
Figure 4.15 eIF4A2/3 knockdown induces ATF4 expression....................68
Figure 4.16 Elatol is tolerated at 20mg/kg daily in mice.........................69
Figure 4.17 20mg/kg elatol daily in tumor bearing mice is toxic...............70
Figure 4.18 Single dose of 65mg/kg elatol is tolerated in mice...............71
Figure 4.19 Elatol significantly delays tumor progression in vivo.............72
LIST OF TABLES

Table 2.1 PIM1 site directed mutagenesis primers...........................................24

Table 3.1 Recurrent PIM1 mutations in DLBLC.............................................45
4EBP: eIF4E binding protein

ABC: Activated B-cell

AID: Activation induced cytidine deaminase

Akt: Protein kinase B

AML: Acute myeloid leukemia

ATF4: Activating transcription factor 4

ATF6: Activating transcription factor 6

ATP: Adenosine triphosphate

BCL2: B-cell lymphoma 2

BCL6: B-cell lymphoma 6

BRAF: B-Raf proto-oncogene

BTK: Bruton tyrosine kinase

c-MYC: cancer- Myelocytomatosis oncogene

C: Carboxy

CARD11: Caspase recruitment domain family member 11

CD79B: Cluster of differentiation 79B

CERT: Cytosine-enriched regulator of translation

CNS: Central nervous system

DDX17: DEAD box protein 17

DDX3: DEAD box protein 3

DDX39A: DEAD box protein 39A

EBV: Epstein-Barr virus
EGFR: Epidermal growth factor receptor

eIF: eukaryotic initiation factors

eIF4F: eukaryotic initiation factor 4F complex

ER: Endoplasmic reticulum

E_{μ}-Myc: IgH enhancer:c-Myc translocation

FLT3: Fms related tyrosine kinase 3

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GCB: Germinal center B-cell

GCN2: General control non-repressible 2

GroEl: Heat shock protein

HER2: Erb-B2 receptor tyrosine kinase B2

HRI: Heme regulated eIF2α kinase

HSP70: Heat shock protein 70

IC50: Inhibitory concentration 50

Ig: Immunoglobulin

IgH: Immunoglobulin heavy chain

IgL: Immunoglobulin light chain

IL-3: Interleukin 3

INK4-ARF: Inhibitor of CDK4-alternate reading frame protein locus

IRES: Internal ribosome entry site

ISR: Integrated stress response

ISRIB: ISR inhibitor

IκBα: NF-κB inhibitor α
JAK2: Janus kinase 2
LD50: Lethal dose 50
MAPK: Mitogen-activated protein kinase
MCL1: Myeloid cell leukemia sequence 1
MEFs: Mouse embryonic fibroblasts
MNK: MAP Kinase interacting kinase
mTOR: mechanistic target of rapamycin
MYD88: Myeloid differentiation primary response 88
N: Amino
NF-κB: Nuclear factor-κB
NOS: Not otherwise specified
NSCLC: Non-small cell lung cancer
p97: Protein 97
PBMC: Peripheral blood mononuclear cells
PDCD4: Programmed cell death 4
PERK: PKR-like ER kinase
PERKi: PERK inhibitor
PI3K: Phosphatidylinositol 3 kinase
PIM1: Proviral integration site for moloney murine leukemia virus 1
PKC: Protein kinase C
PKR: Protein kinase RNA activated
PMBL: Primary mediastinal B-cell lymphoma
PPIB: Peptidyl isomerase B
PTEN: Phosphatase and tensin homology

R-CHOP: Rituximab, Cyclophosphamide, doxorubicin (Hyroxydaunomyc), vincristine (Oncovin), Prednisone

RAS: Rat sarcoma virus proto-oncogene family

S6: Ribosomal protein S6

S6K: Ribosomal S6 kinase

SCID: Severe combined immunodeficiency

SCLC: Small cell lung cancer

SHM: Somatic hypermutation

SYK: Spleen tyrosine kinase

TISU: Translation initiator of short 5'UTR

TNFAIP3: TNF alpha induced protein 3

TOP: Terminal oligopyrimidine

TP53: Tumor protein 53

tRNA: Transfer RNA

TSC2: Tuberous sclerosis complex 2

UTR: Untranslated region

XBP1s: X-box binding protein 1 spliced
Chapter 1
Introduction

1.1  Diffuse large B-cell Lymphoma

Non-Hodgkin's Lymphoma (NHL) is one of the most common cancers diagnosed in both men and women in the United States as well as the western world \(^1\). NHL comprises a large group of different diseases that arise from lymphocytes, and are defined by the type of lymphocyte, the stage of differentiation of the cell from which it arises, as well as other morphologic and pathologic features. Approximately 85\% of NHLs are B-cell lymphomas, of which diffuse large B-cell lymphoma (DLBCL) cases account for approximately one third, making it the most common NHL \(^1\). DLBCL can be further subtyped into DLBCL of the central nervous system (CNS), primary mediastinal (PMBL), Epstein-Barr Virus positive, anaplastic lymphoma kinase (ALK) positive, not otherwise specified (NOS) and others \(^2,3\). DLBCL is an aggressive disease, but considered chemoresponsive and can be cured by the standard of care combination of immune and chemotherapy drugs, sometimes including a bone marrow stem cell transplant, however for patients who do not respond to these treatments the prognosis for survival is dismal \(^4,5\). Addition of the antibody rituximab (R) to the chemotherapy regimen of cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) 15 years ago is the only recent change to the 40-year-old standard of care, despite substantial evidence that ~40\% of patients will fail to be cured \(^5\). The lymphoma research community has acknowledged the need for superior treatments for those patients who don’t respond to chemo and
at least six phase III clinical trials for DLBCL have been reported since 2013 that either modify the standard of care or add molecularly targeted agents\textsuperscript{6}. Unfortunately, none of these studies have shown any improvement over R-CHOP\textsuperscript{6}. This disappointing realization highlights the importance not only of well-designed clinical trials, but also of rational identification of novel treatment approaches based in biological understanding of the disease.

Extensive genome-wide studies have identified clear molecular subtypes of NOS lymphoma that align the gene expression profiles with either germinal center B-cells (GCB) or activated B-cells (ABC) as the cell type of origin\textsuperscript{7}. They differ in many immunologic phenotypes as well as disease progression, with the ABC subtype having a worse prognosis\textsuperscript{8,9}. During normal B-cell development early B-cells rearrange the genes of the immunoglobulin (Ig) locus to create a B-cell receptor (BCR) that is expressed on the cell surface\textsuperscript{10}. Expression of a rearranged BCR marks passage of a major checkpoint and continuation into secondary lymphoid organs for further development. Mature naïve B-cells that enter the germinal center reaction exist in a unique state where mutations are purposefully directed to the variable region of the immunoglobulin heavy and light chain genes (IgH and IgL) locus by the enzyme activation induced cytidine deaminase (AID) to allow for the generation of highly specific antibodies in a process known as somatic hypermutation (SHM) (Fig 1.1). This process is critical for the generation of a diverse repertoire of B-cells able to recognize millions of
different pathogens, but when it goes awry lymphoma can arise. Aberrant SHM, near the Ig locus or elsewhere in the genome, leads to a multitude of genetic abnormalities that are observed in DLBCLs. GCB lymphomas are characterized by expression of the transcriptional regulator B-cell lymphoma 6 (BCL6), the t(14;18) translocation of BCL2, downregulation of phosphatase and tensin homology protein (PTEN) and tumor protein 53 (TP53) mutation, while ABC lymphomas usually have BCL2 overexpression and loss of the inhibitor of CDK4-alternate open reading frame (INK4α-ARF) locus. Additionally, hyperactivation of nuclear factor-kappa B (NF-κB) signaling through various genetic mechanisms is one of the most defining features of the ABC subtype.

Figure 1.1 Germinal center B-Cell development and lymphomagenesis. Mature naive B-cells enter the germinal center (GC) as centroblasts following antigen stimulation by T-cells. In the dark zone, they rapidly proliferate and undergo successive rounds somatic hypermutations to create highly specific antibodies. Strong antigen recognition with T-cell and follicular dendritic cells (FDCs) of the now centrocytes in the light zone promotes positive selection and terminal differentiation into either a plasma or memory B-cell, while negatively selected centrocytes are eliminated through apoptosis. Common aberrations found in lymphoma arising from either GC B-cells (GCB) or activated B-cells (ABC) are shown.
The NF-κB pathway is a well-known survival pathway in cancer and contributes to the chemoresistance of the ABC subtype and the poor prognosis.

1.2 PIM Kinases in cancer

Further identification of targetable oncogenic drivers of the ABC subtype of DLBCL is the key to better clinical outcomes for these patients. Analysis of the mutational landscape of DLBCL has identified a number of recurrently mutated genes, one of the most frequently mutated being proviral integration site for moloney leukemia virus 1 (PIM1)\textsuperscript{11,12}. PIM1 was discovered in a viral-insertion screen for genes that would promote the development of the IgH enhancer: cancer-myelocytomatosis oncogene (E\textsubscript{μ}-Myc) transgenic mouse lymphoma model, and two highly homologous kinases PIM2 and PIM3 were later identified\textsuperscript{13-15}. The PIM kinases are considered functionally redundant, as PIM2 or PIM3 can replace PIM1 in the viral insertion screen when the other genes are absent. The PIM genes are ubiquitously expressed in humans, with some tissue variation, and once expressed these constitutively active kinases phosphorylate many intracellular targets involved in cell cycle regulation, cell growth and survival\textsuperscript{16}. The main mechanism for regulation of PIM is by transcriptional expression as well as protein stability, as there are no known post-transcriptional or localization requirements controlling their activity. Their expression is induced downstream of growth factor and cytokine signaling pathways, and their short half-life is tightly controlled by ubiquitination and proteasomal degradation\textsuperscript{17,18}. 
Despite the PIM kinases being overexpressed in many different cancers of both hematologic and epithelial origin, PIM activity is considered a weakly oncogenic by itself as overexpression alone can only induce T and B-cell lymphomas in mice at a low incidence \(^{19}\). Instead, PIM’s oncogenic activity comes from cooperation with other cancer drivers such as c-MYC, which PIM synergizes potently with in lymphoma and prostate cancer \(^{13,19}\). PIM expression enhances oncogenic capabilities and serves as a marker of aggressiveness in lymphoma, prostate cancer and acute myeloid leukemia (AML) and contributes to chemoresistance in pancreatic, colon and breast cancers \(^{20,21}\). The fact that triple PIM knockout mice have no severe defects apart from smaller body size and impairments in hematopoietic cell response to cytokine combined with the evidence for PIM activity in cancer suggest therapeutic selectivity of PIM inhibition \(^{22}\).

There are several classes of PIM inhibitors under clinical investigation but the major ones that have entered clinical trials are SGI-1776 and AZD1208. SGI-1776 is a pan-PIM kinase inhibitor, although it has over 50 times and 10 times selectivity for PIM1 over PIM2 and PIM3 respectively \(^{23}\). SGI-1776 may be most effective in AML as it also inhibits fms related tyrosine kinase 3 (FLT3), a driver in approximately 30% of AMLs, but unfortunately clinical evaluation with this compound has stalled due to cardiac toxicity \(^{23}\). AZD1208 is another pan-PIM kinase inhibitor with more potent activity for all three PIM kinases than SGI-1776 and is being evaluated in a phase I clinical trial in solid tumors as well as
lymphoma (NCT01588548). The frequent somatic mutation of PIM1 in DLBCL poses the question of whether the mutations are a passenger effect of aberrant SHM, or positively selected due to PIM’s oncogenic properties, which would suggest an opportunity for PIM kinase inhibition.

One of the key outcomes of PIM kinase inhibition in multiple studies is the deregulation of cell growth pathways and loss of expression of pro-apoptotic proteins. We studied the effect of pan-PIM kinase inhibition on translation in ABC DLBCL, as well as the functional consequence of some of the common PIM1 mutations. These studies provided an excellent example of oncogenic signaling pathways feeding into the activation of protein translation in cancer, which will be discussed in detail in the next section.

1.3 Cap-dependent protein translation

Though derived from our own bodies, cancer cells go through a multistep evolution where they acquire genetic aberrations that allow for malignant growth. The existence of a mutation in a single oncogene, such as PIM1, is meaningless until that gene is expressed as a functional protein that can act in combination with the other necessary changes that allow the cell to proliferate in an uncontrolled manner. Countless genome-wide expression studies have highlighted massive changes in the cancer cell transcriptome and cancer research to date has focused heavily on transcription factors and transcriptional reprogramming as the driver of the cancer phenotype. However, gene expression only begins with activation of transcription and creation of messenger RNA.
(mRNA), with many more steps preceding the expression of a protein. Microarray and next generation sequencing technologies have made gene expression studies more easily achievable, but in the last decade advances in proteomics have allowed for comparisons between mRNA and corresponding protein abundance, as well as how the two change in various situations. There is still debate on the exact level of correlation, and the nuances of the experimental design are critical for comparisons between studies, but multiple studies do conclude that post-transcriptional processes such as translation and protein stability exert significant control over gene expression. Additionally, the influence of translation on gene expression, especially of proteins with short half-lives, is amplified when cells respond to stimuli such as growth factors. As cancer cells must respond to countless changes in order to survive from adapting to a hypoxic microenvironment, continual maintenance of proliferation despite depleted nutrients and the dynamic process of invasion and metastasis, these studies suggest that translational control over gene expression would be critical to the cancer cell.

In eukaryotes nearly all nuclear encoded eukaryotic mRNAs are translated through the cap-dependent mechanism of translation, compared to some housekeeping genes and many viral mRNAs that have internal mechanisms for translation. The eukaryotic initiations factors (eIFs) mediate this process, which begins with binding of the 5' methylguanosine cap of the mRNA by the translation initiation factor eIF4E (Figure 1.2). eIF4E is one member of the heterotrimeric eIF4F complex, comprised also of eIF4G and eIF4A. Cap-bound
eIF4E then binds the scaffold protein eIF4G, which also interacts with the adenosine triphosphate (ATP)-dependent RNA helicase eIF4A$^{39,41}$. eIF4A uses its minimal helicase activity, which is enhanced by its interaction with eIF4G and accessory proteins eIF4B and eIF4H, to unwind secondary structures in the 5’ untranslated region (UTR) of the mRNA and facilitate 40S ribosomal subunit

Figure 1.2 Cap-dependent translation initiation. In eukaryotes translation begins with formation of the heterotrimeric eIF4F complex. eIF4E binds the 5’cap of the mRNA as well as the scaffold eIF4G, which then recruits the RNA helicase eIF4A. The 40S ribosomal subunit preloaded with the methionine-charged tRNA bound to GTP-eIF2α, and is recruited to the mRNA by eIF3’s interaction with eIF4G. PABP interacts with the poly-A tail of the mRNA and eIF4G to create the closed loop structure for scanning. With the help of accessory protein eIF4B, eIF4A unwinds secondary structure in the 5’UTR of the mRNA and the ribosome scans for the AUG start codon. Upon recognition GTP is hydrolyzed by eIF2α, which triggers release of eIFs and the transition into elongation.
scanning for the initiation codon. eIF4G also interacts with eIF3 of the preinitiation complex to recruit the ribosome along with the ternary complex containing the methionine charged transfer RNA (tRNA), bound by eIF2, to the 5' end of the mRNA. The poly-A binding protein (PABP) interacts with the poly-A tail of the mRNA as well as eIF4G to create the closed-loop structure for ribosomal scanning, completing the 48S initiation complex for translation initiation.

Recognition of the first in frame AUG codon leads to a series of events including the release of phosphate from the ternary complex, and release of eIFs 1, 2 and 5, and the transition into the elongation phase. Protein synthesis is an exceedingly energetically expensive activity, consuming nearly 75% of a cell’s ATP, and the initiation phase of translation is the primary point for regulation of this process.

1.4 Deregulation of translation in cancer

Perhaps the most basic major mechanism for regulation of translation initiation comes from the cancer cell metabolic phenotype described by Otto Warburg. Deregulation of cancer cell metabolism to consume high levels of glucose and undergo aerobic glycolysis impacts cancer cells in many ways including influencing protein translation by way of amino acid and ATP availability. However, there are several other crucial mechanisms for regulation of translation at the initiation step that become exploited in cancer.

First, overexpression of individual components of the translation initiation machinery. This occurs in almost every type of cancer and sufficient to cause
transformation. eIF4E was the first translation initiation factor to be identified to have cellular transformation capabilities, and cooperate with well-known oncogenes rat sarcoma virus proto-oncogene (RAS) and MYC to promote tumorigenesis. Since then, overexpression of many other translational components has been described and is summarized in these reviews, worth noting is the frequent loss of programmed cell death 4 (PDCD4), the only negative regulator of eIF4A, in a variety of tumor types and its correlation with poor prognosis or aggressive tumor behavior.

Second, nearly all the intracellular signaling pathways that are hijacked in cancer cells converge on activation of translation by eIF4F through the master regulator of cell growth, mechanistic target of rapamycin (mTOR) (Figure 1.3). When activated mTOR in complex 1 (mTORC1) phosphorylates the eIF4E binding proteins (4EBPs), which inhibit eIF4F formation by competing for the same binding site on eIF4E that eIF4G binds. This releases eIF4E to bind the 5' cap of mRNA for initiation to begin. eIF4E activity is further regulated downstream of mitogen-activated protein kinase (MAPK) signaling by phosphorylation by the MAPK interacting kinase (MNK). This phosphorylation event enhances eIF4E recruitment of mRNAs into the eIF4F complex.

Third, phosphorylation of eIF2α of the ternary complex provides another node for regulation of translation initiation. Phosphorylated eIF2α inhibits translation of mRNA by preventing exchange of its guanine nucleotide by the guanine nucleotide exchange factor eIF2B. When levels of phosphorylated
eIF2α are high translation and subsequent cell growth are globally inhibited in normal cells as well as cancer cells, but low levels of phosphorylation are critical for brief inhibition of translation and activation of cellular stress response pathways important for adaption. An example of this is in melanoma cells, where reprogramming of translation through eIF2α drives the invasive phenotype. With all of these mechanisms for deregulation employed by cancer cells, it is impossible to deny the critical role translation plays in driving tumorigenesis.

Global increases in protein translation are necessary to support the rapid growth of cancer cells, but research suggests the effects of elevated translation on cancer cells is more complex than that. Not only do the key cancer signaling pathways converge on the activation of protein translation, but alternative mechanisms of activation of translation through eIF4F can actually confer resistance to targeted inhibitors and chemotherapeutic drugs, which is a major clinical problem. Elevated expression of eIF4E drives acquired resistance to epidermal growth factor receptor (EGFR) inhibitors in lung cancer, while persistent formation of the eIF4F complex by measuring the eIF4E:eIF4G interaction drives resistance to MAPK pathway inhibitors in B-Raf proto-oncogene (BRAF) mutant melanoma, colon and thyroid cancers. In breast cancer cells, chemotherapy resistance is linked to microRNA mediated downregulation of the eIF4A inhibitor PDCD4. These studies suggest that inhibiting translation can synergize with targeted inhibitors and overcome chemo-resistance, and that is exactly what numerous groups have demonstrated.
The unifying feature of these examples lies in the maintained activation of eIF4F that allows for sustained protein translation in spite of the therapy.

The role of eIF2α phosphorylation in cancer drug resistance is not as well understood.

Figure 1.3 Deregulation of translation initiation in cancer. Cellular signaling pathways typically activated by growth factors, hormones and cytokines become hijacked in cancer cells to promote their uncontrolled growth and survival. These pathways converge on the initiation of translation via activation of mTORC1. mTORC1 phosphorylates the inhibitory 4EBPs, releasing eIF4E to join the eIF4F complex to initiate translation. Further deregulation comes from the MNKs downstream of MAPK signaling, which phosphorylate eIF4E to promote oncogenic protein translation. The inhibitor of eIF4A, PDCD4, is also deregulated to promote eIF4A activity in eIF4F both by mTORC1 activation and frequent loss of expression in tumors. Together these alterations promote elevated translation of proteins essential for cancer cell growth, proliferation and survival.
understood, though there is mounting evidence that it is important. eIF2α has the complex job of integrating signals from the four eIF2α kinases: general control nonderepressible 2 (GCN2), protein kinase RNA activated (PKR), heme regulated eIF2α kinase (HRI), PKR-line endoplasmic reticulum kinase (PERK), which all phosphorylate the same serine residue in response to a variety of intracellular stresses, termed the integrated stress response (ISR) (Figure 1.4) 58.

Phosphorylation of eIF2α leads to two broad outcomes. The first is temporary inhibition of global protein translation due to the inability of eIF2 to exchange its nucleotide, but through an upstream open reading frame mechanism the eIF2α effector activating transcription factor 4 (ATF4) is expressed 60. ATF4 induces expression of other stress response genes to allow the cell to attempt to adapt to the stress condition and resuming normal proliferation 39,60. There are other mRNAs that are still translated when eIF2α is phosphorylated through different mechanisms, but the global cellular response is a decline in protein synthesis. If, however, the cell is not able to reverse the stress in a reasonable time, prolonged phosphorylation of eIF2α induces apoptosis 58. The outcomes resulting from eIF2α phosphorylation by the four different kinases are not identical, and in some stress situations multiple eIF2α kinases may be activated 68-72. As cancer cells evolve and generally thrive in stressful cellular environments, some amount of eIF2α phosphorylation is thought to be necessary to allow them to adapt to those conditions, however there is evidence that prolonging eIF2α phosphorylation by inhibiting its phosphatase can resensitize resistant cells to chemotherapy and proteasome inhibition through eIF2α’s pro-apoptotic activity.
As mentioned earlier, nearly all mRNAs are translated through the cap-dependent mechanism, however, they do differ in their requirement of cap-mediated initiation for efficient translation. The dependency on eIF4F for translation was long thought to be linearly correlated with the length and secondary structure in the 5' UTR. Many proteins that control cell growth and

**Figure 1.4 The integrated Stress Response.** The integrated stress response is a cytoprotective pathway with four arms that become activated in response to a variety of intracellular stressors. Amino acid deprivation, viral infection, heme deprivation, and ER stress activate eIF2α phosphorylating kinases GCN2, PKR, HRI, and PERK respectively. When phosphorylated, eIF2α cannot exchange its GDP nucleotide resulting in a decline in global translation. However, through an upstream ORF mechanism ATF4 is produced and activates expression of stress response genes.
the cell cycle as well as apoptosis also have long, complex 5’UTRs and are sensitive to eIF4F activity, explaining why altering eIF4F actually reprograms translation in cancer cells to favor growth and survival\textsuperscript{49,77}. More recent research using mTOR, eIF4E or eIF4A inhibition has identified subsets of mRNAs containing unique sequence motifs that influence their dependency on specific translation initiation factors. While there is still debate on what the exact sequence requirements are, it is clear that this confers an additional level of translational control over gene expression. Initially, a group of proteins involved in translation (ribosomal proteins, elongation factors, etc.) were identified as upregulated following growth factor stimulation and mTOR activation, and were characterized by the near ubiquitous presence of the 5’ terminal oligopyrimide (TOP) motif \textsuperscript{77,78}. Recently, the class of mTOR dependent mRNAs has been expanded to include more genes involved in invasion and metastasis and the presence 5’TOP motif on all mTOR sensitive mRNAs has been called in to question \textsuperscript{78-80}. Other studies have suggested eIF4E can regulate mRNA expression independent of mTOR, linking eIF4E to driving oxidative stress related proteins or mitochondrial structure and pro-survival proteins, and defined the new 5’UTR features cytosine enriched regulator of translation (CERT) and translation initiator of short 5’UTR (TISU) motifs \textsuperscript{81,82}. Discrepancies in the datasets of mTOR/eIF4E regulated mRNAs can be attributed to experimental methods used, either ribosome profiling or RNA sequencing of polysome fractions, which each have their own pitfalls, as well as the cell type and context \textsuperscript{37,83}. Despite these problems, these studies clearly illustrate that mRNAs that
support the oncogenic phenotype are exceedingly sensitive to inhibition of translation.

An elegant study comparing mTOR and eIF4A inhibition side by side using polysome profiling revealed that mTOR dependent mRNAs fall in to three classes: 5’TOP containing which are mainly translational machinery proteins, non-TOP short 5’UTR which are many oxidative stress and mitochondrial proteins, and non-TOP long 5’UTR which are many pro-proliferative and pro-survival proteins. The long 5’UTR indicates a requirement for the helicase activity of eIF4A for efficient translation. In their study they showed that class of mRNAs were also sensitive to eIF4A inhibition.

1.5 Targeting translation for cancer therapy

The growing body of evidence that protein translation is important in cancer suggests that it could be an effective target for cancer therapy. One of the first avenues identified to pharmacologically block protein translation is through the compound rapamycin. It was identified in 1975 for its antifungal properties as it was able to inhibit the highly conserved, growth regulating TOR kinase in yeast, and repurposed in the 1990s as an immunosuppressant. By 2000, the growing evidence for translational deregulation in cancer prompted the use of rapamycin as a pharmacologic inhibitor of translation in cancer. Rapamycin, however only partially inhibits the mTOR kinase and only in the mTORC1 complex. This results in sustained activation of certain mTOR targets as well as feedback activation of protein kinase B (Akt) and has limited rapamycin’s
clinical impact. Development of dual mTOR/PI3K and TOR kinase inhibitors that target both mTORC1 and mTORC2 show superior preclinical efficacy to rapamycin, however eIF4E levels and alternative activation of MAPK signaling have already been identified as likely routes of resistance to these therapies \(^{66,86}\).

Inhibiting eIF4F directly, rather than through the upstream regulator mTOR, would bypass these resistance mechanisms and is an exciting area of cancer research. Compounds that prevent eIF4E from binding the 5’ cap on mRNA and that block the eIF4E/eIF4G interaction have shown efficacy in preclinical studies and anti-sense oligonucleotides targeting eIF4E are currently in clinical trials, but these strategies face pharmacologic hurdles as the drugging of other protein interactions has proven difficult \(^{47}\). eIF4A is the sole enzymatic component of the eIF4F complex and thus the only one targetable by small molecule inhibition. Additional support for eIF4A as the target for translational inhibition comes from the Gandin paper \(^{83}\). While more mRNAs were dependent on mTOR/eIF4E for translation than eIF4A, the decrease in both mitochondrial structure components and pro-survival proteins following mTOR inhibition actually results in a cytostatic effect, while the loss of just the pro-survival proteins with still active mitochondria following eIF4A inhibition was far more cytotoxic to cancer cells \(^{83}\).

In 2004, a screen for inhibitors of cap-dependent protein translation identified three natural compounds that all target eIF4A, but through distinct mechanisms \(^{87}\). Hippuristanol is a steroid compound that binds the c-terminus of eIF4A and allosterically inhibits RNA binding \(^{88}\). Pateamine A stimulates the
RNA-binding of eIF4A outside of the eIF4F complex, thereby sequestering it from a functional eIF4F, but its covalent binding was shown to be toxic. Silvestrol is the best studied eIF4A inhibitor, and similar to pateamine A it stimulates eIF4A RNA binding outside of the eIF4F complex, but to specific polypurine stretches in the mRNA. Silvestrol has been extensively studied in a variety of preclinical cancer models, revealing issues preventing its clinical development. First, silvestrol’s complex chemical structure is difficult and costly to synthesize and leads to poor druglike properties. Furthermore, silvestrol was identified as a target of the multi-drug export pump ABCB1/P-glycoprotein making drug resistance a likely problem.

eIF4A is actually a family of three highly homologous proteins, eIF4A1, 2 and 3, and also represents the founding member of the DEAD-box family of helicases. These helicases share nine conserved sequence motifs required for nucleotide binding and unwinding, including the ATPase B/Walker II motif containing the Asp-Glu-Ala-Asp (D-E-A-D) amino acid sequence. eIF4A1 and eIF4A2 share 90% amino acid sequence homology and both participate in the eIF4F complex in vitro, while eIF4A3 is only 60% homologous and shows weak interaction with eIF4G and helicase activity. eIF4A3 is instead expressed in the nucleus, where it interacts with protein mago nashi homolog (MAGOH) and Y14 of the exon-junction complex (EJC) and acts as an RNA clamp. Though functionally redundant in vitro, knockdown experiments revealed that eIF4A2 is not able to replace a global loss in translation caused by loss of eIF4A1 in vivo. eIF4A2 was reported to participate in micro-RNA mediated mRNA regulation,
but this point has been contested by other groups\textsuperscript{101,102}. Due to the high homology of these proteins specificity of compounds targeting them is an important question. Silvestrol is able to interact with both eIF4A1 and eIF4A2 in vitro, but mutational validation of eIF4A1 as its target in vivo suggests effects of eIF4A inhibitors on translation are driven through eIF4A\textsuperscript{103}.

The rationale for eIF4A inhibition in NHL as well as other cancers is well-established, but despite identification of eIF4A inhibitors over a decade ago no drug has entered clinical evaluation for this group of diseases. With this clear unmet need, we developed a novel screen for inhibitors of eIF4A1’s ATP hydrolyzing activity, a completely unique approach for this target. This approach for drug discovery is supported by the fact that RNA-aptamers that target eIF4A1 inhibited cap-dependent translation by preventing eIF4A from hydrolyzing ATP, and eIF4A\textsubscript{3}’s action as an RNA clamp in the EJC is due to MAGOH-Y14 preventing it from hydrolyzing ATP\textsuperscript{104,105}. 
Chapter 2
Materials and Methods

Cell-free in vitro studies of eIF4A1

Protein purification and assessment of ATPase activity by malachite green were as previously described (9). Helicase assays were performed as per (23).

Isothermal titration calorimetry (ITC): eIF4a was dialyzed against buffer A (20 mM MES-KOH, pH 6.0, 10 mM potassium acetate, 2.5 mM MgCl2, 1% glycerol, and 1 mM DTT) for 12 h. eIF4a was supplemented with 2% DMSO to match the ligand solution, degassed, and loaded in the cell of a nano-isothermal titration calorimeter (TA Instruments). A total of 12-20 injections of 0.2 mM elatol in buffer A were made every 200 s over a 3000 s time frame. NanoAnalyze software (TA Instruments) was used to integrate the peaks of the isotherm. The peaks were then integrated from injection start to 75 s post injection and fit to an independent binding model. Replicate experiments were done using 25 and 10 µM eIF4A to add power to the stoichiometric given by the nanoAnalyze software.

ATPase Activity assays

Proteins were prepared as follows: 1 µM eIF4A1 in buffer A (20 mM MES-NaOH, pH 6.0, 100 mM potassium acetate, 2.5 mM MgCl2, 1% glycerol, and 1 mM DTT); 500 nM eIF4A1-K82R in buffer A; and 500 nM eIF4A1-K238E in buffer A. ATP was added to generate samples of each protein containing a gradient of ATP (2 mM, 1 mM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 0). The assay was carried out at 37°C, and after 1 hour, a 20 µL aliquot of the reactions was added to 40 µL of malachite green solution (9.3 µM malachite green, 53 mM...
(NH₄)₂MoO₄, 1M HCl, 0.04% Tween 20). After 5 min, the OD₆₆₀ was read on a
GEN5 plate reader (BioTek Synergy 2). This was repeated after 2, 3, and 4
hours. The Michaelis–Menten curves were plotted and the Michaelis-Menten
values were calculated (GraphPad Prism Software).

**Molecular modeling**

Modeling of elatol with eIF4A1 (PDB code: 2ZU6) was performed using Glide
docking program (Schrodinger). Initially, the docking grid was created around the
binding site and 1 was docked using extra precision (XP) glide docking. Resulting
poses were evaluated using docking score and hydrogen bonding interaction
with the active site residues.

**Cell Lines and Reagents**

All cell lines were routinely verified by STR fingerprinting and confirmed
mycoplasma negative using the PlasmoTest Kit (Invivogen: REP-PT1). HBL1,
TMD8, U2932, Riva, Toledo, OZ, SU-DHL-4, WSU-DLCL-2, MD901, MDA-MB-
468 and SNU-398 cell lines were grown in RPMI culture media (Corning)
supplemented with 10% fetal bovine serum (FBS, VWR) and
penicillin/streptomycin (P/S, VWR). DB, Farage, SU-DHL-10, SU-DHL-6, Karpas-
422 were grown in RPMI with 20% FBS and P/S. OCI-Ly-2, OCI-Ly3, OCI-Ly-10
and OCI-Ly19 were grown in Iscoves modified DMEM in 20% FBS and P/S, I20
media. The FL5.12 cells were a gift from the Wendel Lab, MSKCC, NY and
grown in RPMI with 10% FBS, ± WeHi-3B supernatant and murine IL-3 (400pM,
eBioscience). MDA-MB-468, HEK-293t and Phoenix packaging cells were grown
in DMEM 10% FBS and P/S. The mouse embryonic fibroblasts eIF2α Serine
A/A and corresponding wildtype were a kind gift of Dr. Randal Kaufman at Sanford Burnham Prebys Medical Discovery Institute. The MEFs AT4 knockout and wildtype were a kind gift of Dr. Peter Johnson at the National Cancer Institute. AT4 wildtype and knockout MEFs were cultured in D10 additionally supplemented with non-essential amino acids (NEAA, ThermoFisher) and 50 μM β-mercaptoethanol. eIF2α wildtype, and Ser51 A/A mutant MEFs were cultured in D10 media supplemented with NEAA. Cell lines were grown at 37 degrees Celsius in 5% CO2.

PIM447 and AEB071 were provided under material-transfer agreement (MTA) with Novartis Oncology. Ibrutinib was provided under MTA with Pharmacyclics. AZD1208 was purchased from Selleck Chemicals. Silvestrol was purchased from Medchem express (HY-12351). The PERK inhibitor GSK2606414 was purchased from Millipore/Calbiochem (516535). Tunicamycin was purchased from Sigma (T7765). Carboplatin was acquired from the University of Miami Sylvester Comprehensive Cancer Center pharmacy. The retroviral shRNA knockdown vectors were a kind gift from Jerry Pelletier.

**Proliferation Assay**

Cells were plated at 1 x 10^5 cells/mL on day 0 and treated with vehicle (DMSO) or indicated concentration of inhibitor and live cells counted every day by trypan blue exclusion. Assessment of elatol’s effects on cell growth for determination of the inhibitory concentration 50 (IC50) in the Harvard/Wellcome cell line collection was carried out as previously described 106.
**FL5.12 PIM2-driven line**

The Phoenix packaging cell line was seeded at 230,000 cells/mL for 16 hours. Then transfected with human PIM2 cDNA cloned into the multiple cloning site of the p-MIG vector using X-treme GENE 9 DNA transfection reagent (Roche). The transfection cocktail consisted of 100μL DMEM media, 1μg DNA and 3μL of X-treme GENE 9. Media on Phoenix cells was changed after 24 hours and at 48 hours collected and filter sterilized through a 0.45μm filter. 100,000 FL5.12 cells were resuspended in 600 μL of viral supernatant and 120 μL of 5X infection solution was added (4mL FL5.12 media, 1mL WeHi supernatant, Polybreen and murine IL-3). The infection was repeated two more times, at least 6 hours apart to achieve maximum viral titer, by adding sterilized viral supernatant and 5X infection solution to the existing plate of cells. Six hours after the final infection cells were plated in fresh FL5.12 media and allowed to recover for 24 hours and initial infection was assessed by flow cytometry using the Guava EasyCyte flow cytomter to check GFP levels. 500,000 cells were withdrawn from cytokine by washing 4 times and plating in cytokine-free media for 24 hours, or until majority of cells have shrunk in size and appear unhealthy, then put back in regular FL5.12 media until recovered healthy appearance then GFP was assessed by flow cytometry. This process was repeated until cells reached >95% GFP then they were gradually split into cytokine-free media until able to grow to confluence. Flow cytometry was used again to ensure cells were now 100% GFP positive.
**PIM1 competition assay ± PIM447**

Site-directed mutagenesis was used to create the PIM1 mutants from the short form of human PIM1 cDNA cloned in to the multiple cloning site of the pMIG vector according to the QuikChange II Site-Directed mutagenesis Kit manufacturers protocol (Agilent Technologies). The primers used are listed in Table 2.1. Mutants were confirmed by sanger sequencing and FL5.12 cells were infected with each mutant individually as described above in FL5.12 PIM2 driven line. After recovery from three infections initial GFP was assessed by flow cytometry and then mixed with uninfected FL5.12 cells to create a stable mix of approximately 20% GFP positive cells. 500,000 cells were then washed out of IL-3 and simultaneously treated with either 300nM PIM447 or vehicle. After 24

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Table 2.1 PIM1 site directed mutagenesis primers. Forward and reverse primers used to create the PIM1 mutants.
hours, cells were put back in normal FL5.12 media with either PIM447 or vehicle. Cells were allowed to recover and then GFP was assessed by flow cytometry.

**Western Blotting**

Cells were treated as indicated and protein isolated using RIPA lysis buffer (50 mmol/L Tris-HCL pH 8.0, 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS), phosphatase inhibitor (Roche), Phosphatase Halt (Thermo), and Triton X-100. Proteins were quantified using the BCA Assay (Thermo) with 30 mg loaded per lane onto 10% SDS-PAGE gel and resolved by electrophoresis, then transferred to PVDF membrane. Protein was detected using autoradiography film (GeneMate).

Cell Signaling Technologies 1:1000: Ribosomal S6 protein, phospho-Ribosomal S6 protein Ser^{240/244}, 4EBP1, phospho-4EBP-1 Ser^{65}, AKT, phospho-AKT Ser^{473}, IκBα, phospho-IκBα Ser^{32}, CyclinD3, Myc, Tsc2, eIF4B, phospho-eIF4B Ser^{422}, eIF4E, phospho-eIF4E Ser^{209} Cyclin D3, MYC, PIM2, MCL1, BCL2, Survivn, S6, phospho-S6 Ser^{240}, 4EBP1, phospho-4EBP1 Ser^{65}, eIF2α, ATF4, PKR, PERK, α-Tubulin, GAPDH, XBP1. Santa-Cruz Biotechnologies 1:200: pTsc2 Ser^{1798}, PIM1, eIF4A2. Sigma 1:2000: α-Tubulin, HRP conjugated anti-rabbit and anti-mouse secondaries. Antibodies for eIF4A1 (31217) and phospho-eIF2α Ser^{51} were purchased from Abcam. PPIB antibody was purchased from Thermo.

**siRNA knockdown**

Control non-targeting siRNA, ATF4 and eIF4A ON-TARGET siRNA pools where purchased from Dharmacon. 5 x 10^{5} cells were plated in D10 media without antibiotics. The following day cells were transfected with 50nM siRNA using
Lipofectamine 3000 (Thermo) following manufacturers protocol. Transfection media was replaced with D10 media after 24 hours and cells were collected for analysis at 48 hours. For ATF4 knockdowns cells were treated with DMSO, elatol or tunicamycin at 48 hours and collected for analysis 56 hours following transfection with siRNA.

**RT-PCR**

Total RNA was isolated from cell lines using the RNeasy Mini Kit (Qiagen). cDNA was generated using the Taqman Reverse Transcriptase Kit (Roche). qRT-PCR was performed using an Applied Biosystems Prism 7000 Sequence Detection system with Taqman probes according to manufacturer specifications. Taqman probes were purchased from Thermofisher: 18s rRNA 4319413E-0810041, Gapdh Hs02758991_g1, eif4a1 Hs00426773, eif4a2 Hs00756996_g1, Ccnd3 Hs00236949_m1, Mcl1 Hs01050896_m1, Myc Hs00153408_m1, Hs0165498_m1 (PIM1), Hs00179139_m1 (PIM2), Hs00420511_m1 (PIM3), and for normalized using the \(2^{-\Delta\Delta CT}\) method.

**Viability Assays**

Cells were plated at 3-5 x 10^3 cells/well in serial dilutions of drug ranging two logs with the top concentration for silvestrol 1 μM and the top concentration for elatol 10 μM. Viability was measured after 72 hours using Cell Titer Glo (Promega) following manufacturer’s protocol. Luminescence was detected on the BioTek HT Synergy plate reader and lethal dose 50 (LD\(_{50}\)) values calculated using nonlinear regression fit in Graphpad Prism7.
**Apoptosis Assays and Annexin Staining**

1-3 x 10^5 cells were plated with indicated drug treatments and washed once with ice cold PBS at indicated time point and stained with PE conjugated Annexin V and 7-aminoactinomycin D (BD Biosciences) according to manufactures protocol. Stained cells were analyzed by flow cytometry on the Attune NxT (Thermo). Data was analyzed using FlowJo v9.9.6 (FlowJo).

**Dual-Luciferase Reporter Assay**

The Dual-luciferase plasmids were a kind gift from Jerry Pelletier at McGill University. 7 x 10^5 HEK-293 cells were plated and transfected with 400 ng of plasmid using Lipofectamine 3000 (Thermo). 36 hours after transfection cells were treated as indicated before they were lysed using the dual luciferase reporter assay kit (Promega) and 100 μL added in triplicate to a white bottom 96 well plate for detection of luciferase following the manufacturers protocol. Luciferase units were normalized to the DMSO treated control.

**O-propargyl puromycin assay**

100,000 cells were seeded and treated with indicated inhibitor or vehicle. In the final 30 minutes of incubation cells were pulsed with 25μM o-propargyl puromycin (OPP) (Jena Bioscience). Cells were then collected, washed once with PBS, fixed in 4% paraformaldehyde in PBS at 4 degrees Celsius for 15 minutes, and permeabilized with 3% BSA .1% Saponin in PBS at room temperature for 15 minutes. CuAAC detection of OPP labeled proteins was then performed using the Click-iT Cell Reaction Buffer Kit according to manufacturer specifications with the Alexa Fluor 488 Azide (Thermo Fisher). After labeling GFP was assessed by
flow cytometry on the Attune Nxt (Thermo) and analyzed using FlowJo v9.9.6 (FlowJo). As a control, a proportion of cells were treated with cycloheximide for 5 minutes before OPP addition and fluorescence of cycloheximide-treated cells was subtracted from all experimental values.

**Polysome Profiling**

20 x 10⁶ cells were treated as indicated and washed with ice-cold PBS with 100 g/mL cycloheximide for 10 minutes prior to lysis. Cells were pelleted at 200xg at 4°C for 10 minutes and lysed in 500 μL of lysis buffer (.3M NaCl, 15 mM MgCl₂, 15 mM Tris-HCl pH 7.4, 1% Triton X-100, 100 g/mL cycloheximide, 100 U/mL RNasin). Lysates were cleared and equal A₂₆₀ units (measured using NanoDrop 2000, Thermo) were loaded onto 10-50% sucrose gradients and centrifuged at 260,343 x g rpm in a SW-41 Ti rotor for 1.5 hours at 4°C. Samples fractionated and 254 nm absorbance recorded using the gradient fractionation system (Brandel).

**Retroviral Complementation Experiments**

Generation of the control and eIF4A stable knockdowns in NIH-3T3 cells was performed as described ¹⁰³.

**In Vivo experiments**

All in vivo experiments were performed following protocols approved by the relevant Institutional Animal Care and Use Committee. The initial non-tumor bearing maximum tolerated dose studies and SU-DHL-6 xenografts were done with female 8-10 week old SCID mice from the University of Arizona Cancer Center and OCI-Ly3 xenografts done with female 8-10 week old SCID mice.
purchased from Charles River Laboratories. Complete blood counts were collected from non-tumor bearing mice dosed as indicated and evaluated on a Hemavet950. For SU-DHL-6 xenografts 2x10^6 cells were washed with ice cold PBS and mixed 1:1 with matrigel (Corning 354234) and injected s.c. into the right flank of SCID mice. Once the tumor reached 60mm^3 mice were pair-matched and divided into two treatment groups: vehicle (sterile water with 5.2% tween-80, 5.2% PEG-400) or 20mg/kg elatol i.p. daily for 5 days. Mice were weighed and tumor volume calculated twice weekly. The maximum tolerated dose study of a single i.p. injection of elatol up to 100 mg/kg was done on cohorts of 5 CD1 mice. H&E pathology slides were prepared using standard techniques and analyzed at the University of Miami. For the OCI-Ly3 xenograft study 10 x 10^6 cells were first washed with ice cold PBS and mixed 1:1 with matrigel and implanted s.c. on the flank of SCID mice. Once tumors reached 500mm^3 tumors were dissected and dissociated using the gentleMACs Dissociator (Miltenyi Biotec) and 1 x 10^6 serially transplanted tumor cells were implanted s.c. into the flank of SCID mice. Once tumors reached 50mm^3 mice were pair matched and split into two treatment groups: vehicle or 40mg/kg elatol twice weekly. Mouse weight and tumor volume were monitored twice weekly.

**Statistics**

All numerical data are based off of biological replicates represented as mean ± SEM. For annexin staining and OPP incorporation unpaired, two-tailed t-tests were performed, with the addition of the two-stage linear setup procedure of Benjamini, Krieger and Yekutieli to compare treatments over time, with p<0.05 as
significant. The OCI-Ly3 xenograft experiment was compared using a two-way ANOVA with p<0.05 significant. All statistical comparisons were performed using GraphPad Prism7. The validity of the malachite green ATPase assay for screening was verified by determining the Z-score with EDTA and DMSO as the positive and negative controls respectively.
Chapter 3

Control of Translational Activation by PIM Kinase in Activated B-Cell Diffuse Large B-Cell Lymphoma Confers Sensitivity to Inhibition by PIM447

3.1 Summary

While the efficacy of PIM kinase inhibition in hematologic malignancies such as myeloid and lymphoid leukemias has been demonstrated, it has not been well studied in DLBCL, despite evidence for PIM1 and PIM2 mutation or overexpression. Novartis developed the compound PIM447, that has high specificity for all three PIM kinases which we used to assess the sensitivity of our NHL cell lines to PIM inhibition \(^{107}\). We found the ABC subtype of DLBCL to be the most sensitive group overall, but high PIM1/2 expression alone was not a marker for sensitivity. When comparing sensitivity of our ABC cells to various B-cell receptor (BCR) signaling inhibitors, we noticed several cell lines that were sensitive only to PIM inhibition and resistant to upstream inhibitors, and several cell lines sensitive to upstream inhibitors but resistant to PIM447. The PIM sensitive cell lines all harbored mutations in TNFα induced protein 3 (TNFAIP3, encoding the protein A20) and caspase recruitment domain family member 11 (CARD11), resulting in downstream activation of the NF-κB pathway and a reliance on PIM to activate cap-dependent protein translation through mTOR. Indeed, in sensitive cell lines only, treatment with PIM resulted in decreased activation of mTOR downstream targets and decreased global translation levels. Interestingly, direct inhibition of translation using the compound silvestrol was
toxic to all ABC DLBCL cell lines, regardless of PIM447 sensitivity. We also
performed a meta-analysis of somatic mutations in PIM1 in DLBCL and
confirmed the pattern to be indicative of aberrant SHM. We functionally validated
the 15 most frequently identified mutants and found all but one retained kinase
activity, but conferred no additional survival upon PIM447 treatment.

3.2 Pan-PIM Inhibition with PIM447 in non-myeloma lymphoid tumor cell
lines

We screened all of our NHL cell lines for sensitivity to PIM447 in a
metabolic viability assay. We noticed a range of sensitivity to PIM inhibition,
which was expected as similar results were obtained using LGB321, the tool
compound from which PIM447 was derived (Figure 3.1A)\textsuperscript{108}. The majority of the
sensitive cell lines were DLBCL, and of the DLBCL the ABC subtype was
significantly more sensitive with 57% (4/7) sensitive below 4uM, while only 29%
(4/14) of GCB were sensitive (p=0.0152) (Figure 3.1B). To confirm these results,
we compared the ABC cell line sensitivities to the other pan-PIM kinase inhibitor
currently in clinical evaluation, AZD1208. We found similar sensitivity with the
exception of the RIVA cell line, which was resistant to AZD1208 (Figure 3.1C).
As PIM1 and PIM2 expression was reported in the gene signature that
differentiates ABC from GCB DLBCL we measured the mRNA levels of all three
PIM kinases in our DLBCL cell lines. We found PIM1 and PIM2 were significantly
higher in the ABC cell lines (p=0.0016, p=0.0025), while PIM3 was similar
between the groups, confirming the gene expression classification data
Despite almost every ABC cell line expressing higher levels of PIM1/2, there were still some cell lines resistant to PIM inhibition by either inhibitor, indicating that PIM expression alone was not a sufficient marker for sensitivity to PIM kinase inhibitors.

Figure 3.1 Pan-PIM inhibition in NHL with PIM447. A) NHL cell line sensitivity to PIM447 was tested in a 72h viability assay. LD_{50} was calculated using nonlinear regression fit analysis in Graphpad Prism7. Mean ± SEM n=4. B) LD_{50} of ABC and GCB subtype of DLBCL to PIM447. Significance calculated using a two-tailed t-test in Graphpad Prism7. C) Comparing ABC cell line sensitivity to PIM447 or AZD1208.
3.3 Comparing PIM447 and upstream signaling inhibition in ABC DLBCL cell lines

To better understand what was driving sensitivity to PIM447 in the ABC DLBCL cell lines we compared PIM treatment to other well-known BCR targeted agents. BCR activation has far-reaching effects, activating MAPK, PI3K and NF-κB signaling pathways. Ibrutinib inhibits bruton tyrosine kinase (BTK), which is activated by spleen tyrosine kinase (SYK) directly downstream of BCR activation, while AEB071 inhibits protein kinase C (PKC), which is activated several steps down from BTK. PKC promotes activation of the NF-κB pathway leading to

![Figure 3.2 PIM kinase expression in DLBCL cell lines.](image). Relative mRNA expression of PIM1, PIM2 and PIM3 in ABC-derived vs. other DLBCL cell lines, measured by qRT-PCR and compared with two-tailed T test. Relative mRNA levels calculated using the ΔΔCT method normalized to GAPDH. Mean ± SEM n=3.

PIM expression and activity, therefore ibrutinib and AEB071 inhibit BCR signaling upstream of PIM. We noticed that two of our PIM447 sensitive cell lines, OCI-Ly3 and U2932, were completely resistant to the upstream inhibitors, while the other two sensitive cell lines, OCI-Ly10 and RIVA, were sensitive to both PIM447 and the upstream inhibitors, and so we termed these our PIM sensitive cell lines (Figure 3.3A). The TMD8 and HBL1 lines were resistant to PIM447 but sensitive
to both upstream inhibitors and SU-DHL2 was resistant to all drugs, and these we called our PIM resistant lines (Figure 3.3A).

Numerous studies have characterized the somatic mutations in these cell lines, and so we used that information to try to better understand the different sensitivities we observed to BCR signaling inhibitors. We noted that all of the PIM447 sensitive cell lines harbored mutations in either CARD11 or TNFAIP3, the gene encoding the protein A20 (Figure 3.3B)\textsuperscript{112-114}. CARD11 is the structural component of the CBM complex, which also contains mucosa-associated

![Graph showing differential sensitivity of ABC DLBCL cells to BCR signaling inhibitors.](image)

**Figure 3.3 Differential sensitivity of ABC DLBCL cells to BCR signaling inhibitors.** A) Cell viability of ABC DLBCL cells treated for 72h with increasing concentrations of inhibitors up to 10 μM (PIM447 and AEB071) or 1 μM for ibrutinib. Best fit line shown using nonlinear regression fit analysis in Graphpad Prism7. Mean ± SEM n=4. B) Summary of sensitivity of ABC DLBCL to signaling inhibitors and known BCR activating mutations.
lymphoid tissue lymphoma 1 (MALT1) and BCL10, and upon formation triggers phosphorylation of IκBα and subsequent release of NF-κB to activate transcription of target genes. Meanwhile, A20 is an inhibitor of the NF-κB complex’s signaling activity, and so either of these mutations result in direct activation of NF-κB and PIM expression. Conversely, the PIM447 resistant cell lines lack these downstream mutations and instead have activating mutations in cluster of differentiation 79B (CD79B), which associates with the BCR at the plasma membrane to transduce signaling, and myeloid differentiation primary response 88 (MYD88), a component of toll-like receptor signaling that results in NF-κB activation independent of the CBM complex (Figure 3.3B). These upstream mutations allow for broad activation of NF-κB as well as MAPK and PI3K signaling and a decreased dependence on PIM activity for proliferation and survival, and thus resistance to PIM inhibitors. The sensitive cell lines OCI-Ly3 and OCI-Ly10 and the dual-resistant SU-DHL2 have both upstream and downstream activating mutations, and so additional factors must be influencing their sensitivities.

3.4 Loss of mTORC1 activation in PIM-447 sensitive ABC DLBCL cell lines

PIM activity promotes survival and proliferation through multiple targets, but activation of eIF4F has been reported as an important output by many groups. PIM promotes eIF4F activation by phosphorylating the negative regulator of mTORC1, tuberous sclerosis complex 2 (TSC2), and we found that following
PIM447 treatment TSC2 phosphorylation was decreased in the sensitive cell lines, while the phosphorylation status of TSC2 in the resistant cell lines was unchanged (Figure 3.4A). This resulted in a much stronger decrease in

![Western blot analysis of protein expression in ABC DLBCL cells following a 16h treatment with 5μM PIM447. Representative images, n=3. (B) Western blot analysis of protein expression in ABC DLBCL cells following a 16h treatment with 1μM Ibrutinib. (C) ABC DLBCL cell lines were treated with 4μM PIM447 and PIM kinase mRNA and protein expression was analyzed at the indicated time points. Relative mRNA expression measured by qRT-PCR and compared with two-tailed T test. Relative mRNA levels calculated using the ΔΔCT method normalized to GAPDH. Mean ± SEM n=3.]

Figure 3.4 PIM447 treatment results in mTOR deactivation and PIM stabilization in sensitive cell lines.
phosphorylation of mTORC1 targets 4EBP1 and ribosomal protein S6 in the sensitive cell lines compared to the resistant cell lines. PIM also phosphorylates the translation initiation factor eIF4B to activate translation and that was strongly downregulated by PIM447 only in the sensitive cell lines, while translation initiation factor eIF4E phosphorylation, a MAPK target, was unaffected by PIM447 in all cell lines. The combined deregulation of translation resulted in a decrease in expression of the oncogene MYC in PIM sensitive cell lines only. PIM and Akt share many targets, and interestingly we observed a decrease in phospho-Akt at serine 473 following PIM447 treatment in some cell lines regardless of sensitivity to the drug. Expression of all three PIM kinases was also increased in all cell lines in response to PIM447 treatment in all cell lines indicating that despite resistance to PIM inhibition, the compound was having an on-target effect.

We then compared the effects of ibrutinib treatment, to which only the OCI-Ly3 cell line is resistant (Figure 3.4B). Ibrutinib treatment resulted in absolutely no change in signaling in OCI-Ly3 cells, while sensitive cells showed a decline in phosphorylation of S6 and eIF4B, while phosphorylation of 4EBP1 was unaffected. This resulted in a reduction of MYC expression. IκBα, a signaling mediator downstream of BTK but upstream of PIM, was potently dephosphorylated following ibrutinib treatment, and led to a decline in expression of all PIM kinases. Akt phosphorylation was also decreased due to ibrutinib treatment, as BTK is upstream and feeds into PI3K signaling. Together these results highlight the strong input of activated BCR signaling into mTORC1 and
protein translation in ABC DLBCL, and the role of PIM447 and ibrutinib treatment in quenching those signals. The observation of increased PIM kinase expression following treatment with PIM inhibitors (Figure 3.4A) has been observed by others and is linked to an autophosphorylation degradation pathway. We monitored PIM protein and mRNA expression over a time course of PIM447 treatment and observed that PIM protein levels increased independently of increased mRNA in accordance with an increase in protein stability rather than transcriptional activation (Figure 3.4C).

To interrogate the effect of PIM447 treatment in a system driven by PIM activity, we used the FL5.12 murine pro-B-cell line with constitutive BCL2 expression, whose survival is dependent on the cytokine interleukin 3 (IL-3). We retrovirally transduced these cells with human PIM2 cDNA and withdrew them from IL-3 to create a novel pro-B-cell line whose proliferation is completely driven by PIM2 (Figure 3.5A). The FL5.12/BCL2/PIM2 cells were significantly more sensitive to PIM447 than the FL5.12/BCL2 parent cells (LD$_{50}$ = 37 and 249 nM respectively) (Figure 3.5B). In the FL5.12/BCL2 cells grown in cytokine PIM2 is expressed and Akt is strongly phosphorylated resulting in activation of mTORC1 and phospho-S6 and 4EBP1 (Figure 3.5C). PIM447 treatment led to a stabilization of PIM2 and partial decrease in phospho-4EBP1 but not S6 or Akt, due to the maintained upstream activation of signaling by IL-3. In the FL5.12/BCL2/PIM2 cells exogenous PIM2 was detected by the D1D2 antibody which only reacts with human, while the 1D12 antibody reacts with both human
and mouse. Akt was not as strongly phosphorylated due to the lack of IL-3, and was unaffected by PIM447 treatment. mTORC1 targets S6 and 4EBP1, however, were both potently dephosphorylated by PIM447 treatment, indicating PIM2 activity is driving translational activation in these cells.

Figure 3.5 PIM447 shuts off translational activation by PIM2 in independent system A) Cell proliferation of FL5.12/BCL2 cells in IL-3 and PIM2-driven FL5.12/BCL2 cells. 50,000 cells were plated in media with or without IL-3 as indicated and counted every day for 5 days with trypan blue exclusion. Mean ± SEM n=3. B) Cell viability of untransformed FL5.12/BCL2 cells in normal media and the transformed FL5.12/BCL2 PIM2 cells in media without cytokine treated with increasing concentrations of PIM447 was assessed after 72h. Best fit line shown using nonlinear regression fit analysis in Graphpad Prism7. Mean ± SEM n=4. C) Western blot analysis of protein expression in FL5.12 parental and PIM2 driven cells treated as indicated for 16h. Representative images n=3.
3.5 **PIM447 treatment leads to a reduction in cap-dependent protein translation in sensitive cell lines**

PIM inhibition by PIM447 clearly deregulates activation of translation in sensitive ABC DLBCL cell lines, but as PIM has many cellular targets we wanted to determine whether shutting off translation was the primary effect driving loss of viability. Silvestrol is a natural compound that inhibits the RNA helicase eIF4A, the enzymatic component of the eIF4F complex, and therefore directly inhibits translation.

**Figure 3.6 PIM447 treatment inhibits global protein translation in sensitive ABC DLBCL cells.** A) Cell viability of ABC DLBCL cells treated for 48h with increasing concentrations of inhibitors up to 10 μM PIM447 (blue line) or 1 μM for silvestrol (green line), or the combination (red line). Mean ± SEM n=4. B) OPP levels following 24h drug treatment measured by flow cytometry. Representative images n=3. C) Western blot analysis of translationally regulated protein expression after 16h treatment. Representative images n=3.
translation initiation downstream of PIM. When we combined PIM447 treatment with silvestrol in our ABC DLBCL cell lines we saw no additive effect of decreased viability (Figure 3.6A). Interestingly, all of the ABC DLBCL cell lines, including the dual-resistant line SU-DHL2, were highly sensitive to silvestrol, with LD_{50}s below 20nM. This highlights the activation of translation as a critical node for DLBCL survival. Using flow cytometry to measure o-propargyl puromycin (OPP) incorporation in ABC cell lines, we found that in the sensitive cell lines PIM447 treatment reduced global translation similar to silvestrol treatment (Figure 3.6B). Additionally, PIM447 reduced expression of known translationally regulated oncoproteins Cyclin D3 and MYC similar to silvestrol in the sensitive cells (Figure 3.6C). Therefore, the primary effect of PIM447 treatment in ABC DLBCL is loss of protein translation.

3.6 Recurrent somatic mutations in PIM1 are missense mutations that preserve kinase function

Our data along with the evidence for overexpression of PIM in DLBCL suggest an important role for PIM activity in ABC DLBCL specifically. *PIM1* has been reported to be one of the most frequently mutated genes in DLBCL, but the role of these mutations on PIM1 function has not been addressed. We performed a meta-analysis of somatic mutations in *PIM1* in DLBCL and found 92 reported PIM1 amino acid altering mutations, 86 of which were missense mutations (Figure 3.7A and Appendix 1)\textsuperscript{12,116-121}. The infrequency of deleterious frameshift
or nonsense mutations suggests a selection for mutations that at least preserve the function of PIM1.

To determine if these mutations could be driving PIM1 activity in DLBCL, we chose to further analyze those mutations found in at least two independent samples, of which there were 15 frequently mutated amino acid residues with 53 total mutations between them. As the majority of PIM1 mutations were clustered towards the 5’ end of the gene, consistent with the mutational pattern of aberrant AID activity, we analyzed the sequences for the AID hotspot motif WYRC, where W= adenine or thymine, R= purine, C= cytosine and Y= pyrimidine, and found that all but the E135 substitutions were in consensus AID hotspots (Figure 3.7B and Table 3.1). At each frequently mutated amino acid, either the most frequent substitution or the one predicted to be most damaging to PIM1 structure by Polyphen2 was retrovirally introduced into FL5.12 cells. As expected, wildtype

Figure 3.7 Somatic mutations in PIM1 in DLBCL. A) Representation of the type of 92 total amino acid altering mutations identified in DLBCL patient samples. Missense mutations are in blue, nonsense are in purple and frameshift mutations are in red. B) Schematic showing the alignment of recurrent missense mutations in PIM1. Nonsense mutations are shown as pink squares while missense mutations and in frame deletions are orange triangles placed in the appropriate exons of the RNA sequence. The two protein isoforms are shown with the kinase domain is dark blue, nucleotide interacting residues are light blue, and the unannotated domains in green.
PIM1 enriched strongly following IL-3 withdrawal and the kinase dead mutant did not (Figure 3.8A). When we treated cells with PIM447 concurrently with cytokine withdrawal wildtype PIM1 did not enrich, indicating the growth advantage conferred by PIM1 is dependent on its kinase activity. We assessed each of the PIM1 mutants in our FL5.12 assay and found all but the L182/184F mutants were able to enrich following cytokine withdrawal, but none could overcome PIM447.

Figure 3.8 Recurrent PIM1 mutations preserve kinase function, but don't overcome PIM447. A) PIM1 enrichment in FL5.12 cells upon cytokine withdrawal is dependent on kinase function. GFP expression in FL5.12 cells infected with empty vector, wildtype PIM1 or a kinase dead mutant following withdrawal from IL-3 and treated with vehicle or PIM447 measured by flow cytometry. B) FL5.12 cells treated as in A, but infected with PIM1 mutants from meta analysis. Mean + SEM n=3.
treatment (Figure 3.8B and Table 3.1). Therefore, in DLBCL mutations that preserve PIM1 function are selected for, and mutational status of PIM1 should not impact efficacy of pan-PIM inhibition with PIM447. These studies confirm the frequent aberrant SHM of \textit{PIM1} in DLBCL, and uncover the context dependence on PIM for activation of translation and survival in ABC DLBCL summarized in Figure 3.9.

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Table 3.1 Functional analysis of recurrent PIM1 mutations in DLBCL. Amino acid altering mutations found at least two times in separated clinical samples, with their amino acid substitutions and the number of times that change was found in parenthesis. The results of enrichment assays in FL.512 cells after cytokine withdrawal and treatment with PIM447, and whether that mutation is in at an AID hotspot motif are also shown.
Figure 3.9 BCR mutational activation as a biomarker for targeted therapy. Mutational activation of BCR signaling dictates sensitivity to various targeted signaling inhibitors under clinical evaluation. DLBCL mutated upstream, either CD79A/B or MYD88, can be targeted with upstream inhibitors targeting BTK or PCK (ibrutinib or AEB071) and have multiple routes for activation of translation. DLBCL mutated downstream, CARD11 or A20, miss the activation of translation through PI3K and are dependent on PIM kinase for activation of mTOR and thus sensitive to PIM447.
Chapter 4
Targeted Screen Identifies Elatol as a Novel eIF4A Inhibitor with Potent Antitumor Activity

4.1 Summary

Inhibition of translation through the helicase eIF4A is a promising area for cancer therapy, and while several eIF4A inhibitors have been well-studied pre-clinically, they do not show promise for reaching clinical evaluation. With this in mind, we designed a screen for inhibitors of eIF4A’s ATPase activity, which drives consecutive rounds of unwinding, a completely unique mechanism for this target. From our screen, we identified elatol as a specific inhibitor of eIF4A1’s ATP hydrolysis, with potency against a broad range of cancer cell lines. In NHL cells, elatol induced apoptosis at low micromolar doses, and inhibited both global and cap-dependent protein translation. Curiously, elatol treatment induced a relatively immediate upregulation of the stress response factor ATF4 that was independent of ER stress and not essential for elatol’s antitumor activity, while eIF4A1 was, confirming elatol’s activity against its target in cells. Finally, elatol was tolerated at high doses in vivo and significantly delayed DLBCL tumor progression. This work establishes a pipeline for further development of eIF4A inhibitors for cancer therapy.

4.2 Elatol identified in a novel screen for inhibitors of eIF4A ATPase activity

To screen directly for inhibitors of eIF4A1 ATP hydrolysis our collaborators employed the ingenious malachite green assay, which uses colorimetric detection of inorganic phosphate (P_i) without requiring radiation or deconvolution.
of the signal, making it amenable to high-throughput development \(^{122}\) (Figure 4.1A). From a small library of natural products, several compounds were able to inhibit eIF4A, but only the compound elatol was active against four initial NHL cell lines tested (Figure 4.1A-B). Elatol showed no activity when counter-screened in the same malachite green assay against other unrelated ATP hydrolyzing enzymes, bacterial chaperonin GroEL, and human chaperone heat shock protein 70 (HSP70) (Figure 4.1C). We then tested another walker A/B motif containing

![Diagram](image1)

**Figure 4.1 Elatol is a specific inhibitor of eIF4A1 ATPase activity.** A) Schematic of malachite green screen for inhibitors of eIF4A1’s ATPase activity and the chemical structure of the top hit from the screen, elatol. B) LD\(_{50}\) of DLBCL cell lines treated with the top 4 eIF4A1 ATP hydrolysis inhibitors based on the malachite green screen. Mean± SEM, n=4. C) Sensitivity of indicated ATP-hydrolyzing enzymes to elatol determined by malachite green based ATP hydrolysis assay. Mean± SEM, n=3 D) Results of elatol’s activity in a commercial kinase screen.
protein, p97, and three other DEAD-box family helicases: DDX3, DDX17 and DDX39A, which elatol also showed no activity against (Figure 4.1C). As ATP hydrolyzing kinases would be a concerning off-target in cancer, we then submitted elatol to a commercial kinase screen containing 97 common kinases including Erb-B2 receptor tyrosine kinase (HER2), janus kinase 2 (JAK2), MAPK5 and mTOR, and found no significant inhibition of any of the kinases in the screen (Discovery Rx) (Figure 4.1D). These results rationalized elatol’s specificity as an eIF4A inhibitor and further preclinical evaluation.

4.3 Elatol is potent against a broad range of cancer cell lines

Inhibition of translation through eIF4A may be favorable in DLBCL, as our studies with PIM447 suggest (Chapter 3), but also in many other cancers where oncogenic signaling drives translational activation. We used Harvard and the Wellcome Trust’s collection of over 900 cancer cell lines to comprehensively evaluate elatol’s potential in cancer therapy. We found that 37% (344/924) of the cell lines tested were sensitive to elatol below 1µM, measured by growth inhibition (Figure 4.2). Though there were both sensitive and resistant cell lines from every tumor type tested, leukemia and lymphoma were the most sensitive groups overall, followed by breast and lung, confirming previous studies of eIF4A or eIF4F inhibition in these cancers. With these results, as well as our previous studies identifying ABC DLBCL sensitive to silvestrol, we assessed proliferation and apoptosis in one GCB (SU-DHL6) and two ABC (OCI-Ly3 and RIVA) cell lines. A single treatment with elatol completely halted proliferation in
all three cell lines, and induced apoptosis in a dose and time dependent manner, with greater than 50% apoptosis in all cell lines by day 3 (Figure 4.3A-B).

4.4 Elatol interacts with two key lysines of eIF4A1 with a 2:1 stoichiometry

Competitive inhibition of ATP binding is a common pharmacologic mechanism for enzymes with well-defined substrate pockets, eIF4A, however, lacks such a pocket and ATP binding is instead promoted by cooperative binding with RNA leading to a closure of the amino (N) and carboxy (C) RecA-like
domains. We, therefore, wanted to better interrogate the interaction of elatol with eIF4A. Isothermal titration calorimetry not only confirmed binding of elatol to eIF4A ($K_D = 1.98 \pm 0.31$), but revealed a 2:1 stoichiometry with two molecules of elatol per one of eIF4A (Figure 4.4A). A computer generated molecular model of elatol with eIF4A also confirmed this stoichiometry and suggested elatol
binding in the inter-domain cleft, where each elatol molecule interacts with a lysine from either the N or C domains (Figure 4.4B). We then performed mutational analysis to confirm these interactions. Lysine 82 (K82) of the N terminal domain is part of the Walker A ATPase motif that functions in nucleotide binding and is conserved in all DEAD-box helicases. Mutation of this residue to glutamate (E) resulted in a catalytically inactive protein, however the

![Figure 4.4 Elatol interacts with two key lysines in eIF4A1 with 2:1 stoichiometry. A) Isothermal titration calorimetry of eIF4A1 and elatol. Data fit to an independent binding model using NanoAnalyze software from TA instruments. Mean ± SEM n=3 B) Putative elatol binding with eIF4A1 based on molecular modeling experiments shows two elatol molecules (blue and pink) interacting with key lysines (yellow) in the RNA binding groove between the two helicase domains of eIF4A (gray and purple). C) Malachite green assay for ATP hydrolysis showing IC50 for elatol treatment against wildtype eIF4A1 or proposed lysine mutants in the proposed binding sites. Mean ± SEM n=3]
conservative mutation to arginine (R, K82R) was functional and was significantly less sensitive to elatol compared to wildtype (Figure 4.4C). Lysine 238 (K238) is part of the linker region and the non-conservative K238E mutant was functional and resistant to elatol treatment, although less so than K82R (Figure 4.4C). These results confirm elatol's interaction with eIF4A and highlight the Walker A motif as a promising target for eIF4A drug development.

4.5 Elatol inhibits global as well as cap-dependent protein translation similar to silvestrol

Since the compound silvestrol has been extensively studied and its activity against eIF4A is well understood, we used it to better understand elatol's mechanism of action. First, we compared the sensitivity of our NHL cell lines

![Figure 4.5 NHL cell line sensitivity to elatol and silvestrol](image)

**Figure 4.5 NHL cell line sensitivity to elatol and silvestrol.** Sensitivity of a collection of Non-Hodgkin Lymphoma cell lines to known eIF4A inhibitor silvestrol or elatol. Viability measured after 72-hour treatment using Promega Cell Titer Glo reagent. LD_{50} calculated using nonlinear regression fit analysis in Graphpad Prism 7. Mean ± SEM n=4.
to either compound and found silvestrol to be far more potent than elatol, with their average LD\(_{50}\)'s being 45 and 1181 nM, respectively (Figure 4.5). As these drugs likely inhibit eIF4A through distinct mechanisms, and noting elatol's 2:1 binding stoichiometry, we were not initially deterred by this difference in potency. We do note however that both compounds were toxic to healthy human donor peripheral blood mononuclear cells (PBMCs), elatol at doses similar to sensitive cancer cell lines (Figure 4.5). With this information, we proceeded to evaluate elatol's activity on translation in a variety of cellular assays comparing it to silvestrol at similar concentrations relative to either drug’s LD\(_{50}\) for the cell lines tested. Polysome profiling showed a high concentration of silvestrol potently inhibited global translation at two hours, while elatol required a longer treatment to achieve the same effect (Figure 4.6A). We then used OPP incorporation to measure effects on translation over a range of concentrations and times, which confirmed that by four hours in the most elatol sensitive cell line (OCI-Ly3) elatol had inhibited translation similar to silvestrol. Elatol also significantly reduced translation in the SU-DHL6 cell line, but it required longer treatments to achieve the same effect as silvestrol. Dead cells are excluded in OPP analysis, but to confirm these effects on translation are specific to translational inhibitors we compared the DNA-damaging agent carboplatin at a lethal dose and found it had no significant effect on OPP incorporation (Figure 4.6B). Silvestrol was discovered in a screen for compounds that preferentially block cap-dependent translation over internal ribosome entry site (IRES) driven translation. Using
Figure 4.6 Elatol inhibits global and cap dependent translation. A) Polysome profiling of OCI-Ly3 cells treated with DMSO or 100nM silvestrol for 2 hours or 2 μM elatol for 2 or 16 hours. B) Mean fluorescent intensity of live cells labeled with OPP in DLBCL cells treated with indicated concentrations of silvestrol, elatol, or carboplatin for four (green), 16 (blue) or 24 (red) hours. Normalized to DMSO control. Mean ± SEM n=3. * = p<0.05, ** = p<0.001. C) Dual luciferase reporter assay measuring cap-dependent versus IRES mediated luciferase expression following an eight-hour treatment with the indicated translational inhibitors. Relative luciferase units normalized to DMSO-treated cells. Mean -/+ SEM, n=3
this reporter system we found elatol also significantly reduced cap-dependent translation with minimal effects on IRES translation (Figure 4.6C).

As discussed in the introduction, toxicity following translational inhibition in cancer cells is due to the high dependence of many oncogene’s mRNA on eIF4F for efficient translation. Proliferative drivers such as MYC or Cyclin D3, as well as pro-survival proteins like myeloid cell leukemia sequence 1 (MCL1) and Survivin, are preferentially lost upon eIF4F inhibition\(^{83,90,124}\). We evaluated the expression of some of these well-known translationally regulated proteins following silvestrol or elatol treatment and found they were strongly downregulated in all of our cell lines, and surprisingly elatol actually had a greater effect on inhibiting expression of PIM2, MCL1, BCL2 and Survivin in the OCI-Ly3 cell line (Figure 4.7). Elatol therefore affects global protein translation, though with a slightly delayed

**Figure 4.7 Elatol reduces expression of translationally regulated oncogenes.** Western blot showing protein expression of translationally regulated genes in DLBCL cells treated with DMSO (D), 50 nM silvestrol (S) or 5 μM elatol for 16h. Representative images, n=3.
response compared to silvestrol, and effectively downregulates cap-dependent protein translation.

### 4.6 Elatol does not induce eIF4A2 transcriptional response

With the clear evidence elatol is affecting translation, we wanted to confirm its action was in fact by inhibiting eIF4A as our *in vitro* studies suggest it should. Interestingly, when eIF4A1 is knocked down or pharmacologically inhibited, a cellular response increases eIF4A2 mRNA and protein expression,

![Graphs showing mRNA expression](image)

**Figure 4.8 Elatol does not induce eIF4A2 transcriptional response.** Relative mRNA expression of eIF4A1 and eIF4A2 following 24h treatment with silvestrol or elatol. Expression calculated using the ΔΔCT method normalized to DMSO. Mean +/- SEM, n=3.
providing a useful tool for evaluating eIF4A1 inhibitors. When we evaluated eIF4A1/2 mRNA expression we found silvestrol treatment strongly induced eIF4A2 levels at near LD₅₀ concentrations in each cell line, while elatol treatment only caused a modest increase in eIF4A2 levels at LD₅₀ concentrations in the SU-DHL6 and OCI-Ly3 lines, but not in RIVA (Figure 4.8). Furthermore, in SU-DHL6 and OCI-Ly3, the higher concentration of elatol actually led to a decrease in both eIF4A1 and eIF4A2 mRNA.

4.7 Elatol’s effects on translation preceed off-target effects on transcription at high doses

To determine if any of the gene expression changes we observed with elatol were actually transcriptionally mediated we evaluated mRNA levels of three of the proteins in Figure 4.7 under the same treatment conditions. Under these conditions, RIVA cells actually increased MCL1 and MYC mRNA levels with no change in Cyclin D3 following silvestrol or elatol treatment (Figure 4.9A). In SU-DHL6 Cyclin D3 was decreased in response to both silvestrol and elatol treatment, while MYC was only decreased in response to elatol and MCL1 increased in response to silvestrol but was stable in response to elatol. In OCI-Ly3 all three transcripts went up in response to silvestrol and dropped by at least half in response to elatol (Figure 4.9B). This can be partially explained by the sensitivities of the different cell lines tested to elatol. The treatment concentrations were the same for each cell line but actually most similar to the LD₅₀ of the RIVA cell line, while higher than the LD₅₀’s of OCI-Ly3 and SU-
DHL6. Such global decreases in mRNA could be attributed to excessive cell death in the more sensitive cell lines in response to the high treatment concentration. We then evaluated mRNA and protein side by side with escalating

![Graph showing mRNA expression of translation targets](image)

**Figure 4.9 Elatol affects mRNA expression at high concentrations, after translation.** A) Relative mRNA expression of translationally regulated targets following 24h treatment with silvestrol or elatol. Expression calculated using the ΔΔCT method normalized to DMSO treated cells. Mean +/- SEM, n=3 B) Protein and relative mRNA expression in OCI-Ly3 cells treated with the indicated concentration of elatol for 24 hours. Expression calculated using the double delta CT method normalized to DMSO. Mean +/- SEM, n=3.
doses in the OCI-Ly3 cell line to determine where the gene expression changes originate from, and found that a decline in protein expression precedes decreases in mRNA levels, which only occur at concentrations much higher than the LD$_{50}$ (Figure 4.9B).

4.8 Elatol induces early ATF4 expression

Because of elatol's delayed effects on translation relative to silvestrol, we wanted to evaluate other factors that could be mediating elatol's activity in cells. Inhibition of translation can be achieved indirectly through the kinase mTOR, which phosphorylates the negative regulator of eIF4F complex formation 4EBP1. In hepatocellular carcinoma (HCC), malignant mTORC1 activation is achieved by the loss of its negative regulator TSC2. As expected, the TSC2$^{-/-}$ HCC cell line SNU-398, was extremely sensitive to translation inhibitors, but response to the mTOR inhibitor rapamycin is cytostatic rather than the cytotoxic effect of silvestrol and elatol (Figure 4.10A). Silvestrol and elatol treatment strongly reduce expression of MYC and Cyclin D3 in SNU-398 cells, and have no effect on phosphorylation of mTOR targets S6 and 4EBP1, while rapamycin does, suggesting elatol is inhibiting translation downstream of mTOR (Figure 4.10B). Interestingly, when we evaluated phosphorylation of the translation factor eIF2$\alpha$, another mechanism for inhibition of translation independent of eIF4F, we did not
observe much change from baseline, but we did see a massive increase in the transcription factor ATF4 (Figure 4.10B). We then performed a time course of elatol treatment in our DLBCL cell lines, and observed a cycling of eIF2α phosphorylation and again a massive increase in ATF4 expression within 1-2 hours.

![Graph showing cell viability](image)

**Figure 4.10 Elatol causes immediate upregulation of ATF4**

A) Cell viability of SNU-398 cells measured after 72-hour treatment with translation inhibitors silvestrol, rapamycin or elatol. Mean ± SEM n=4. B) Western blot showing protein expression SNU-398 cells treated with DMSO (D), 200 nM rapamycin (R), 100 nM silvestrol (S) or 1 μM elatol (E) for 16 hours. Representative images. C) Time course showing protein expression in DLBCL cell lines following elatol treatment. Representative images. n=2. D) Protein expression in MDA-MB-468 cells treated with DMSO (far left), 500nM, 2.5 μM, 10 μM elatol or 10 μM tunicamycin (far right) for six hours.
hours (Figure 4.10C). To further validate this surprising effect, we used the MDA-MB-468 breast cancer cell line, which was sensitive to elatol in the Harvard/Wellcome Trust screen (Figure 4.2A). When we treated these cells with increasing concentrations of elatol, we again saw a massive increase in ATF4 expression, even more so than what was caused by treatment with the known eIF2α phosphorylation inducer tunicamycin (Figure 4.10D). These results suggest that ATF4 expression is a general response to elatol treatment, not cell type specific.

4.9 Elatol induction of ATF4 is not mediated by PERK or the UPR

eIF2α is phosphorylated on serine 51 by four known kinases, PERK, GCN2, HRI, and PKR, which become activated in response to various intracellular stresses in the ISR pathway (Figure 1.4)\textsuperscript{58}. The expression of ATF4 we observed suggested that elatol may be directly or indirectly activating the ISR. PERK activates the ISR in response to endoplasmic reticulum (ER) stress caused by accumulation of unfolded proteins in the ER lumen, and seemed like the most likely candidate for triggering the ISR in response to drug treatment\textsuperscript{58}. To test whether elatol was triggering the ISR via the PERK arm, we used the highly specific PERK inhibitor GSK2606414 (PERKi)\textsuperscript{129}. Combining the PERKi with the known ER stress inducer tunicamycin significantly rescued apoptosis in DLBCL cells, measured by annexin V staining (Figure 4.11A). As expected, addition of the PERKi to silvestrol treated cells had no effect on apoptosis measured, nor did it cause a significant rescue of the apoptosis induced in
response to elatol treatment (Figure 4.11A). Addition of the PERKi to tunicamycin treated cells eliminated the induction of ATF4 protein expression and rescued MYC and Cyclin D3 protein expression to near baseline levels (Figure 4.11B).

We found no similar effect on ATF4 expression when PERKi was combined with elatol and no rescue of MYC or Cyclin D3 levels. We did not observe an overall induction of the unfolded protein response indicated by either XBP1s expression
or ATF6 cleavage, therefore ruling out off-target activation of the unfolded protein response or PERK as the cause of ATF4 expression due to elatol treatment (Figure 4.11B).

**4.10 Elatol induction of ATF4 is dependent on eIF2α phosphorylation, but not necessary for tumor cell toxicity**

To get a more general understanding of the role of eIF2α phosphorylation in driving elatol’s toxicity in cancer cells we acquired mouse embryonic fibroblasts (MEFs) homozygous for the nonphosphorylatable alanine at serine 51 (S51A/A) \(^{130}\). The S51A/A MEFs showed no induction of ATF4 in response to elatol or tunicamycin treatment, while their wildtype counterparts (S51S/S) did (Figure 4.12A). There was, however, no significant difference in sensitivity to elatol between the mutant and wildtype MEFs (LD\(_{50}\) = 1,029nM (S51A/A), 1399 (S51S/S); \(p=0.9845\)) (Figure 4.12B). We also tested Atf4 knockout MEFs and

![Figure 4.12 Upregulation of ATF4 following elatol is dependent on eIF2α phosphorylation. A) Western blot showing protein expression in eIF2α wildtype and Ser\(^{51}\) A/A mutant MEFs treated with DMSO, 5 \(\mu\)M elatol or 5 \(\mu\)M tunicamycin for 8 hours. B) Cell viability in MEF cells wildtype or homozygous mutant for alanine at serine 51 of eIF2α and wildtype or homozygous knockout of ATF4 measured using Cell Titer Glo following 72h treatment with elatol. Mean ± SEM n=4 * = \(p<0.05\), ** = \(p<0.001\).]
found the cells lacking ATF4 were more sensitive to elatol treatment (LD50 = 2,785nM (Atf4/-), 4,038nM (WT); p=0.0138) (Figure 4.12B).

To confirm these results in human cancer cells we first knocked down ATF4 by siRNA in the MDA-MB-468 cells and found it had no effect on elatol’s toxicity (Figure 4.13A-B). Similarly, the drug ISRIB, which acts downstream of

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**Figure 4.13 eIF2α phosphorylation and ATF4 are not important for elatol’s potency against cancer cells**

A) Western blot analysis of MDA-MB-468 breast cancer cells transfected with 50nM ATF4 siRNA or a non-targeting control (NT). 48 hours after transfection cells were treated with DMSO, 5 μM elatol or 5 μM tunicamycin for 8 hours and samples were collected for analysis. Representative images, n=2. B) MDA-MB-468 cells were transfected with 50nM ATF4 siRNA or NT control for 48 hours and then treated with DMSO, 5 μM elatol or 5 μM tunicamycin for 24 hours. Then cell death was measured by flow cytometry following annexin V staining. Mean +/- SEM n=3. C) Western blot showing protein expression in cells treated for four hours with DMSO, 100 nM silvestrol, 5 μM elatol or 5 μM tunicamycin with or without the combination with 200 nM ISRIB. Representative images. n=3. D) Cell death measured by flow cytometry following annexin V staining in DLBCL cell lines treated with indicated inhibitors with or without the combination of the 200 nM ISRIB. Mean +/- SEM n=3. * = p<0.05, ** = p<0.001.
eIF2α phosphorylation to maintain translation during ISR, did not rescue DLBCL cells from apoptosis induced by elatol (Figure 4.13C). ISRIB treatment did, however, prevent the induction of ATF4 in response to elatol treatment and partially rescued the loss of MYC and Cyclin D3, but nowhere near as much as it did in response to tunicamycin treatment, indicating elatol’s maintained inhibition of translation is likely through eIF4A1 (Figure 4.13D). ATF4 expression in response to elatol is therefore dependent on eIF2α phosphorylation, but does not drive elatol’s toxicity in cancer cells, and ATF4 expression may in fact be triggered to protect cells from elatol’s activity against eIF4A as MEFs lacking Atf4 were more sensitive.

4.12 Knockdown of eIF4A1 further sensitizes cells to elatol treatment similar to silvestrol

As ISR activation is not driving elatol’s effects on protein translation, inhibition of eIF4A1 is still the likely cause, though not yet confirmed in a cellular context. To verify this we used a knockdown system previously published as verification of eIF4A1 as silvestrol’s cellular target 103. Treatment of cells with either silvestrol or elatol on top of eIF4A1 (sh4A1) knockdown caused a significant increase in sensitivity to the drugs compared to control (shRluc) (LD50= 6nM (shRluc silvestrol), 3.8nM (sh4A1 silvestrol) p<0.0001; LD50= 3,547nM (shRluc elatol), 2,382nM (sh4A1 elatol) p=0.0026) (Figure 4.14A-B). When we treated the eIF4A1 knockdown cells with carboplatin we found no change in drug sensitivity, confirming eIF4A1 loss is not a general sensitizer to
cytotoxic agents and these results are specific to eIF4A1 inhibitors (Figure 4.14).

These results validate elatol as an eIF4A1 inhibitor, but the effects on mRNA levels at high concentration and ATF4 induction suggest there may be some off targets. The eIF4A family member eIF4A2 is thought to function in micro-RNA mediated mRNA decay, while eIF4A3 acts as an RNA clamp in the EJC, and both share high homology to eIF4A1, making them likely off target.

Figure 4.14 Elatol’s activity is dependent on eIF4A1. A) Western blot showing eIF4A1 and eIF4A2 protein levels in NIH-3T3 cells stably expressing shRLuciferase (shRLuc) control or eIF4A1 shRNA transfected with the indicated proteins. Representative images, n=2. B) Viability of NIH-3T3 cells stably expressing control or eIF4A1 shRNA measured by Cell Titer Glo after being treated with the indicated compounds for 5 days. Mean ± SEM n=4. * = p<0.05, ** = p<0.001, ***=p<0.0001 C) 5 day viability of NIH-3T3 cells stably expressing shRNA targeting control (RLuc) or eIF4A1 treated with carboplatin measured using Cell Titer-Glo. Nonlinear regression fit analysis in Graphpad Prism 7. Mean +/- SEM n=4.
candidates. Interestingly, when we knocked down eIF4A2 and eIF4A3 in MDA-MB-468 cells using siRNA, we saw an increase in eIF2α phosphorylation and ATF4 expression, possibly explaining the induction of ATF4 we see in response to elatol treatment (Figure 4.15).

![Western blot analysis of MDA-MB-468 cells transfected with siRNA targeting eIF4A1, eIF4A2, eIF4A3 or a non-targeting control (NT). Protein expression analyzed 48h after knockdown. Representative image n=2.]

**Figure 4.15 eIF4A2/3 knockdown induces ATF4.**

**4.13 Elatol in vivo**

Finally, we wanted to confirm elatol’s activity as an anticancer drug *in vivo*. Elatol was reported to be used in mice in only one previous study, in which they tested both oral and intraperitoneal (i.p.) administration and found i.p. dosing up to 30mg/kg to be more effective at inhibiting tumor growth. We evaluated elatol in non-tumor bearing SCID mice at 10, 20, and 30mg/kg daily by i.p. injection for five days, and contrary to the previous study we found 30mg/kg to be toxic causing greater than 10% body weight loss in the mice by day three, while 10 and 20 mg/kg showed no signs of morbidity up to five days (Figure 4.16A).
day five, six hours after the final dose, complete blood counts in the mice given 20mg/kg showed no significant differences to vehicle treated control mice (Figure 4.16B). We therefore xenografted SU-DHL6 cells in SCID mice and once the tumors reached $100 \text{mm}^3$ began treatment with 20mg/kg elatol daily (Figure 4.17A). In tumor bearing mice however, 20mg/kg daily was not well tolerated, and we had to halt treatment after 5 days due to weight loss (Figure 4.17B). The five-day cycle was able to stop tumor progression, but not indefinitely, and by day

Figure 4.16 Elatol is tolerated at 20mg/kg daily in mice. A) Mouse weights measured twice weekly during maximum tolerated dose study. 10 and 20 mg/kg doses were given daily for five days and 30mg/kg was stopped after three days due to toxicity. Mean +/- SEM n=5. B) Complete blood counts for white blood cells, hemoglobin and platelets of SCID mice following treatment with 20 mg/kg elatol daily for 5 days. Mean +/- SEM n=17 and 7 vehicle and elatol respectively.
15 the tumors in the mice that had been given elatol started to progress (Figure 4.17A).

These results, along with our initial apoptosis data (Figure 4.3B), suggest that elatol may be quite stable, but without any pharmacokinetic data we decided to better establish the maximum tolerated dose of elatol. The first cohort of five

![Figure 4.17 20mg/kg elatol daily dosing in tumor bearing mice is toxic](A) SCID mice were engrafted with $2 \times 10^6$ SU-DHL6 cells. Once tumors reached $60 \text{mm}^3$ treatment began with 20 mg/kg i.p. daily for 5 days and tumor volume was measured twice per week. Mean ± SEM, n=8. B) Mouse weights measured twice weekly during SU-DHL6 xenograft study. Mean ±/SEM, n=8.
mice was given a single i.p. dose of 50mg/kg elatol and monitored for signs of toxicity, but none were observed (Figure 4.18A). The next group was given 100mg/kg, which was lethal and all five mice had to be sacrificed by day 5. A single dose of 80mg/kg was also lethal, with three of five mice having to be sacrificed, while 65mg/kg was well tolerated with no signs of toxicity observed. At day 14 after dosing (or at the time of sacrifice) organs were collected for tissue pathology and we observed that in the mice given the lethal 100mg/kg dose there were clear signs of acute damage to the heart as well as inflammation and congestion in the liver (Figure 4.18B). Therefore, elatol is well tolerated as a

*Figure 4.18 Single dose of 65mg/kg elatol is tolerated in mice.* A) Maximum tolerated dose study in normal CD1 mice. Cohorts of 5 mice were given a single indicated dose of elatol and observed daily for signs of morbidity. At time of death due to toxicity or at day 14 if no toxicities were observed mice were sacrificed for organ pathology. B) H&E staining of the heart and liver of mice treated with 50 or 100 mg/kg elatol. Representative images n=5.
single dose up to 65mg/kg, but as our previous experiment showed it is less tolerated in tumor bearing animals. This is dramatically higher than silvestrol, which is typically dosed between 0.5-1.5mg/kg, offsetting the potency differences between the two compounds we observed in vitro\textsuperscript{67,92,93}. We then performed another xenograft experiment with OCI-Ly3 cells and an optimized dosing schedule of 40mg/kg twice weekly and found that elatol treatment significantly

![Graph A](image1.png)

**Figure 4.19 Elatol significantly delays tumor progression in vivo.** A) SCID mice were implanted with 1x10\textsuperscript{6} serially transplanted OCI-Ly3 xenograft cells. When tumors reached at least 50 mm\textsuperscript{3} mice were pair matched and treatment began with either vehicle or 40 mg/kg of elatol i.p. twice weekly. Mean ± SEM, n=8. * = p< 0.05. B) Mouse weights measured three times weekly during OCI-Ly3 xenograft study. Mean -/+ SEM, n=8.
delayed tumor progression and extended the survival of the animals (Figure 4.19A). Together, these experiments establish elatol as an inhibitor of ATP hydrolysis by eIF4A, with potent in vitro and in vivo antitumor activity, and present a pipeline for further discovery of inhibitors.
Chapter 5
Discussion and Future Directions

5.1 Control of translational activation by PIM kinase in activated B-cell

_diffuse large B-cell lymphoma confers sensitivity to inhibition by PIM447_

Currently, there is still no FDA approved PIM kinase inhibitor, despite substantial preclinical studies supporting its use hematologic malignancies, as well as some solid tumors. In 2008 phase 1 trial with the PIM1 preferential inhibitor SGI-1776 in prostate cancer and lymphoma (NCT00848601) was prematurely terminated due to cardiac toxicity, and at the same time the field shifted toward pan-PIM kinase inhibition due to their functional redundancy in cancer and the lack of serious abnormalities in PIM triple knockout mice. The pan-PIM kinase inhibitor AZD1208 showed strong preclinical activity in AML and multiple myeloma (MM), but a phase 1 trial in solid tumors as well as lymphoma showed significant toxicities (NCT01588548). PIM447, another pan-PIM kinase inhibitor developed from the tool compound LGB321 is the only PIM inhibitor currently under clinical evaluation with two completed trials in MM and hematologic malignancies and three more trials still active. In the phase 1 study in heavily pretreated MM patients, PIM447 was tolerated up to 500mg with limited toxicity and some evidence for single agent efficacy.

These promising results for PIM inhibition in MM then raise the question of where else PIM kinase inhibition may be useful, which is still very unclear. Though overexpression of the PIM kinases is prevalent, they are not driver oncogenes and instead synergize strongly with c-MYC to promote an aggressive
tumor phenotype. Additionally, the PIM kinases have few unique targets, meaning activation by redundant kinases promotes easy resistance to PIM inhibitors. We assessed PIM kinase inhibition with PIM447 in non-myeloma NHL cell lines and found the ABC subtype of DLBCL to be the most sensitive group (Figure 3.1). ABC DLBCLs are derived from post-germinal center B-cells on the path to terminal differentiation into plasma cells, which is the same precursor cell for MM, suggesting the shared sensitivity to PIM kinase inhibition may be due to the similar stage of the B-cell from which these diseases arise. We found that high expression of PIM1 and PIM2 alone in our ABC lines, a distinguishing feature relative to GCB DLBCL, did not determine sensitivity to PIM447 as 3/7 cell lines were resistant (Figure 3.2). Instead we found sensitivity to PIM447 to segregate based on the mutational activation of BCR signaling in the cell lines, where downstream mutations in the CBM complex conferred sensitivity to PIM447 and mutations upstream conferred resistance. In the sensitive cell lines PIM447 treatment shut off translation activation through mTORC1, while resistant cell lines had alternative mechanisms to maintain translational activation, highlighting the redundant activity of the PIM kinases (Figure 3.3-4).

These findings suggest a niche for PIM kinase inhibition beyond MM in ABC DLBCL, which has one of the poorest prognoses in DLBCL. The key for improving outcomes for patients with ABC DLBCL is targeting their unique drivers, such as NF-κB signaling, but as there are no direct inhibitors of NF-κB other means need to be evaluated. The BTK inhibitor ibrutinib activates NF-κB upstream of the CBM complex and therefore inhibits NF-κB signaling driven by
upstream mutations or chronic antigen stimulation and is currently in clinical trials. However, our results suggest that for those cases where signaling is activated downstream, by loss of \textit{TNFAIP3} or \textit{CARD11} mutation, inhibition of BTK is irrelevant. Evidence for this has already been reported in the clinic where ABC patients with mutations in \textit{TNFAIP3} or \textit{CARD11} did not respond to ibrutinib\textsuperscript{110}. Highly activated autocrine signaling complicates complete elucidation of the signaling machinery in this disease, and indeed we found the OCI-Ly10 cell line which harbors \textit{CD79B} and \textit{MYD88} upstream and \textit{TNFAIP3} and \textit{CARD11} downstream mutations to be sensitive to both PIM447 and ibrutinib, while the SU-DHL2 cell line containing an upstream and a downstream mutation was resistant to both inhibitors (Figure 3.3). It is possible that in these instances combining multiple signaling inhibitors could be effective. In spite of this, with continued research into precision medicine in DLBCL there can be a role for PIM inhibition.

Our study unveiled very context specific indications for inhibitors targeting specific intermediates of activated BCR signaling, but it also emphasized the importance of activation of cap-dependent protein translation downstream of oncogenic signaling. In their respective sensitive cell lines, treatment with either PIM447 or ibrutinib shut off mTOR targets and protein expression, while all ABC cell lines tested regardless of mutational status were extremely sensitive to direct inhibition of translation by silvestrol (Figure 3.6). Moving forward DLBCL should be included in the evaluation of new strategies for targeting translation in cancer.
We also unveiled the first meta-analysis and functional characterization of \textit{PIM1} mutants found in DLBCL. \textit{PIM1} has long been known to be one of the most frequently mutated genes in DLBCL, and the hypothesis has been that \textit{PIM1} was a target of aberrant SHM by the germinal center expressed enzyme AID, but the significance of these mutations remained unknown. Either \textit{PIM1} mutations, along with other mutated genes, are merely a marker of the aberrant SHM that occurs during the pathogenesis of DLBCL, or \textit{PIM1} mutations are selected for by the tumor because they somehow enhance \textit{PIM1}'s oncogenic function. Evidence for the former theory came from two mechanistic studies in 2015 that described \textit{PIM1}'s location within a super-enhancer and site of frequent convergent transcription, an ideal setting for targeting by AID\textsuperscript{133,134}. Our data also support the idea that \textit{PIM1} mutation is a side effect of DLBCL biology as the vast majority of mutations were located in AID hotspot motifs (Table 3.1, Appendix 1). Importantly though, there does seem to be a selection for mutations that preserve \textit{PIM1} function. Of the 92 mutations identified only 6\% were nonsense or frame shift changes, and nearly all of the recurrent missense mutations we characterized were able to rescue FL5.12 cells from cytokine withdrawal similar to the wildtype protein (Figure 3.7-8). Therefore, while PIM activity may not be a driver in ABC DLBCL, in general, activity of PIM1 is preferentially maintained.
5.2 Targeted screen identifies elatol as a novel eIF4A inhibitor with potent anti-tumor activity

The era of genome-wide sequencing has identified specific drivers of cancer and led to the paradigm of targeted therapy dictating that the targets should be something unique to the cancer cells in order to avoid the toxicities seen with traditional chemotherapeutic treatments. Though this notion is compelling, some oncogenic drivers, such as transcription factors or formation of protein complexes, are notoriously difficult to target with current pharmacologic approaches. To overcome this problem drug delivery techniques are rapidly evolving, but the persistent issues of redundancy of signaling pathways and intra-tumor heterogeneity are more profound hurdles for targeted therapy. Treatment of patients with identified BRAF driving mutations with the targeted agent vemurafenib show great initial responses but shortly succumb to relapse via activation of alternate signaling pathways. This same problem is seen with targeted inhibitors in breast, lung, prostate, and many other cancers where targeting a single signaling molecule is easily overcome. Additionally, solid tumors display high levels of intra-tumor heterogeneity lending to the inevitable failure of targeted inhibitors as single agents. Activation of the eIF4F complex lies downstream of the commonly deregulated signaling pathways in cancer serving as a bottleneck for the transmission of growth and adaption signals into the cellular phenotype and its function is essential to normal and cancer cells alike so expression of eIF4F components is homogenous.
(Figure 1.3). Therefore, targeting eIF4F directly sidesteps these major hurdles for targeted cancer therapy.

In 2012, omacetaxine was the first translation inhibitor actually approved by the food and drug administration (FDA) for the treatment of chronic myelogenous leukemia (CML) that has failed treatment by standard TKIs. Omacetaxine is derived from a natural Chinese herbal medicine and inhibits translation at the elongation step by interfering with tRNA binding in the ribosome A site. The result is similar to inhibition of eIF4F in cancer cells with a general decline in protein synthesis and loss of translationally regulated proteins important for cancer cells such as MYC, BCL2 and the fusion protein BCR-ABL which is a driver of CML. This successful approval of a translation inhibitor validates preclinical studies demonstrating a therapeutic window for inhibition of translation in cancer and further development of this area of cancer research.

There are several mechanisms for eIF4F inhibition currently being pursued including eIF4E antisense oligonucleotides, eIF4E:eIF4G interaction inhibition and MNK inhibitors, but the RNA helicase eIF4A is a particularly promising target. It is the only enzymatic component of the eIF4F complex and therefore the only one easily drugable by small molecules, and inhibition of eIF4A has been shown to have a more cytotoxic effect compared to inhibition of mTOR/eIF4E. Since pharmacologic issues have halted eIF4A inhibitors in the preclinical phase, we pursued a target based screen for eIF4A1 inhibitors to advance this therapeutic approach for cancer.
Elatol was identified as the top hit from our screen, a compound that had been previously described for its anticancer and antiparasitic properties but its target was unknown \(^{131,136}\). Elatol was highly specific for eIF4A1 compared to other DEAD box family members in the ATP hydrolyzing assay, and showed no off-targets in a small commercial kinase screen (Figure 4.1). Molecular modeling and subsequent mutational analysis identified lysines 82 and 238 as critical for elatol's effect on eIF4A1 (Figure 4.4). K82 is a part of the walker A ATPase motif conserved in all DEAD-box helicases, and is a potentially important site for future drug development.

Elatol showed broad antitumor activity, but as has been previously reported with other translational inhibitors the most sensitive tumor types were hematopoietic, breast and lung cancers (Figure 4.2). A single treatment with elatol completely halted proliferation and induced apoptosis in DLBCL cells (Figure 4.3). In the most sensitive cell line, OCI-Ly3, elatol treatment reduced global protein synthesis as well as expression of translationally regulated oncogenes similar to silvestrol, but required slightly longer treatment times to achieve the same effects as silvestrol in SU-DHL6 cells (Figure 4.6-7). At high concentrations, with the effect delayed relative to effects on protein expression, elatol does reduce expression of mRNAs, but this is off-target effect is avoidable with lower dosing (Figures 4.8-9). Unlike silvestrol, elatol treatment is followed by an immediate increase in ATF4 expression in all cell types tested that is mediated by eIF2\(\alpha\) phosphorylation but not the PERK arm of the ISR (Figure 4.10-2). This response, however, is not important for elatol's toxicity in cancer
cells as treatment with ISРIB or knockdown of ATF4 did not rescue the effects of elatol treatment, suggesting future studies are needed to identify the exact mechanism of ATF4 upregulation (Figure 4.13). Knockdown of eIF4A1, however, further sensitized cells to both silvestrol and elatol treatment, indicating the effects we observe from elatol are due to its action against eIF4A1 (Figure 4.14). We established twice weekly dosing of elatol at 40mg/kg as able to significantly delay tumor progression in a DLBCL xenograft model (Figure 4.19). Overall elatol is far less potent in cancer cells compared to silvestrol (Figure 4.5), but the discrepancy is recovered in vivo as elatol can be dosed safely in tumor bearing animals up to 40mg/kg, while silvestrol is typically administered between 0.5-2mg/kg.

This work establishes elatol as an eIF4A inhibitor, but there are clear off targets that we still do not fully understand. When we silenced eIF4A2 and eIF4A3 in breast cancer cells we observed an increase in ATF4 expression, similar to what we see with elatol treatment (Figure 4.15). Additionally, eIF4A2 and eIF4A3’s reported diverging roles in in micro-RNA mediated gene repression and mRNA export in the EJC could explain the effects we see on mRNA expression following elatol treatment. These observations, along with their high homology to eIF4A1 make them likely off-target candidates for elatol, and further experiments confirming this will be critical to the development of elatol as an eIF4A1 inhibitor.

Our research has revealed some important points for regarding eIF4A as a therapeutic target that should be noted for future drug development. In the
majority of studies with eIF4A inhibitors and on eIF4A expression in cancer, research has not differentiated between the paralogs, but it is clear that that will be important moving forward. eIF4A1 expression seems to be exclusively correlated with poor prognosis in cancer, but a few studies have reported high eIF4A2 to inversely correlate. Additionally, it has also been reported that they are differentially expressed within a tumor, where eIF4A1 is highly expressed on the exterior in the invading and rapidly dividing cells and eIF4A2 expression is restricted to the quiescent tumor core. A better understanding of their biology in vivo as well as development of compounds that can discern between the two will be critical to the success of eIF4A inhibition. Our results highlight the importance of counter-screening all three eIF4A paralogs early in the drug development pipeline to avoid off-targets and accurately interpret the effect of treatment in vivo. Inhibiting eIF4A’s ATPase activity is a novel mechanism for eIF4A inhibition in cancer, and one that poses interesting new questions for the biology of eIF4A1 especially, as it is the primary paralog active in eIF4F. Whether the mechanism of eIF4A1 inhibition (inhibition of RNA binding by hippuristanol, sequestration by silvestrol or pateamine A, or inhibition of ATP hydrolysis by elatol) differentially affects the translational output has not yet been addressed and could have important clinical implications.

Taken together, we have reinforced targeting translation directly as a therapeutic strategy for DLBCL, identified elatol as a novel inhibitor of eIF4A’s ATPase activity, and presented a pipeline for future development of eIF4A inhibitors.
## Appendices

### PIM1 mutational analysis

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Table 6.1 Meta analysis of somatic mutations in PIM1 DLBCL. A summary of identified amino acid altering somatic mutations in the PIM1 gene in DLBCL clinical samples. Effect on protein function was predicted using Polyphen2 and AID hotspot motif analysis with the c/g nucleotide that was mutated included.
References


