Targeting Aberrant STAT3 Signaling as a Therapeutic Strategy for Multiple Myeloma

by

Danielle Croucher

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Department of Medical Biophysics
University of Toronto

© Copyright by Danielle Croucher 2013
Targeting Aberrant STAT3 Signaling as a Therapeutic Strategy for Multiple Myeloma

Danielle Croucher
Masters of Science
Department of Medical Biophysics
University of Toronto
2013

Abstract
The oncogenic transcription factor STAT3 is aberrantly activated in over 70% of human tumours, including Multiple myeloma (MM). The present studies use both genetic and chemical tools to validate STAT3 as a therapeutic target, and demonstrate the anti-MM activity of a novel small molecule STAT3 inhibitor, BP-4-018. We show that shRNA-mediated STAT3 knockdown induces apoptosis in human myeloma cell lines (HMCLs). We translate these findings to a therapeutically relevant setting by demonstrating the broad anti-MM activity of BP-4-018 against HCMLs and primary patient samples, and demonstrate that BP-4-018 remains active against HMCLs co-cultured with bone marrow stroma. Inhibiting STAT3 via shRNA knockdown and BP-4-018 suppresses STAT3 transcriptional activity and down-regulates anti-apoptotic and proliferative STAT3 target genes. Finally, we show that BP-4-018 has activity in vivo, both alone and combined with subtherapeutic doses of bortezomib, without significant toxicities. Taken together, these data support the utility of STAT3 inhibitors for MM treatment.
Acknowledgments

The advice, support and encouragement of many people have been integral to my graduate education and the completion of this thesis, both at the University of Toronto and in my personal life. I am especially grateful for the tireless support of Dr. Suzanne Trudel, whose mentorship has been invaluable to my graduate education. I admire your enthusiasm, dedication and curiosity towards medicine and science, and your compassionate and empathetic approach to teaching is infinitely appreciated. I am grateful to have had the opportunity to train in a diverse learning environment with such admirable individuals, particularly ZhiHua Li and Ellen Wei, whose proficiency and expertise in the lab have been immeasurably helpful to me. I will always be grateful for your kind help and patience throughout my time in Dr. Trudel’s lab. I would also like to thank my committee members, Dr. Mark Minden and Dr. David Hedley, for your thoughts, insight and guidance throughout my project.

Without a doubt, my studies would not have been possible without the love and support from my family. To my Mom and Dad, thank you for always having late-night dinners ready for me, even if was just a saran-wrapped plate of leftovers in the fridge. You have kept me grounded and balanced, and it is your patience, encouragement and flexibility that have made this experience possible. We have been through a lot as a family, but knowing that you will always stand behind me gives me courage everyday. For all of these reasons, I am proud to be your daughter and I love you. To Simon, you will never know the appreciation that I have for you, it reaches far beyond the past two years of grad school. Thank you for always being my voice of reason when I needed it most and always reminding me of the light at the end of this tunnel. Through the ups and downs, you have been there for me, pushing me, and believing in me, and I am so grateful to have someone like you in my life. Finally, to my sister Nikky for whom this work is dedicated to. Even though you left us before I began my journey at grad school, I know that you have been with me the whole time. You taught me to trust that I am exactly where I am meant to be and so I know that you would be proud of how far I have come. I should have said it more, but I am grateful to have had you as my sister and I miss you everyday.
# Table of Contents

Abstract ........................................................................................................................................... ii
Acknowledgments ................................................................................................................................. iii
List of Tables ......................................................................................................................................... vi
List of Figures ......................................................................................................................................... vii
List of Appendices ................................................................................................................................. ix
List of Abbreviations .............................................................................................................................. x

**Chapter 1 Introduction** ......................................................................................................................... 1
  1.1. Multiple Myeloma ....................................................................................................................... 1
  1.2. Therapeutic Approaches for MM ............................................................................................. 1
  1.3. Molecular Pathology of MM ...................................................................................................... 3
  1.4. STAT3: Signal Transducer and Activator of Transcription ...................................................... 11
  1.5. STAT3 in MM: Aberrant Expression and Functional Role ....................................................... 14
  1.6. Targeted Therapeutics Aimed at STAT3 ............................................................................... 18
  1.7. Rationale ..................................................................................................................................... 21
  1.8. Hypothesis and Experimental Aims ......................................................................................... 21

**Chapter 2 Validating STAT3 as a Therapeutic Target in MM** ............................................................ 23
  2.1. Objectives .................................................................................................................................. 23
  2.2. Methods ...................................................................................................................................... 24
  2.3. Results ...................................................................................................................................... 29
  2.4. Summary ..................................................................................................................................... 35

**Chapter 3 In vitro Pre-clinical Evaluation of Small Molecule STAT3 Inhibitors in MM** ............. 36
  3.1. Objectives .................................................................................................................................. 36
  3.2. Methods ...................................................................................................................................... 38
  3.3. Results ...................................................................................................................................... 41
Chapter 4 In vivo Efficacy Studies of BP-4-018 Alone and in Combination with Bortezomib Using a Xenograft MM Mouse Model

4.1. Objectives
4.2. Methods
4.3. Results
4.4. Summary

Chapter 5 Discussion

Chapter 6 Conclusions and Future Directions

References

Appendices
List of Tables

Table 3.3.1 Structure and growth inhibitory properties of screened small molecule STAT3 inhibitors .......................................................... 42
List of Figures

Figure 1.3.1 Step-wise pathogenesis of MM ................................................................. 5
Figure 1.3.2 Model for molecular pathogenesis of MM ............................................... 7
Figure 1.4.1 IL-6/JAK/STAT3 signaling pathway .......................................................... 12
Figure 1.4.2 Implications of STAT3 in the hallmark characteristics of cancer ............... 14
Figure 1.6.1 Schematic representation of the STAT3 structural domains targeted by STAT3 inhibiting molecules ................................................................. 20
Figure 2.3.1 Analysis of STAT3 activation status in HMCLs ......................................... 29
Figure 2.3.3 Effects of STAT3 knockdown on STAT3-driven luciferase expression ........ 31
Figure 2.3.4 Analysis of STAT3 knockdown-induced apoptosis ..................................... 32
Figure 2.3.5 Analysis of HMCL-specific modulation of STAT3 targets following STAT3 knockdown ....................................................................................... 34
Figure 3.1.1 Inhibitory strategy of BP-series of small molecules ..................................... 37
Figure 3.3.1 Initial screening of small molecule STAT3 inhibitors and their derivatives against HMCLs ......................................................................................... 41
Figure 3.3.2 Extended analysis of BP-4-018-mediated growth inhibition against a panel of HMCL ......................................................................................... 43
Figure 3.3.3 Flow cytometric analysis of BP-4-018-induced apoptosis ........................... 44
Figure 3.3.4 Activity of BP-4-018 against malignant plasma cells from primary MM patient BM aspirates ........................................................................................................................................... 45

Figure 3.3.5 Activity of BP-4-018 against non-malignant BM cells from primary MM patient samples ........................................................................................................................................... 46

Figure 3.3.6 Activity of BP-4-018 against HMCLs in the presence of a recapitulative BM microenvironment ........................................................................................................................................... 47

Figure 3.3.7 BP-4-018-mediated inhibition of STAT3 signaling in HMCLs ........................................ 49

Figure 3.3.8 Subtherapeutic dose combinations of BP-4-018 and bortezomib ............................... 51

Figure 4.2.1 Study design for evaluating the *in vivo* efficacy of single agent BP-4-018 and BP-4-018 in combination with subtherapeutic doses of bortezomib ........................................................................................................ 57

Figure 4.3.1 BLI analysis of *in vivo* STAT3 transcriptional activity prior to treatment initiation ........................................................................................................................................... 60

Figure 4.3.2 Effect of BP-4-018 alone or in combination with bortezomib in MM xenograft tumour model ........................................................................................................................................... 62

Figure 4.3.3 BLI analysis of short-term BP-4-018 treatment effects on STAT3 transcriptional activity *in vivo* ........................................................................................................................................... 63
List of Appendices

Appendix A. STAT3 inhibitory activity of small molecule analogues as assessed by FP assay . 92

Appendix B. Inhibiting Activated STAT3 Proteins with Tetrapodal, Small Molecule SH2 domain binders: Promising Agents Against Multiple Myeloma ................................................................. 93

Appendix C. BLI kinetics study for in vivo STAT3-driven luciferase expression in MM xenograft mouse model.......................................................... 115

Appendix D. Pilot study to establish tolerable dose of BP-4-018 in MM xenograft mouse model .................................................................................................. 116
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT (PKB)</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BCL</td>
<td>B cell CLL/lymphoma</td>
</tr>
<tr>
<td>BCL-xL</td>
<td>BCL2-like 1</td>
</tr>
<tr>
<td>BID</td>
<td><em>Bis in die</em> (twice daily)</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescence imaging</td>
</tr>
<tr>
<td>BLIMP1</td>
<td>B-lymphocyte-induced maturation protein 1</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescence polarization</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>gp130</td>
<td>Glycoprotein130</td>
</tr>
<tr>
<td>h36B4</td>
<td>Human ribosomal protein h36B4</td>
</tr>
<tr>
<td>HMCLs</td>
<td>Human myeloma cell lines</td>
</tr>
<tr>
<td>HRD</td>
<td>Hyperdiploid</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgH</td>
<td>Ig heavy chain</td>
</tr>
<tr>
<td>IgL</td>
<td>Ig light chain</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-6Rα</td>
<td>IL-6 receptor alpha</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IMiDs</td>
<td>Immunomodulatory agents</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IRF4</td>
<td>Interferon regulatory factor 4</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus-activated kinase</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid cell leukemia sequence 1</td>
</tr>
<tr>
<td>MGUS</td>
<td>Monoclonal Gammopathy of Undetermined Significance</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>MNCs</td>
<td>Mononuclear cells</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NHRD</td>
<td>Non-hyperdiploid</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non-obese diabetic/severe combined immunodeficient</td>
</tr>
<tr>
<td>pProtein</td>
<td>Phosphorylated protein</td>
</tr>
<tr>
<td>(c)PARP</td>
<td>(cleaved) Poly ADP-ribose polymerase</td>
</tr>
<tr>
<td>PAX5</td>
<td>Paired box protein 5</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>pTyr</td>
<td>Tyrosine phosphorylation or phosphorylated tyrosine</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2</td>
</tr>
<tr>
<td>shRFP</td>
<td>RFP-targeting shRNA</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>shSTAT3</td>
<td>STAT3-targeting shRNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STAT3C</td>
<td>Constitutively active STAT3 mutant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween 20</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein 1</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

1.1. Multiple Myeloma

Multiple myeloma (MM) is a mature B cell malignancy characterized by the latent expansion of malignant plasma cells in the bone marrow (BM). In the majority of cases, MM is epitomized by the hypersecretion of monoclonal immunoglobulin (Ig) molecules in the serum, which can be detected as a sharp peak (M-spike) by serum protein electrophoresis, or as Bence Jones proteins in the urine when Ig light chains are secreted. Among hematological malignancies, MM tumour cells are unique in their ability to cause significant bone destruction, which can in turn lead to osteoporosis and hypercalcemia. Together, the MM tumour, its products, and its effect on the surrounding environment, collectively contribute to the clinical manifestations of MM, which include persistent lytic bone disease, renal insufficiency, immune suppression and anemia.

Although MM accounts for only 1.3% of all new cancer cases, it is the second most common blood cancer after non-Hodgkin lymphoma, representing approximately 15% of hematological malignancies.\(^1\) Although significant advances have been made in the clinical management of MM, it remains almost uniformly incurable, with the currently available therapeutic strategies producing a mean 5-year survival rate of 44.5%.\(^2\)

1.2. Therapeutic Approaches for MM

Conventionally, the therapeutic regimens implemented for the treatment of MM included alkylating agents such as melphalan, and corticosteroids, which can extend patient survival by an average of 3-4 years.\(^3\) Although these figures have been modestly improved by combining high-dose chemotherapy with autologous hematopoietic stem cell transplantation, not all patients are eligible for this regimen, and even in those that are, treatment is not curative, not all patients respond and disease relapse commonly prevails with complex drug resistant phenotypes.\(^4,5\)

Consequently, treatment strategies for MM are being reevaluated, and a major focus in the clinical landscape of this disease has now become the incorporation of regimens more suited for
the era of targeted therapeutics. The use of targeted therapeutic strategies for the treatment of human cancers is based largely on the premise that a comprehensive suppression of tumour burden cannot be achieved solely by aiming at the classical targets of cytotoxic chemotherapeutic drugs (e.g. deoxyribonucleic acid (DNA) or cytoskeleton) but also requires the targeting of other molecular pathways specifically identified to drive the behavior of tumour cells. Indeed, success has been, and continues to be achieved using molecular based therapies for various human tumours including breast and lung cancers, as well as certain subtypes of leukemia, which in turn provides evidence for the utility of therapeutic agents that explicitly target molecular vulnerabilities identified to drive the malignant behavior of tumour cells.

Within the past decade, ongoing efforts have significantly enhanced what is known about the underlying molecular pathogenesis of MM, providing a host of new therapeutic targets and vulnerabilities. As a result of such efforts, several novel classes of therapeutics have emerged including thalidomide and its more potent immunomodulatory analogs (IMiDs), the proteosome inhibitors bortezomib (also known as PS-341, Velcade®) and carfilzomib, and histone deacetylase inhibitors such as vorinostat. The in vitro pre-clinical studies that led to the identification of these classes of drugs as candidates for clinical development in MM involved classical drug sensitivity studies using both cultured tumour cells and BM stroma co-culture experiments, as well as subsequent efficacy studies in murine models of MM. Indeed, the promising pre-clinical results observed for molecular-based therapeutics such as IMiDs and bortezomib have since been translated from the bench to the bedside in Phase I, II and III clinical trials where consistently, these agents have demonstrated marked clinical activity even in patients with refractory and relapsed disease.

The success of these agents in the treatment of MM can, for the most part, be attributed to their ability to specifically target one, or more of the molecular aspects that drive the complex behavior of MM tumour cells. For example, reports of increased BM angiogenesis in MM, coupled with the known anti-angiogenic properties of thalidomide provided the empirical rationale for evaluating the utility of this agent as a treatment for MM, and remarkably, clinical responses were observed in nearly one-third of MM patients who had relapsed with disease or that were refractory to conventional therapies. Likewise, the rationale for evaluating the preclinical activity of bortezomib for MM came from an understanding of MM tumour biology, particularly as it pertains to the inhibitory action of bortezomib on nuclear factor-kappa B (NF-
κB) signaling, and the known role of this pathway in promoting the growth and survival of MM cells.\textsuperscript{11,12}

The use of these novel agents has significantly improved the outlook for patients with MM and their increased efficacy over traditional, non-targeted therapies provides the proof-of-concept that an enhanced understanding of MM can dramatically improve the management of this disease. Despite this, there remains a paucity of durable responses in the majority of patients, as shown by the lack of plateau in survival curves from clinical trials that evaluate the efficacy of these currently available molecular therapeutics.\textsuperscript{5,13} Furthermore, cumulative experimental evidence suggests that the anti-tumour activity of these agents is mediated through much more complex molecular mechanisms than previously anticipated, and although a number of putative molecular targets have since been identified, unified molecular mechanisms have not been defined. While substantial therapeutic challenges remain, a deeper understanding of MM molecular pathology is expected to greatly improve clinical response by guiding the rational development and implementation of novel targeted therapeutics.

### 1.3. Molecular Pathology of MM

**Normal and Malignant Plasma Cell Biology**

In normal B cell development, hematopoietic stem cell commitment begins in the BM, where the rearrangement of Ig heavy (IgH) and light (IgL) chain genes leads to the expression of cell surface Ig molecules, and subsequent migration of the resulting immature B cells from the BM to lymphoid organs. Upon exposure to antigenic stimulation, immature B cells can either differentiate into short-live plasma cells, which generally produce low-affinity IgM antibody responses, or alternatively, enter the germinal center (GC) of lymphoid organs. Within the GC, B cells undergo a series of DNA remodeling processes including somatic hypermutation (SHM) of IgH and IgL chain genes, as well as IgH chain class switching from IgM to IgG, IgA, IgD or IgE. These post-GC, isotype-switched B cells then migrate to the BM, where they terminally differentiate into mature and long-lived plasma cells that secrete large amounts of high-affinity antibody.\textsuperscript{14}
The process of B cell development and differentiation is highly dependent on a coordinated set of molecular regulators that ultimately dictate commitment to plasma cell fate. The guardian protein of the B-cell phenotype is paired box 5 (PAX5), a transcription factor that induces B cell-specific genes, and represses the expression of genes that are critical for the generation and survival of plasma cells, including B lymphocyte-induced maturation protein 1 (BLIMP1) and X-box binding protein 1 (XBP-1). Similarly, the B cell CLL/lymphoma (BCL) 6 transcription factor maintains the B cell phenotype and induces proliferation by down-regulating BLIMP1 expression. Activation of B cells in the GC leads to the up-regulation of interferon regulatory factor 4 (IRF4), which has been said to “license” B cells for plasma cell development by inducing the initial proliferative burst required for terminal differentiation, and the downregulation of BCL6 with concomitant loss of BLIMP1 repression. BLIMP1 is as a transcriptional repressor of PAX5, which as mentioned previously, serves to maintain B cell identity, and thus the overexpression of BLIMP1 results in the full engagement of B cell differentiation into plasma cells.

The insight that these observations have provided into the processes and regulation of normal plasma cell biology has been indispensable for elucidating the normal cellular counterpart of MM tumour cells. For example, DNA sequence analysis of IgH chain genes has revealed that MM tumour cells have undergone extensive SHM but lack intraclonal variation. This, combined with the fact that in the vast majority of cases these cells harbor an Ig isotype other than IgM, indicates that MM tumour cells have passed through the GC, but are no longer undergoing continuous influence of the SHM process. Moreover, microarray analyses of MM tumour cells reveal increased BLIMP1 expression, but low levels of PAX5, which is consistent with repression of the “B cell program” and enforcement of the “plasma cell program”. Finally, malignant plasma cells from MM patients and established human myeloma cell lines (HMCLs) generally express immunophenotypic markers that are reminiscent of long-lived plasma cells, including cluster of differentiation (CD) 138 and CD38. Taken together with the localization of MM tumour cells in the BM, these observations are highly indicative that the major identifiable myeloma tumour population consists of long-lived, antibody-secreting plasma cells.
Molecular Pathogenesis of MM

Like many other human cancers, the pathogenesis of MM seems to occur in a stepwise manner (Figure 1.3.1), with the initial transforming event producing a monoclonal plasma cell population that may stay quiescent for several years\textsuperscript{23}. This non-accumulating clonal population is recognized pathologically as a pre-malignant monoclonal gammopathy of undetermined significance (MGUS), and although debated for many years, it is now thought that MGUS consistently precede all cases of MM.\textsuperscript{24,25} The risk of transformation from MGUS to MM is approximately 1-2\% per year,\textsuperscript{26} and is hallmarked by the progressive accumulation of plasma cells within the BM (intramedullary myeloma), osteolytic bone lesions and end organ damage. Although patients with intramedullary disease may not present with clinical features (asymptomatic or smoldering MM), disease progression can lead to clinical features diagnostic of symptomatic MM (lytic bone disease, anemia, immunodeficiency and renal impairment), and in a fraction of patients, the occurrence of extramedullary tumours in sites such as blood, pleural fluid and skin. Generally representing the end-stages of disease with more aggressive and drug-resistant features, these extramedullary MM tumours are no longer dependent on signals from the BM microenvironment, and when involving the blood, are called primary or secondary plasma cell leukemia, depending on whether preceding intramedullary myeloma has been recognized.\textsuperscript{27} With very rare exceptions, extramedullary tumours are the source of most HMCLs.\textsuperscript{28}

\textbf{Figure 1.3.1 Stepwise pathogenesis of MM.} The progression of MM through different disease stages is generally associated with a distinct set of molecular characteristic and disease manifestations. The horizontal lines illustrate the timing of these events, with the dashed lines indicating some uncertainty.\textsuperscript{29,30} Although the major identifiable myeloma tumour population consists of terminally differentiated plasma cells, the initial transforming event likely occurs in a mature B cell during the GC reaction.
Molecular analyses and cytogenetic profiling have provided evidence that almost all cases of MM are characterized by extreme karyotypic instability and thus numerous chromosomal abnormalities.\textsuperscript{31} Furthermore, many of these molecular alterations are highly associated with the multistep process of MM tumourigenesis, as they are found to be present in both MGUS and MM cells from paired patient samples.\textsuperscript{32} Although karyotypes from MM cells are usually very complex, careful analyses of large patient datasets have shown that the disease can be subdivided into several subtypes, each associated with unique clinico-pathological features (Figure 1.3.2).\textsuperscript{33} Accounting for approximately 50\% of MM tumours, the hyperdiploid (HRD) group is associated with recurrent trisomies involving eight odd chromosomes (3, 5, 7, 9, 11, 15, 19, 21), a low incidence of structural chromosomal abnormalities, and a more favorable prognostic outcome.\textsuperscript{34} In contrast, non-hyperdiploid (NHRD) tumours are usually associated with a more aggressive malignant phenotype, and are characterized by a high prevalence of IgH translocations between five recurrent partners, which can further subdivide the classification of this malignancy. Those recurring translocations include Cyclin D1 (CCND1-11q13), fibroblast growth factor receptor 3/multiple myeloma, SET domain containing protein (FGFR3/MMSET-4p16), c-MAF (16q23), Cyclin D3 (CCND3-6p21), and MAFB (20q11).\textsuperscript{35} Although HRD and NHRD pathways are, for the most part non-overlapping,\textsuperscript{36} their associated genetic defects are thought to represent early events in the pathogenesis of MM that initiate transition to a recognizable tumour, with subsequent secondary translocations and/or acquisition of additional genetic events contributing to the progression of overt MM.\textsuperscript{29,30}
Figure 1.3.2 Model for molecular pathogenesis of MM. In a recently updated model for the molecular pathogenesis of MGUS and MM, Bergsagel et al. suggest a number of temporal oncogenic events involved in the progression of MM through different disease stages. NHRD and HRD, which are mostly non-overlapping pathways, are proposed to be involved in the initial transition from a GC B cell to a recognizable tumour population, with various secondary events associated with disease progression (Tx: translocation). Figure adapted from Bergsagel and Kuehl, *J Clin Invest*, 2012.

The classification of human tumours into molecular subtypes has undoubtedly had highly relevant clinical implications, particularly in the proper selection of treatment strategies. A classic example of this would be the classification of breast cancers based on receptor-status, and the resulting impact this has had for targeted therapeutics such as endocrine-based therapies for receptor-positive breast cancers, or trastuzumab (Herceptin) against HER2/neu-positive breast cancers. Although targeted therapeutics used for the treatment of MM are primarily based on molecular vulnerabilities that are specific to the malignancy, many demonstrate superior therapeutic efficacy in certain molecular subtypes, including bortezomib, which overcomes poor prognostic outcomes for t(4;14) tumours. Furthermore, there are clinical trials underway for subset-specific therapeutics, including the FGFR3 inhibitor TKI-258, which would presumably have clinical benefit for MM patients with t(4;14) translocations. Although ongoing studies in MM continue to highlight the intrinsic heterogeneity of these tumours and in turn the importance
of subtype-specific therapeutics, they have also shown that the malignant behavior of MM tumour cells is not solely determined by genetics alone.

MM and the BM Microenvironment

It is well accepted that most tumour cells do not exist in isolation, but are rather dynamically interacting with components of their microenvironment. This is particularly evident for MM tumours, which are hallmarked by a highly organized biological network collectively referred to as the BM stroma. Included in this molecular framework is a heterogeneous cellular population comprised of fibroblasts, macrophages, adipocytes, osteoblasts, osteoclasts and endothelial cells, as well as non-cellular components that include extracellular matrix (ECM) glycoproteins and a diverse milieu of soluble factors. The interactions between myeloma cells and components of the BM microenvironment activate a multitude of signaling pathways that contribute directly to the malignant behavior of MM tumours, influencing growth, survival and therapeutic response. Furthermore, non-cellular components such as growth factors and cytokines mediate cellular cross-talk between MM tumours and BM stroma that not only optimizes this microenvironmental niche for tumour cell growth, but also maintain a perpetual loop of autocrine and paracrine communication (Figure 1.3.3).

![Figure 1.3.3. Interactions between MM tumour cells and stromal cells in the BM microenvironment.](image)

The malignant behavior of accumulating MM tumour cells are critically influenced by components of the BM microenvironment including direct tumour-host interactions, adhesion to ECM proteins and a variety of soluble factors such as cytokines and growth factors. Collectively, these interactions perpetuate autocrine and paracrine loops of communication that optimize the BM niche for MM tumour growth.
Although the molecular components that comprise the BM microenvironment continue to be investigated for their hierarchical importance in MM tumour cell biology, accumulating evidence in both normal and malignant plasma cell biology indicates that the effect of one molecule in particular, the cytokine interleukin-6 (IL-6), has significant implications for the malignant behavior of MM tumours and is regarded as one of the most important cytokines in MM disease progression.

Human IL-6 was originally identified as a factor that stimulates antibody production in transformed B lymphoblastoid cell lines, supporting its direct involvement in the induction of B cell differentiation to antibody-secreting plasma cells. However, IL-6 has been further identified as a pleiotropic cytokine that participates in several hallmark processes during plasma cell terminal differentiation including down-regulation of surface major histocompatibility class II expression, increased expression of the cellular secretory apparatus, and cell cycle arrest. The direct involvement of IL-6 in the pathogenesis of plasma cell tumours was first suggested from experiments involving the mineral oil pristine. When injected into certain strains of mice, pristine was found to induce the formation of chromic granulomatous tissue, which under certain conditions, can lead to plasmacytosis and eventually the development of plasma cell neoplasms. The direct correlation between IL-6 and plasma cell tumours was subsequently demonstrated when pristine oil was found to induce the expression of interleukin-hybridoma/plasmacytomas-1, the murine homologue to human IL-6. In support of these observations, it was subsequently demonstrated that overexpression of human IL-6 in transgenic mice induces polyclonal plasmacytosis, and depending on the genetic background of the mice, the development of plasmacytomass.

Although IL-6 in the BM microenvironment is predominantly produced by stromal cells, it can also be secreted by MM cells in an autocrine manner. Regardless of the source, IL-6 has been shown to be a critical mediator of MM tumour cell growth and survival. In contrast to normal terminally differentiated antibody-secreting plasma cells that are usually non-proliferative and short lived, IL-6 has been shown to act as the primary growth factor for freshly isolated MM plasma cells, as well as several established HMCLs. For example, about 40-60% of HMCLs increase $^3$H-thymidine incorporation after stimulation with IL-6, and in fresh MM samples, IL-6
induces a similar proliferative response. Moreover, malignant plasma cells from primary MM patient samples cannot survive alone in vitro, and must be supplemented with exogenous IL-6, or co-cultured with IL-6-producing feeder cells, such as BM-derived stromal cells for long term culture. In addition to its effects on growth and survival, IL-6 has also been implicated in protecting MM cells from the apoptotic effects of certain anti-MM drugs such as dexamethasone. Accordingly, high levels of serum IL-6 have been reported in MM patients with aggressive or progressive disease and have in turn been associated with poor prognostic outcomes.

It is therefore clear that the malignant behavior of MM tumours is driven by both genetic background and tumour-host interactions, highlighting the heterogeneity and complex molecular etiology of this disease. This has in turn presented a significant barrier to identifying unified molecular vulnerabilities for therapeutic targeting that are inclusive of both genetic and microenvironmental influences. As such, it has been proposed that an integrated therapeutic strategy could be derived by considering the points of molecular convergence that mediate these aberrant signals. For this reason, oncogenic transcription factors have become appealing therapeutic targets in cancer since, ultimately, one effective anti-transcription factor drug could have the potential to combat the aberrant activity of various upstream molecules.

**Oncogenic Transcription Factors**

The aberrant activity of oncogenic transcription factors is most often mediated by an intrinsic genetic aberration such as an activating mutation or chromosomal translocation. Indeed, transcription factors such as c-Myc, Fos and Jun, whose constitutive activation is generally mediated by activating mutations, were among the earliest oncogenes to be identified. Similarly, the combined efforts of cytogenetic analysis, gene expression profiling and whole genome sequencing have revealed several oncogenic transcription factors that are intrinsically dysregulated in MM, including BLIMP1, IRF4, HOXA9, c-MAF, MAFB, and members of the NF-κB family of proteins. It is however, becoming increasingly apparent that even in the absence of activating mutations or chromosomal translocations, transcription factors can still become dysregulated and serve as a key step in the molecular pathogenesis of cancer. One such transcription factor proposed to be an important oncogenic regulator in MM is signal transducer
and activator of transcription (STAT) 3, which evidentially, has been shown to be universally activated by IL-6 in MM tumour cells.

1.4. STAT3: Signal Transducer and Activator of Transcription

STAT3 belongs to the STAT family of latent cytoplasmic transcription factors, of which there have been six other mammalian members described to date (STAT1, STAT2, STAT4, STAT5A, STAT5B, STAT6). Each STAT protein respectively responds to a defined set of signals emanating from specific receptor-ligand interactions at the cell surface. However, unlike other signal-responsive proteins that may rely on several downstream effector molecules for each step of a particular signal transduction pathway, STAT proteins, as their name suggests, play a dualistic role in the transmission and interpretation of intracellular messages.

STAT3 Signaling Pathway

In the context of STAT proteins, the specific cellular responses that are elicited by extracellular signals can be explained, at least in part, by the nature of the receptor that is stimulated and in turn, the particular STAT protein which is activated. For STAT3, this predominantly includes the IL-6 family of cytokines that signal through a heterodimeric receptor complex composed of two subunits: a common membrane-spanning, signal-transducing component, glycoprotein 130 (gp130), and a ligand-specific, non-signaling component, which for IL-6, has been identified as IL-6 receptor α (IL-6Rα). IL-6-stimulated engagement of the IL-6Rα/gp130 receptor complex triggers activation of downstream tyrosine kinases, particularly those of the Janus-activated kinase (JAK) family of which there have been four members described to date (JAK1, JAK2, JAK3, TYK2). Activated JAK proteins subsequently phosphorylate tyrosine residues (pTyr) within the cytoplasmic domain of gp130, which in turn serve as docking sites for Src homology 2 (SH2)-domain containing proteins. Although preferential recruitment of these downstream effector molecules is dependent on which gp130 tyrosine residue is phosphorylated, extensive studies have established that STAT3 plays a central role in mediating signals from IL-6 (Figure 1.4.1). Under resting conditions, STAT3 proteins are primarily located in the cytoplasm where they exist as monomeric molecules, but upon recruitment to the pTyr residue of cell surface
gp130, STAT3 is activated by JAK-mediated phosphorylation of a single tyrosine residue (Tyr705) within the carboxyl terminus. Once phosphorylated, STAT3 proteins dimerize through reciprocal interactions between the pTyr residue of one monomeric STAT3 and the SH2 domain of another, permitting nuclear localization and binding to specific response element sequences within the DNA regulatory regions of direct target genes. Interestingly, promoter studies have also revealed that many of these STAT3-binding sites are often in close proximity to binding sites for other transcription factors such as NF-κB and c-Jun, supporting potential cooperativity between these factors for transcriptional regulation of STAT3 target genes.

Figure 1.4.1 IL-6/JAK/STAT3 signaling pathway. Under basal conditions, STAT3 monomers are located within the cytoplasm. Upon IL-6 receptor engagement, JAK proteins mediate the phosphorylation (P) of Tyrosine (Y) residues within the cytoplasmic tail of gp130, which serve as docking sites for STAT3 monomers. When STAT3 proteins localize to the gp130 pY residues at the cell surface, they are in proximity to JAK proteins, which in turn mediate the phosphorylation of STAT3 Tyr705 residues. This leads to formation of STAT3 dimers, which translocate to the nucleus to induce the transcription of direct target genes. It is also worth noting that although STAT3 is predominantly phosphorylated by members of the JAK family of tyrosine kinases in the context of IL-6-mediated pathway activation, a growing number of additional proteins have also been implicated, including other cytoplasmic kinases, and several growth factor receptors with intrinsic kinase activity.
Oncogenic Potential of STAT3

STAT3 was first implicated in the oncogenic process following the observation that STAT3 DNA binding activity is significantly elevated in malignant breast carcinoma samples compared to normal breast tissue.66 The causal relationship between STAT3 and oncogenesis was subsequently confirmed by demonstrating the requirement for intact STAT3 signaling during neoplastic transformation by the cytoplasmic tyrosine kinase v-Src.67 To further solidify STAT3 as an oncogene, ensuing studies have shown that ectopic expression of a constitutively active mutant form of STAT3 (STAT3C) is sufficient to transform a variety of non-malignant cell types.68,69 Based on these early findings, STAT3 was initially thought to contribute to the tumourigenic process by triggering the expression of anti-apoptotic and pro-proliferative genes. However, there is now strong evidence that implicates aberrant STAT3 activation in oncogenic processes beyond survival and growth.

Over a decade ago, Hanahan and Weinberg suggested six essential alterations in cellular physiology that are fundamentally required for the malignant growth of cancer cells: (I) self-sufficiency in growth signals, (II) insensitivity to anti-growth signals, (III) evasion of apoptosis, (IV) limitless replicative potential, (V) sustained angiogenesis and (VI) tissue invasion and metastasis.70 As illustrated in Figure 1.4.2, STAT3 has been implicated in all six of these hallmarks, as well as several additional aspects of tumour biology, including tumour-promoting inflammation,71 evasion of tumour immune surveillance,72 and reprogramming of cellular energy metabolism.73 It is therefore clear that STAT3 represents a central node in the malignant process, and accordingly has been shown to be constitutively active in nearly 70% of human cancers,74 including prostate, breast, pancreatic, ovarian and brain tumours, as well as several hematologic malignancies such as lymphoma, leukemia, and MM. However, its role in the initiation, development and progression of tumours has been shown to vary with each type of tumour analyzed75, and as such, should be defined in the context of that particular cancer.
STAT3 and the Hallmarks of Cancer

- Evasion of apoptosis
  - Bcl-2, Bcl-X, Mcl-1, Survivin
- Sustained angiogenesis
  - VEGF, FGF2, HIF1α
- Limitless replicative potential
  - HTERT
- Dysregulation of cellular energetics
  - Hsp70, Hsp90, HIF1α
- Tumour-promoting inflammation
  - IL-6, IL-1β, IL-11, CC/2, CXCL12
- Self-sufficiency in growth signals
  - cyclinD1, c-myc, cdc2
- Insensitivity to anti-growth signals
  - p53, Smad7
- Tumour invasion and metastasis
  - Twist, MMP2, MMP9, ICAM-1
- Evasion of immune surveillance
  - IL-10, IFNβ, IFNγ

Figure 1.4.2 Implications of STAT3 in the hallmark characteristics of cancer. STAT3 has been shown to regulate the expression of numerous target genes, which are indicated below the original (blue) and emerging (green) hallmark characteristics of cancer that they are associated with. There are likely additional targets relevant to these STAT3-mediated biological outcomes that remain unidentified.

1.5. STAT3 in MM: Aberrant Expression and Functional Role

Given that IL-6 is thought to act as the primary growth factor for MM tumours, it is not surprising that aberrant STAT3 signaling has in turn been implicated in the disease pathology of MM tumour cells. Indeed, whereas blood or BM cells from healthy donors generally fail to show evidence of STAT3 activation, analyses of primary tumour cells from patients with MM, as well as established HMCLs, have consistently revealed that STAT3 is constitutively active in approximately 40-60% of MM tumours. These observations in turn raise two fundamental questions. 1) Given that, at present, there are no reported naturally occurring STAT3 gene mutations in MM, what is the genetic basis for its constitutive activation in MM tumour cells, and more importantly, 2) does the persistent state of STAT3 signaling directly contribute to the underlying pathogenesis of MM, or does it merely reflect a bystander effect with little physiological consequences.
In normal cells, the transient nature of STAT3 activation is mediated, in part, through a number of negative regulators, including cytoplasmic tyrosine phosphatases, and cytokine-induced proteins called suppressors of cytokine signaling (SOCS). Thus, it has been proposed that the persistent state of STAT3 activation observed in MM may be driven by mutations and/or deletions in the proteins that negatively regulate STAT3 activation. This is supported by promoter methylation studies that have revealed that key negative regulators of STAT3 phosphorylation such as the protein tyrosine phosphatase SHP-1 and SOCS3 are often inactivated in MM cells through epigenetic silencing.\textsuperscript{84,85}

Alternatively, and perhaps one of the more widely discussed mechanisms that may account for constitutive STAT3 activation in MM tumour cells involves the interaction between myeloma tumour cells and components of the BM microenvironment. This includes cell-to-cell and ECM adhesion, as well as soluble factors such as IL-6 and vascular endothelial growth factor (VEGF), which interestingly are both target genes of STAT3.\textsuperscript{76,78,86} For example, both IL-6 and adhesion to fibronectin results in a dramatic amplification of STAT3 phosphorylation, nuclear translocation and DNA-binding in MM tumour cells.\textsuperscript{87} Furthermore, the direct interaction between MM tumour cells and BM stroma has been shown to augment IL-6 secretion from stromal cells.\textsuperscript{47,88} In turn, IL-6-mediated activation of STAT3 in MM tumour cells upregulates the expression and secretion of VEGF, which acts in a paracrine manner to further potentiate the secretion of IL-6 from neighboring BM stroma.\textsuperscript{89} This ultimately leads to a persistent feedforward loop of paracrine and autocrine STAT3 activation, and in turn dysregulation of downstream STAT3 target genes that control proliferation, survival and drug resistance (Figure 1.5.1).
Figure 1.5.1. BM microenvironment-mediate mechanisms of constitutive STAT3 activation in MM tumour cells. Through the actions of autocrine signaling and paracrine communication between BM stroma and surrounding MM tumour cells, STAT3 signaling is proposed to be persistent activated, which in turn induces tumour cell survival by upregulating the expression of anti-apoptotic and pro-proliferative proteins.

In particular, IL-6-mediated activation of STAT3 has been shown to induce tumour cell survival by upregulating the expression of anti-apoptotic and pro-proliferative proteins such as Bcl-2, myeloid cell leukemia 1 (Mcl-1), BCL2-like 1 (Bcl-xL) and c-Myc in MM cells. These results are indeed consistent with the observation that among the 25 Bcl-2 members described to date, Bcl-xL, Bcl-2 and Mcl-1 have all been identified as direct target genes of STAT3. In addition, the role of Bcl-2 family members in MM have been subject to multiple studies, which have found that increased levels in Bcl-2, Bcl-xL and Mcl-1 expression are linked to MM cell survival and resistance to chemotherapeutic agents. In turn, the dysregulated expression pattern of these Bcl-2 family members has been said to separate the malignant phenotype of MM tumours cells from that of normal plasma cells.

The role of STAT3 in MM has been investigated in the context of tumour cell survival and proliferation using a number of direct and indirect methods. One of the earliest studies focusing on STAT3 in MM found that expression of a dominant-negative STAT3 mutant induced apoptosis in the U266 MM cell line, and that the anti-apoptotic effects of STAT3 in this context were mediated, at least in part, by the downregulation of key anti-apoptotic proteins Bcl-xL and Mcl-1. A number of other studies have alternatively investigated the role of STAT3 in MM by employing the specificity of ribonucleic acid (RNA) interference (RNAi) technology. For
example, Scuto et al. demonstrated that small interfering RNA (siRNA)-mediated knockdown of STAT3 in the KMS11 MM cell line resulted in 50% inhibition of tumour cell proliferation compared to cells transfected with control siRNA.\textsuperscript{96} Similarly, Ge et al. showed that RNAi-mediated knockdown of STAT3 in RPMI-8226 MM cells had a robust effect on tumorigenicity, significantly reducing soft agar-plated colony formation and marked inhibition of tumour growth in immunocompromised mice.\textsuperscript{97} Unfortunately, the downstream target genes of STAT3 that could potentially mediate these effects were not investigated in either study.

Emerging reports are now suggesting that STAT3 may also be involved in the process of MM tumourigenesis because of its implications in the generation and maintenance of the normal myeloma cell counterpart. As discussed previously, commitment to a plasma cell lineage is ultimately dependent on the synchronized modulation of transcription factors that are involved in the repression of a “B cell program” and enforcement of a “plasma cell program”. Interestingly, although these molecular circuits have been well studied in the context of B cell biology, STAT3 has recently been implicated in the transcriptional regulation of proteins that are necessary for committing to the plasma cell fate by regulating the balance between BCL6 and BLIMP1 expression.\textsuperscript{98} Specifically, it was shown that activation of STAT3 triggers the upregulation of BLIMP1, and induces the phenotypic and functional features of mature plasma cells such as Ig secretion.\textsuperscript{99} As these data support an important role for STAT3 in the generation of terminally differentiated plasma cells, it has been postulated that aberrant activation of this pathway may play a similar, and perhaps even universal role in the development and/or maintenance of MM tumour cells.

It is certainly clear that aberrant STAT3 signaling contributes significantly to underlying behavior of tumour cells in several human cancers. However, in MM, a number of inconsistencies have emerged and controversy still exists over the precise role, if any, that STAT3 plays in this malignancy. For example, although both primary MM tumour cells and a number of HMCLs express high levels Bcl-xL and Mcl-1, it has been demonstrated that in many cases, this occurs regardless of IL-6 levels or STAT3 activation status.\textsuperscript{100} From this observation, it has been suggested that MM cells may become independent of the IL-6/JAK/STAT3 pathway in the presence of BM stromal cells\textsuperscript{101}, and that in turn, STAT3 may not be a key player in anti-apoptotic and survival signaling that operates in MM tumour cells. Consistent with this, a large-scale RNAi screen aimed to identify therapeutic targets in MM was recently performed using the
same KMS11 MM cell line previously shown by Scuto et al. to be dependent on STAT3 for proliferation. Although this study’s screen of over 6000 genes identified several previously unknown molecular vulnerabilities in MM, STAT3 was not among them.\(^{102}\)

Nonetheless, these observations in MM, and those that have been outlined in numerous other human tumours, provide strong evidence that aberrant activation of STAT3 represents a critical mediator of the neoplastic process in cancer. This, coupled with evidence that STAT3 inhibition is well tolerated in normal cells,\(^{103,104}\) suggests that STAT3 inhibition would represent an excellent therapeutic strategy, with the potential of having broad clinical impact.

### 1.6. Targeted Therapeutics Aimed at STAT3

In contrast to many of the proteins found to be constitutively activated in cancer, naturally occurring activating mutations directly within the STAT3 gene have only been identified in a small subset of human tumours.\(^{105,106}\) Alternatively, the persistent activation of STAT3 observed in human tumours is predominantly driven by aberrant activity or expression of upstream kinase molecules. Consequently, major efforts to silence aberrant STAT3 activation have employed strategies that either directly target STAT3 itself, or indirectly target those dysregulated kinase proteins that are upstream of STAT3.

**Indirect Strategies to Inhibit STAT3**

Numerous upstream oncoproteins have been identified to constitutively activate STAT3 and consequently, have been targeted in efforts to develop cancer therapeutics. For example, Garcia et al. employed tyrosine kinase selective inhibitors in human breast cancer cell lines to demonstrate that c-Src family tyrosine kinases cooperate to mediate constitutive STAT3 activation. Upon treatment with the c-Src-selective inhibitor PD180970, STAT3 transcriptional activity was dose-dependently suppressed, which correlated with the inhibition of cell growth and the induction of apoptosis.\(^{107}\) Alternatively, aberrant activation of JAK2 that is driven by activating mutations (V617F) or chromosomal translocations (TEL-JAK2) has lead to the development of several small molecule JAK inhibitors such as AG490 and AZD1480, both of
which have demonstrated promising anti-tumour activity in a number of human cancers, including MM.\textsuperscript{96,108}

In contrast to constitutive kinase activation by activating mutations, the STAT3-activating oncoproteins described above can also be dysregulated by the aberrant expression of growth factors and cytokines. To address this, several groups have focused on inhibiting the activity of STAT3 by directly neutralizing these upstream soluble factors. For example, in light of the critical role that IL-6 is known to play in MM, and its ability to induce STAT3 activation, a tremendous amount of focus has been placed on developing monoclonal antibodies to IL-6. These efforts are further supported by reports of high serum IL-6 levels in patients with aggressive or progressive MM.\textsuperscript{109} The clinical translation of these observations have led to the development of a murine monoclonal antibody directed against IL-6, and its subsequent evaluation in clinical trials for MM patients. Although none of the studied participants from this trial achieved complete remission, a handful of patients exhibited marked inhibition of plasmablastic proliferation.\textsuperscript{110} The results of this trial therefore demonstrates the potential utility of IL-6 monoclonal antibody therapy in MM, and suggest that pharmacological targeting of aberrant STAT3 activity may have promise for the treatment of this disease.

Although strategies that target upstream regulators as a means to interfere with STAT3 signaling have provided valuable insight into the complexities of STAT3 signaling, they can also be associated with limitations. Given that these upstream proteins and soluble factors also regulate several other downstream targets in addition to STAT3, concerns have arisen regarding their ability to truly be targeted, and thus potentially warrant unwanted off-target effects. Conversely, since STAT3 itself can be activated by multiple intracellular and extracellular components, selected inhibition of these upstream molecules may not necessarily guarantee the abrogation of STAT3 signaling. Thus, a great deal of effort has been focused at identifying molecules that can directly block the function of STAT3.

**Direct Strategies to Inhibit STAT3**

Several different approaches have been explored to directly inhibit STAT3 and although the goal of these strategies is the same, they are each accomplished by targeting different regions within
the STAT3 protein. There are several distinct structural domains that comprise the STAT3 protein including the N-terminal, coiled-coil, DNA-binding, SH2, and the transactivation domains. As outlined in Figure 1.6.1, the majority of strategies to inhibit STAT3 have been targeted to the SH2 domain, the DNA-binding domain, or the N-terminal domain. Indeed, early reports of antisense oligonucleotides, dominant-negative STAT3 mutants and peptide-based mimetics provided proof-of-principle that STAT3 is a rational therapeutic target, however these approaches are hindered by limited cell permeability, poor in vivo stability, and immunogenic potential. To overcome these physiochemical and pharmacological barriers, many groups have shifted their focus to the development of non-peptidic small molecules that are likely to demonstrate properties more suited for potential clinical applicability. Among these small molecules are Withanacin and analogs, FLLL31 and FLLL32 from Curcumin, CJ-863 and analogs, STA-21 and analogs, and OPB-31121 (Otsuka), to name a few. Although these small molecules appear to have superior potency, the mechanisms by which they inhibit STAT3 remains unclear, and their anti-tumour effects are often argued to be mediated by other mechanism of actions, including inhibition of tyrosine kinases.

Figure 1.6.1 Schematic representation of the STAT3 structural domains targeted by STAT3 inhibiting molecules. The modular structure of STAT3 consists of an N-terminus domain that mediates dimer interactions and stabilizes DNA-dimer formation, while the coiled-coil domain participates in interactions with other proteins. The DNA-binding domain makes physical contact with STAT3-response elements in the promoters of target genes and is linked to the SH2 domain by a linker domain. The functional significance of the STAT3 SH2 domain was described previously to mediate recruitment and binding of STAT3 monomers to the pTyr residues of activated receptors and also to participate in reciprocal interactions with STAT3 pTyr resides for the formation of dimers. The pTyr705 residue of STAT3 is located within the C-terminal transactivation domain, which mediates the transcriptional activation of target genes. Currently identified STAT3 inhibitors and their predicted sites of interaction are indicated below.

Surprisingly, although many of these compounds have demonstrated antitumor effects in vitro and in vivo, there are currently only two known clinical trials evaluating the therapeutic potential
of STAT3 inhibition. This includes a STAT3 decoy oligonucleotide (University of Pittsburgh), however, because of their physiochemical characteristics, decoy oligonucleotides have several limitations, particularly as it pertains to their pharmacokinetic and pharmacodynamic properties. Alternatively, the small molecule STAT3 inhibitor, OPB-31121 (Otsuka), has reached clinical assessment, and is currently being evaluated under a Phase I trial in subjects with advanced solid tumours (MD Anderson Cancer Center: NCT00955812). However, since it has yet to be demonstrated whether OPB-31121 inhibits STAT3 directly or indirectly, its precise mechanism of action remains unclear.

1.7. Rationale

The use of molecular tools that inhibit proteins upstream of STAT3 or inhibit STAT3 activity directly have provided further insight into the role of this oncogenic transcription factor in MM. However, the conclusions drawn from such investigations should be interpreted with caution, as it is unclear whether the tools employed for inhibition were in fact explicitly specific for STAT3. It is therefore paramount that the functional effects of STAT3 inhibition in MM cells be performed using specific and targeted molecular tools. Furthermore, given that conflicting reports have emerged regarding the role of STAT3 in MM, it is clear that additional studies are needed to truly validate this protein as a therapeutic target in this malignancy. Finally, despite substantial efforts to develop suitable candidates, none of the available small molecule STAT3 inhibitors with clearly defined mechanisms of inhibition have been evaluated in a clinical setting. Thus, if STAT3 truly is a therapeutic target in MM, novel and clinically relevant strategies to inhibit its aberrant activity are needed.

1.8. Hypothesis and Experimental Aims

We therefore hypothesize that STAT3 signaling contributes to survival of MM tumour cells predominantly through the activation of anti-apoptotic and survival signals and thus, novel small molecule STAT3 inhibitors will demonstrate potent anti-MM activity.
The aims of this study are:

1. **Validate STAT3 as a therapeutic target in MM:** Determine whether small hairpin RNA (shRNA)-mediated knockdown of STAT3 affects survival of HMCLs that express constitutively phosphorylated STAT3. Evaluate the resulting changes in downstream survival-related STAT3 target genes that may mediate the effects of STAT3 knockdown on MM cell survival.

2. **Pre-clinically evaluate the anti-MM activity of small molecule STAT3 inhibitors in vitro:** Perform a preliminary screen of novel small molecule STAT3 inhibitors for their anti-tumour activity against HMCLs that harbor constitutively phosphorylated STAT3. The lead compound to emerge will be further evaluated in extensive in vitro pre-clinical efficacy studies involving both classical drug sensitivity assays on a panel of genetically heterogeneous HMCLs and primary patient samples, as well as compound activity assessment in BM stroma co-culture models.

3. **Translate findings from Aim 2 into in vivo efficacy studies using a STAT3-driven luciferase reporter xenograft model of MM.** Establish xenograft model using MM cells that harbor a STAT3-driven luciferase reporter construct to evaluate the ability of the lead compound to inhibit STAT3 transcriptional activity in vivo. Evaluate the in vivo anti-MM activity of the lead compound as a single agent and in combination with subtherapeutic doses of bortezomib.
Chapter 2
Validating STAT3 as a Therapeutic Target in MM

2.1. Objectives

The first aim of this investigation is to validate STAT3 as a therapeutic target in MM. To establish the functional significance of constitutive STAT3 activation in MM, we will employ RNAi technology to specifically knockdown STAT3 expression in a selection of HMCLs found to harbor constitutively phosphorylated STAT3. RNAi-mediated gene silencing is an evolutionarily conserved process that has become a powerful tool for investigating gene function. For the purposes of efficient, stable and long-term gene silencing studies, RNAi typically involves the generation of shRNA, whose antisense strand is complementary to the gene-of-interest’s mRNA transcript, followed by delivery of these shRNA to cell populations through lentiviral expression cassette vectors. This strategy can therefore be used to directly knockdown, or silence, the expression of specific endogenous proteins, and in turn, is particularly valuable for the application of therapeutic target validation.

As a transcription factor, the oncogenic effects of STAT3 are mediated, at least in part, through the expression of its target genes. Thus, to verify that the degree of STAT3 knockdown is sufficient to abrogate its activity as a transcription factor, knockdown experiments will be performed in HMCLs engineered to contain a STAT3-driven firefly luciferase reporter construct, thus enabling the assessment of STAT3 transcriptional activity.
2.2. Methods

**Culture of HMCLs.** All HMCLs (ATCC-H929, RPMI-8226, JJN3, UTMC2, XG6, XG7, MM1.S, SKMM2) were grown and maintained in Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 µg/ml penicillin and 100 µg/ml streptomycin (Gibco, Grand Island, NY, USA). Cell lines were maintained routinely in a humidified chamber at 37˚C and 5% carbon dioxide.

**Generation of stable STAT3 reporter construct cell lines.** Target cells were infected with replication incompetent, VSV-g pseudotyped lentiviral particles containing the STAT3-driven firefly luciferase reporter constructs (pCignal Lenti-STAT3TRE-FLuc). Transductions were performed with polybrene (8 µg/µl) in accordance with the Cignal Lenti Reporter Assay Kit (SA Biosciences, Frederick, MD, USA). The pCignal Lenti-STAT3TRE-FLuc reporter construct is under the control of a basal promoter element (TATA box) joined to tandem repeats of a specific STAT3 transcriptional response element (TRE), and regulates the expression of the mammalian codon-optimized, non-secreted form of the firefly luciferase gene. Stably transduced cells were selected using puromycin (2 µg/µl) for 2 weeks.

**Analysis of STAT3-driven luciferase activity.** STAT3 transcriptional activity was quantified using the ONE-Glo™ + Tox Luciferase Reporter and Cell Viability assay (Promega, Madison, WI, USA). Target cell suspensions (100 µl of 1-2 x 10^4 cells) were plated in a 96-well, white opaque-walled assay plate. To control for variability in cell numbers/viability, cells were incubated with 20 µl of Cell Titer-Fluor™ reagent for 30 minutes at 37˚C and fluorescence quantified (380 nmEX/505 nmEM). The Cell Titer-Fluor™ reagent is a cell permeable substrate that produces a fluorescent signal only following protease-mediated cleavage in live cells. To measure luciferase expression, 100 µl of ONE-Glo™ reagent was added to each well, incubated for 3 minutes and light output quantified with a luminescence plate reader (Molecular Devices FlexStation 3). Relative luciferase units (RLU) were calculated by normalizing luminescent to fluorescent signals and background subtracted (medium plus serum only).

**Production of shRNA-containing lentivirus.** Transfection quality plasmid DNA (pLKO.1-shSTAT3, pLKO.1-shRFP, pMD2.6-VSV-G, and psPAX2) was kindly provided by Dr. Jason
Moffat (Banting & Best Department of Medical Research, Toronto, ON, CAN). HEK293T cells (2 x 10⁶ cells in 3 ml Dulbecco’s Modified Eagle Medium (DMEM) + 10% FBS) were plated in 60 mm culture dishes the day before transfection. The cells were co-transfected with 3.65 µg of pLKO.1-shSTAT3 (TRCN0000329887; 5′-CCGG-GCACAACTCTACGAAAGAATCAA-CTCGAG-TTGATTTCTCGTAGATTGTG C-TTTTTG-3’, STAT3 mRNA target sequence underlined), or control plasmid containing red fluorescent protein (RFP)-targeting shRNA (pLKO.1-shRFP), 2.75 µg of psPAX2 packaging plasmid and 1.1 µg of pMD2.6-VSV-G envelope plasmid using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA). The following day, cells were washed once with sterile phosphate buffered saline (PBS) and fed with 3 ml of fresh DMEM medium containing 10% FBS. Medium containing virus was collected 48 hours after initial transfection, and centrifuged to remove residual cells/debris. Viral supernatants were cleared using a 0.45 µm pore-size filter, aliquoted and stored at -70°C.

To determine the titer of viral supernatants, HEK293T cells were seeded in 24-well plates and cultured until 70-80% confluent. The cells were transduced with serial dilutions of viral stock (1 x 10¹ to 1 x 10⁴) in the presence of 4 µg/ml polybrene. The following day, culture medium was replaced with fresh medium containing puromycin (2 µg/ml). Puromycin-resistant colonies were counted 10 days after transduction and used to calculate the number of transducing units/ml of viral supernatant.

**Lentiviral shRNA-mediated knockdown of STAT3.** XG6, RPMI-8226 and JJN3 myeloma cell lines were plated at 5 x 10⁵ cells/ml in 6 ml of culture medium (10% FBS) containing 8 µg/ml of polybrene. Titered lentiviral supernatants were added to cells at an optimal multiplicity of infection (XG6 - 5:1, RPMI-8226 – 8:1, JJN3 – 10:1) and incubated overnight at 37°C. The following day, cells were washed and resuspended in fresh growth media. Assays employed for the analysis of knockdown experiments were performed 48 hours post-transduction for the XG6 cell line, and 72 hours post-transduction for both RPMI-8226 and JJN3. Although the pLKO.1 plasmid contains a puromycin resistance gene for selection of cells harboring stable shRNA integration, our knockdown experiments were performed in cell lines containing the STAT3-driven firefly luciferase reporter constructs, which also contain a puromycin resistance gene. Although we were therefore unable to select for stable shRNA-mediated target gene knockdown, transduction of the same cell lines with green fluorescent protein (GFP)-expressing plasmids resulted in >90% GFP⁺ expressing cells, and thus we were confident that the majority of cells
transduced would express the shRNA of interest. Furthermore, functional assays to evaluate the biological consequence of STAT3 knockdown were performed within 48-72 hours of transduction, reducing the likelihood of untransduced cells outgrowing those that were successfully transduced.

**Real-time polymerase chain reaction (RT-PCR).** RNA from XG6, RPMI-8226 and JJN3 cells were extracted using the Trizol method, according to the manufacturer’s instructions (Invitrogen Corporation, Carlsbad, CA, USA), followed by quality/quantity assessment by spectrophotometric analysis (Nanodrop ND-1000). First strand complementary DNA synthesis of isolated RNA (200 ng) was performed using the GeneAmp® Gold RNA PCR Kit with MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). The thermocycler conditions were as follows: 1 x 25˚C for 10 minutes; 1 x 42˚C for 12 minutes; hold at 4˚C for 5 minutes.

RT-PCR reactions were performed with Power SYBR® Green Master Mix and Reagents Kit (Applied Biosciences, Foster City, CA, USA) using the Applied Biosystems Sequence Detection System (SDS7900HT). The amplifications were initiated at 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 60 seconds, with a final step of 95˚C for 15 seconds, 60˚C for 15 seconds and 95˚C for 15 seconds. Amplifications were performed using the SABiosciences RT² qPCR Primer Assay for Stat3 (PPH00708F; Frederick, MD, USA), and with custom primer pairs from Sigma for c-Myc, glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and human ribosomal protein h36B4 as follows:

**c-Myc**
Forward primer 5’ – AAGGGTCAAGTTGGACAGTGTC – 3’
Reverse primer 5’ – TCCTGCAATTTCGGTGTGG – 3’

**Gapdh**
Forward primer 5’ – GAAGGTGAAGGTCGGAGTC – 3’
Reverse primer 5’ – GAAGATGGTGATGGGATTTC – 3’

**h36B4**
Forward primer 5’ – TGGAGACGGATTACACCTTCC – 3’
Reverse primer 5’ – TCTTCCTTGGCTTCAACCTTA – 3’
Gapdh and h36B4 were used as housekeeping genes to normalize Stat3 and c-Myc expression, respectively, while RNA from the OCI-MY5 and PE-1 HMCLs were used as control samples validated to have low expression of Stat3 and c-Myc, respectively. All samples were run in triplicate and data analyzed using the following formula:

\[ \Delta Ct = Ct(\text{target gene}) - Ct(\text{Gapdh/h36B4}) \]

\[ \Delta\Delta Ct = \Delta Ct - \Delta Ct(\text{control sample with low expression}) \]

\[ \text{Copy number} = 2^{\Delta\Delta Ct} \]

**Immunoblot analysis.** Target cells were harvested and washed twice in ice cold PBS. The resulting cell pellets were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetra acetic acid (EDTA), and 1% NP-40), supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM sodium vanadate (Na₃VO₄) and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) for 30 minutes on ice. Protein lysates were collected by centrifugation at 14,000 rpm for 15 minutes. Protein concentration was determined by Bradford Assay (Thermo Scientific, Rockford, IL) and normalized with lysis buffer before the addition of β-Mercaptoethanol-supplemented Lamelli sample buffer (Bio-Rad Laboratories, Hercules, CA). Proteins (10-30 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7-10% gels, and transferred to polyvinylidene fluoride (PVDF) membranes using wet transfer at 70 V for 1 hour. Membranes were rinsed in Tris-buffered Saline with 0.01% Tween-20 (TBST) and blocked for 1 hour at room temperature in TBST containing 5% bovine serum albumin (BSA) powder, followed by overnight incubation with primary antibodies at 4°C. Primary antibodies against the indicated proteins were diluted in TBST with either 5% BSA or 5% milk, as specified by manufacturer. Following three 15-minute washes in TBST, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Thermo Scientific Pierce, Rockford, IL) diluted 1:4000 in TBST for 1 hour at room temperature. Membranes were developed using the enhanced chemiluminescence kit (Perkin Elmer, Waltham, MA) according to the manufacturer’s instructions and visualized by autoradiography. Resulting autoradiographs were analyzed by densitometry using the Gel Doc XR station and Quantity One Software (Bio-Rad, Hercules, CA).
**Cell death assay.** Cell death was assessed by flow cytometric analysis of apoptosis. Target cells were washed once in PBS and resuspended at 5-10 x 10⁵ cells/ml in apoptosis binding buffer (100 mM HEPES/NaOH, pH 7.5 containing 1.4 M NaCl and 25 mM CaCl₂) from the TACS Apoptosis Detection Kit (R&D Systems, Minneapolis, MN, USA). Approximately 100 µl of the cell suspension was transferred to a polystyrene tube and incubated with 2 µl of Annexin V-Fluorescein isothiocyanate (FITC) conjugate and 5 µl Propidium Iodide (PI) solution. Following incubation of tubes for 10 minutes at room temperature, 100 µl of binding buffer was added and samples analyzed by flow cytometry. All samples were analyzed on a BD FACSCalibur™ flow cytometer (BD Biosciences) programmed to collect a minimum of 10,000 events. Results were analyzed using Flowjo v7.6 (Tree Star, Ashland, OR, USA) and data are presented as the sum of percent Annexin V+/PI⁻ and Annexin V+/PI⁺ cell populations.

**Statistical analysis.** Statistical analysis of this section was not performed, as experiments had not been independently repeated. Graphs with error bars represent the mean of triplicate replicates ± standard deviation (SD).
2.3. Results

Evidence for aberrant activation of STAT3 in myeloma

Using Tyr705 phosphorylation as a surrogate marker of STAT3 activation, the extent of baseline STAT3 activation was first examined in a panel of genetically heterogeneous HMCLs. Whole cell lysates prepared from HMCLs in log growth conditions were subject to immunoblot analysis and probed with antibodies against pSTAT3 (Tyr705) and total STAT3 protein. Although STAT3 protein was expressed in all HMCLs, albeit to varying degrees, constitutively phosphorylated STAT3 was only detected in 6 of 8 HMCLs, with two cell lines, MM1.S and SKMM2, lacking detectable pSTAT3 (Figure 2.3.1, left). Densitometric analysis performed on immunoblots to quantitate the ratio of pSTAT3 to total STAT3 protein confirmed variability in baseline STAT3 phosphorylation in HMCLs (Figure 2.3.1, right), indicating that the STAT3 pathway might only be activated in a subset of MM tumour cells.

![Figure 2.3.1 Analysis of STAT3 activation status in HMCLs](image)

**Figure 2.3.1 Analysis of STAT3 activation status in HMCLs.** Cells were harvested during the exponential growth phase and whole cell lysates subject to immunoblot analysis of Tyr705 pSTAT3 and total STAT3 (left). Relative expression of pSTAT3 and total STAT3 is indicated below respective immunoblots and was calculated as the ratio of the densitometry signal for pSTAT3 or total STAT3 relative to GAPDH in each sample. GAPDH-normalized densitometry signals of the pSTAT3 to STAT3 ratio were calculated, and represented graphically (right) for HMCLs that demonstrated phosphorylation of STAT3 by immunoblot analysis.

Development and verification of STAT3 knockdown in HMCLs

To establish the functional significance of pSTAT3 protein expression in HMCLs, RNAi was employed to specifically knockdown STAT3 expression in cell lines harboring the highest levels
of STAT3 activation, which according to Figure 2.3.1 included XG7, JJN3, XG6 and RPMI-8226. Lentiviral-based pLKO.1 vectors were developed to express an RNAi-inducing shRNA transcript under the control of the human U6 promoter. The shRNA sequence used to target STAT3 (shSTAT3) was designed to specifically target a region within the coding sequence of STAT3 mRNA, and by sequence alignment analysis, was able to target all isoforms of the STAT3 transcript. To control for non-specific effects of RNAi, a second shRNA sequence expressed from the same pLKO.1 vector was included, but instead targeting RFP (shRFP). Unfortunately, the efficiency of STAT3 knockdown was extremely poor in XG7 cells as assessed by immunoblot analysis of STAT3 protein and the STAT3-driven firefly luciferase assay (data not shown), and thus, this cell line was not included in our analysis.

To verify STAT3 silencing in XG6, RPMI-8226 and JJN3 cell lines, shRNA-transduced cells were first subject to RT-PCR analysis, which revealed a 69-75% decrease in STAT3 mRNA from shSTAT3-transduced cells, compared to shRFP control (Figure 2.3.2A). For further confirmation of the knockdown, immunoblot analysis of total STAT3 protein was performed on whole cell protein lysates collected following shRNA-transduction. As depicted in Figure 2.3.2B and consistent with the observed decrease in STAT3 mRNA, cell lines transduced with shSTAT3 constructs demonstrated a marked reduction in STAT3 protein (61-71%), compared to shRFP control cells.

![Figure 2.3.2](image)

**Figure 2.3.2 Lentiviral shRNA-mediated knockdown of STAT3 mRNA and protein.** Following lentiviral transduction with STAT3 (shSTAT3) or control (shRFP) shRNA constructs, total RNA and protein were extracted from HMCCLs for respective analysis of STAT3 mRNA by RT-PCR (A) and protein expression by western blot (B). Relative STAT3 mRNA expression was calculated according to formula outlined in methods, and normalized to shRFP control, with results presented as the mean of triplicate replicates ± SD. Total STAT3 protein was evaluated by immunoblot analysis and quantified by densitometry. GAPDH-normalized densitometry signal intensity from immunoblot analysis shown below (shSTAT3 relative to shRFP control).
To evaluate whether the observed degree of STAT3 mRNA and protein suppression was sufficient to abrogate STAT3 transcriptional activity, cell lysates collected from the same shRNA-transduced cells were subject to analysis using the STAT3-driven firefly luciferase assay. Although shRNA-mediated knockdown of STAT3 was shown to reduce STAT3 mRNA and protein levels by at least 60%, STAT3 transcriptional activity was only moderately suppressed, with an approximately 30% (27-34.5%) reduction in STAT3-driven firefly luciferase expression compared to shRFP control cells (Figure 2.3.3).

Figure 2.3.2 Effects of STAT3 knockdown on STAT3-driven luciferase expression. Lysates from shRNA-transduced cells were subject to analysis using the Cell Titer-Fluor™ (to control for cell viability) and ONE-Glo Luciferase Assay Reagent™ System. Data are presented as relative luciferase units (RLU), which were calculated as ratio of luciferase to fluorescence signal intensities, and are plotted as the mean RLU from duplicate readings ± SD (relative to shRFP controls).

Characterizing the functional effects of STAT3 knockdown on myeloma cell survival

It is well reported that aberrant activation of STAT3 is implicated in promoting cell survival and anti-apoptotic signals in a variety of solid tumours and hematological malignancies, including MM. To determine whether STAT3 plays a similar role in this particular set of HMCLs, the effects of STAT3 knockdown on cell survival and apoptosis were evaluated. Cells transduced with lentiviral shRNA were first subject to flow cytometric analysis of apoptosis using Annexin V staining to identify cells undergoing apoptosis. Cells were also stained with the non-vital dye PI to distinguish between apoptotic and necrotic cell populations. The scatterplots derived from this analysis demonstrated an increased percentage of cells located in the lower right quadrant from shSTAT3 cells, indicating an increase in apoptotic cells upon knockdown of STAT3.
(Figure 2.3.4A). As represented graphically in Figure 2.3.4B, the percentage of cells staining positive with Annexin V-FITC (PI⁺ and PI⁻) increased in all cell lines when STAT3 was knocked down, however the degree of shSTAT3-induced apoptosis was variable across the three HMCLs. Compared to the shRFP control, knockdown of STAT3 in RPMI-8226 induced the highest levels of apoptosis, as demonstrated by a 3.06-fold increase in the percentage of Annexin V⁺ cells, whereas in XG6 and JJN3, STAT3 knockdown induced a 2.03-fold and 1.3-fold increase in apoptosis, respectively.

To further confirm the induction of apoptosis following knockdown of STAT3, whole cell lysates from shRNA-transduced cells were collected and subject to immunoblot analysis for the detection of an additional marker of apoptosis, cleaved Poly ADP-ribose polymerase (cPARP). During the process of programmed cell death, PARP proteins are cleaved by proteases such as caspase-3, with the resulting cleavage fragment facilitating cellular disassembly, and thus serving as a marker of cells undergoing apoptosis.113,114 In all three HMCLs, immunoblot analysis of shRFP-control samples revealed the presence of a 116 kDa band representing the full-length PARP protein (Figure 2.3.4C). However, in STAT3 knockdown samples, both the full-length PARP protein and the 89 kDa cPARP fragment were detectable, consistent with flow cytometry experiments and the induction of the apoptotic program.

Figure 2.3.3 Analysis of STAT3 knockdown-induced apoptosis. Following mock (polybrene), control (shRNA) or STAT3 (shSTAT3) infection, HMCLs were incubated with Annexin V-FITC and PI for analysis of apoptosis by flow cytometry. Scatterplots of Annexin V/PI staining (A) were used to calculate the percentage of cells staining positive for Annexin V (PI⁺/PI⁻) from flow cytometric analysis (B). Whole cell lysates collected from the same lentiviral transfection experiments were subject to immunoblot analysis and probed with a primary antibody able to detect the full-length PARP protein (116 kDa) and the 89 kDa cPARP fragments (C).
Knockdown-induced modulation of STAT3 target gene expression

The results thus far have demonstrated that shRNA-mediated knockdown of STAT3 reduces the level of STAT3 transcriptional activity, and induces a pro-apoptotic phenotype in MM cells. Although STAT3 is known to regulate the expression of numerous downstream target genes that are implicated in a variety of biological processes, the observed effect that STAT3 knockdown had on MM cell survival directed our focus to elucidating the effects of silencing STAT3 signaling on anti-apoptotic, survival and pro-proliferative genes, including Bcl-2, Bcl-xL, Mcl-1, Survivin and c-Myc. It should be further noted that many of the genes regulated by STAT3 have extremely short protein half-lives, and that variability in the kinetics of protein turnover can be a confounding factor when evaluating knockdown-induced changes in gene expression. This has been particularly apparent in evaluations of c-Myc expression, and thus, in acknowledgement, shRNA-transduced cells were first subject to RT-PCR analysis to assess the effects of STAT3 knockdown on c-Myc mRNA expression. Compared to shRFP-transduced cells, c-Myc mRNA expression was reduced in shSTAT3-transduced XG6 and RPMI-8226 cells by 29% and 22%, respectively (Figure 2.3.5A), which is consistent with the previously demonstrated effects of shSTAT3 on STAT3 transcriptional activity in these cells. In contrast, c-Myc mRNA expression remained unchanged in shSTAT3-transduced JJN3 cells (Figure 2.3.5A), despite having demonstrated a significant decrease in STAT3-drive firefly luciferase expression. We speculate that these cell type-dependent differences may reflect differences in how STAT3 regulates the expression of its target genes, and in turn, may account for the more modest pro-apoptotic effects that STAT3 knockdown had in JJN3 cells compared to RPMI-8226 and XG6.

To further assess the effects of STAT3 knockdown on other survival and anti-apoptotic target genes, whole cell lysates from shRNA-transduced cells were subject to immunoblot analysis. As depicted in Figure 2.3.5B, the effects of STAT3 knockdown on Mcl-1 and Bcl-2 protein expression were similar in all three HMCLs, with respective decreases of 22-39% and 30-54%, compared to shRFP control. Conversely, although Bcl-xL protein expression was reduced in three HMCLs, the extent of this decrease was variable. While shSTAT3-transduced RPMI-8226 cells demonstrated nearly complete absence of Bcl-xL protein expression (96% suppression), only modest reductions of 30% and 27% were observed for respective shSTAT3-transduced XG6 and JJN3 cells, compared to shRFP-transduced controls (Figure 2.3.5B). Interestingly,
although the pro-survival protein Survivin has been identified as a direct gene target of STAT3, shSTAT3-transduced HMCLs consistently resulted in an increased level of Survivin protein compared to shRFP control cells.

Figure 2.3.4 Analysis of HMCL-specific modulation of STAT3 targets following STAT3 knockdown. Total RNA and protein were extracted from shSTAT3- or shRFP-transfected cell lines for respective analysis of STAT3 target gene/protein expression by RT-PCR and immunoblot. The expression of c-Myc mRNA was quantified by RT-PCR (A), with resulting data normalized according to the formula provided in the methods section. Results of this analysis are presented as the mean of triplicate replicates ± SD, relative to shRFP control. Whole cell lysates from shRNA-transduced cells were subject to immunoblot analysis and probed with antibodies for Mcl-1, Bcl-2, Bcl-xL and Survivin, with GAPDH-normalized densitometric intensities relative to shRFP control indicated below (B).
2.4. Summary

The first section of this study provides evidence that STAT3 is constitutively active in a subset of HMCLs, and that in three cell lines (XG6, RPMI-8226 and JJN3), shRNA-mediated silencing of aberrant STAT3 signaling significantly impacts MM cell survival through apoptotic cellular responses. Lentiviral transduction of STAT3-targeting shRNA demonstrated the ability to reduce STAT3 mRNA and protein, with concomitant suppression of STAT3 transcriptional activity as detected using a stably expressed STAT3 reporter construct and analyzed by luciferase assay. By abrogating STAT3 signaling by lentiviral shRNA, molecular markers of apoptosis were evident in all three HMCLs, including cell surface Annexin V staining and cleavage of PARP proteins, as detected respectively by flow cytometric and immunoblot analysis. These studies also provide mechanistic insight into how aberrant STAT3 signaling in MM can maintain tumour cell survival, with knockdown of STAT3 consistently reducing the levels of anti-apoptotic Bcl-2 proteins, including Mcl-1, Bcl-2 and Bcl-xL. Noteworthy results from these studies also include the potential cell-type dependent regulatory mechanisms for c-Myc expression, and the possible STAT3-independent mechanism regulating the expression of Survivin. Nonetheless, this section has demonstrated the validity of STAT3 as a therapeutic target in at least a subset of MM tumour cells, and provides the rationale for evaluating the efficacy of molecular therapeutics that target aberrant STAT3 activation.
Chapter 3

*In vitro* Pre-clinical Evaluation of Small Molecule STAT3 Inhibitors in MM

3.1. Objectives

The collective results obtained from RNAi studies provide strong rationale for evaluating the efficacy of STAT3-targeting therapeutics in MM. As previously outlined, several strategies have been evaluated to directly target aberrant STAT3 activation, including antisense oligonucleotides and peptide-based pTyr mimetics. However, the majority of these strategies have failed to pass animal testing for reasons such as limited cell permeability, poor metabolic stability and other pharmacokinetic liabilities. Alternatively, the physiochemical properties of small molecules have been said to confer pharmacological properties more suitable for clinical application, and as such, there is a critical need for the development and optimization of novel small molecule STAT3 inhibitors.

In collaboration with Dr. Patrick Gunning from the Department of Medicinal Chemistry (University of Toronto), we aimed to evaluate the anti-MM activity of a novel series of small molecule STAT3 inhibitors. This BP-series of compounds was synthesized rationally by performing extensive structure activity relationship analysis of a known STAT3-SH2 domain binder, S31-201. S31-201 was identified through an *in silico* high-throughput screen, and led to the development of first generation tolyl-N-alkyl and perfluorobenzene-N-alkyl compounds SF-1-066 and BP-1-102, respectively. Both SF-1-066 and BP-1-102 contain salicylic acid functional groups, which have been shown to act as a pTyr mimetic. Molecular docking studies have shown that both compounds bind the SH2 domain of STAT3 and thus, adopt the inhibitory strategy of selectively inhibiting the phosphorylation and dimerization of STAT3 monomers (Figure 3.1.1). These parental compounds have also been evaluated *in vitro*, where they demonstrate potent antitumour activity against prostate, breast and leukemic cell lines.\(^{115}\)

In an effort to optimize potency and pharmacokinetic properties, Dr. Gunning’s group has synthesized a novel library of second-generation salicylic acid-based inhibitors derived from the parental tolyl-N-alkyl and perfluorobenzene-N-alkyl small molecules (SF-1-066 and BP-1-102, respectively). To ensure that these derivatives retained the ability to inhibit STAT3, fluorescence
polarization (FP) assays were employed to screen those small molecules that bind to the STAT3 SH2 domain and thereby inhibit STAT3 activity. The results of this analysis can be found in Appendix A.

Figure 3.1.1 Inhibitory strategy of BP-series of small molecules. By binding to the STAT3 SH2 domain, the BP class of small molecule inhibitors is predicted to inhibit multiple levels of the STAT3 signaling pathway. Small molecule-mediated blocking of the STAT3 SH2 domain will inhibit STAT3 recruitment to pTyr residues of activated receptors, which in turn will inhibit the ability of JAK proteins to mediated STAT3 Tyr705 phosphorylation. Together, this will abrogate STAT3 dimerization and thus ultimately, inhibit STAT3 nuclear localization and activation of target gene expression.

The aim of the current study is to evaluate the pre-clinical efficacy of these small molecule STAT3 inhibitors by first screening five candidate compounds that have been shown to potently bind the STAT3 SH2 domain (FP assay), including BP-1-102 and its derivatives BP-3-163 and BP-4-018, as well as two SF-1-066 derivatives, BP-2-061 and BP-2-047. The anti-tumour activity of these compounds will be assessed using the HMCLs described in Chapter 2, and from this initial screen, the most potent small molecule will be further evaluated in extensive in vitro pre-clinical efficacy studies.
3.2. Methods

**Test compounds.** The small molecule STAT3 inhibitors included in this study were developed and kindly provided by Dr. Patrick Gunning (University of Toronto, Mississauga, ON). All compounds were dissolved in dimethyl sulfoxide (DMSO) to 20 mM, and further diluted to 10 mM stock solutions in DMSO, both of which were stored at -20°C. Prior to *in vitro* treatment, 10 mM stock solutions were thawed and diluted to 500 µM in culture medium (DMSO vehicle controls were prepared at the same volumes, such that the final solutions contained the same percentage DMSO). A detailed protocol of compound synthesis can be found in Appendix B as part of the manuscript submitted to the Journal of Medicinal Chemistry. Clinical grade bortezomib/PS-341 (Velcade®; Millennium Pharmaceuticals, Cambridge, MA, USA) was obtained from the Princess Margaret Cancer Center Pharmacy and dissolved in 0.9% saline solution to 2.6 mM stock solutions.

**Cell viability assay.** Cells were seeded into 96-well plates at a density of 2-3 x 10⁴ cells per well in 90 µl of fresh culture medium. Prior to the addition of cell suspensions, 10 µl of test compound (or vehicle control) was added to wells in triplicate. Treated cell cultures were then incubated for 72 hours at 37°C, 5% CO₂. Following treatment, cell viability was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance (Roche Molecular Biochemicals, Boehringer, Germany). Briefly, 10 µl of the tetrazolium salt MTT was added to each well and left to incubate for 4 hours at 37°C, after which time, 100 µl of solubilization buffer (10% sodium dodecyl sulfate) was added to solubilize the MTT reaction product. Following an overnight incubation period at 37°C, absorbance was read at 570 nm (reference wavelength; 650 nm) using the OptiMax microplate reader (Molecular Devices, Sunnyvale, CA).

**Cell death assay.** Cells were seeded at a density of 5-10 x 10⁵ cells per well in 24-well plates and treated with the indicated concentrations of test compound. Cells were harvested after 24-48 hours and cell death was assessed by flow cytometric analysis of apoptosis as describe in Section 2.2.

**STAT3 reporter construct and luciferase assay.** STAT3-driven luciferase activity of established reporter construct cell lines was analyzed as described in Section 2.2.
**Immunoblot analysis.** Drug-treated cells were harvested and washed twice in ice-cold PBS prior to lysing. Processing of cell lysates, SDS-PAGE and immunoblot analyses were performed as described in Section 2.2.

**Primary MM patient samples.** BM aspirates were obtained from MM patients with consent under a protocol approved by the Research Ethic Board of University Health Network, Toronto, Canada. Samples were diluted with PBS, and mononuclear cells (MNCs) isolated by density-based cell separation (Ficoll-Paque™ PLUS, GE Healthcare, Uppsala, Sweeden). Briefly, Ficoll-Paque solution was added at one third the volume and samples then centrifuged for 30 minutes at 1500 rpm to isolate the interphase MNC population. The resulting interphase layer was collected with a sterile Pasteur pipette and washed once with sterile PBS. Residual contaminating red blood cells were subsequently depleted from the MNC interphase fraction with ammonium-chloride-potassium lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.3). After 3-5 minutes, cells were washed once with PBS and once with pre-warmed IMDM medium supplemented with 10% fetal calf serum (FCS). Cells were counted and suspended to a final concentration of 5-10 x 10⁵ cells/ml in IMDM medium + 10% FCS. A small aliquot of isolated MNCs (5-10 x 10⁶ cells) was sampled and stained for the cell surface antigen CD138 using a mouse anti-human CD138-Phycoerythrin (PE) antibody (BD Biosciences, Mountain View, CA, USA). The aliquoted sample was washed in PBS and re-suspended in 50-100 µl staining buffer (PBS + 3% FBS) prior to the addition of CD138-PE (5-10 µl). Samples were incubated with CD138-PE antibody for 30 minutes, washed with PBS and resuspended in staining buffer for analysis by flow cytometry. The remaining cells were treated with the indicated drug concentrations. After 24 hours, cells were washed in PBS and stained with CD138-PE as outline above, but with the addition of Annexin V-FITC conjugate following the final wash. Briefly, cell pellets were resuspended in 100 µl of apoptosis binding buffer and incubated for 10 minutes with 2 µl of Annexin V-FITC conjugate (R&D Systems, Minneapolis, MN, USA). Samples were subsequently analyzed by flow cytometry as described in Section 2.2 and cytotoxicity evaluated based the percentage of CD138⁺/Annexin V⁻ cells (viable MM cells) in vehicle treated samples compared to drug treated samples.

**Colony formation assay.** MNCs from primary MM patient BM aspirates were suspended in culture medium containing 2% FBS (2-10 x 10⁴), and combined 1:10 with methylcellulose
medium (MethoCult® H4434 Classic, Stem Cell Technologies, Vancouver, BC, CAN) containing BP-4-018 (final concentration of 15 µM). Samples were then plated in sterile 35 mm culture dishes, placed inside an additional dish containing 3-4 ml of sterile water and incubated at 37°C in 5% CO₂. After 14 days, formed colonies were manually counted using a 60 mm gridded dish under an inverted light microscope. Note that total progenitor colonies were counted, but not discriminated by progenitor type.

**BM stroma co-culture.** To derive primary BM stromal cells, isolated MNCs were plated in 60 mm tissue culture-treated dishes until adherent cell monolayers developed. After removal of non-adherent cells in culture medium, adherent cells were trypsinized, collected by centrifugation and re-plated at 2.5 x 10³ cells/well in 96-well plates. To establish a recapitulative BM stroma microenvironment *in vitro*, BM stromal cells were grown until cell density reached approximately 80%, at which time medium was discarded and replaced by HMCLs in suspension (or medium alone for control). Co-cultures were left to adhere for 2 hours prior to treatment with vehicle control or indicated test compound. For comparison, HMCLs were also concurrently plated in the absence of BM stroma. Cells were treated with indicated doses of BP-4-018 for 72 hours and analyzed by MTT assay as described above.

**Statistical analysis.** Statistical comparisons of means were made using the non-paired two-tailed students’ t test, but only when experiments had been independently repeated. The half maximal inhibitory concentration (IC₅₀) for each HMCL treated with BP-4-018 was calculated using non-linear regression of log-transformed data from MTT experiments to generate a sigmoidal dose-response curve. Graphs with error bars represent the mean ± SD for each data point. All statistical analyses were performed using Prism Software version 5.0 (GraphPad) at the 95% confidence interval (α=0.05).
3.3. Results

Preliminary screening of small molecule STAT3 inhibitors for growth-inhibition of HMCLs

The response of XG6, RPMI-8226 and JJN3 to increasing concentrations of BP-1-102, BP-2-047, BP-2-061, BP-3-163 and BP-4-018 were first investigated by evaluating tumour cell viability using an MTT assay at 72 hours following compound addition. As revealed by the MTT assay results depicted in Figure 3.3.1, all compounds demonstrated anti-MM activity at concentrations up to 10 µM. Overall, XG6, RPMI-8226 and JJN3 were least sensitive to the parental perfluorobenzene-N-alkyl compound, BP-1-102, and the tolyl-N-alkyl derivative BP-2-061. The activity of BP-2-047 and BP-3-163 were somewhat variable in this selection of HMCLs, with the former being more active against XG6 and RPMI-8226, and the latter against JJN3. As expected, the perfluorobenzene-N-alkyl derivative BP-4-018 consistently demonstrated superior potency against this subset of HMCLs, compared to its parental compound BP-1-102. BP-4-018 was also more potent and broad acting than both of the tolyl-N-alkyl derivatives, and was therefore selected for further study. Note that the remaining compounds from this screen continue to be evaluated for their activity against MM, as well as other human tumours. The structural properties of each compound are provided in Table 3.3.1, along with a comparison of their growth-inhibitory potential.

Figure 3.3.1 Initial screening of small molecule STAT3 inhibitors and their derivatives against HMCLs. XG6, RPMI-8226 and JJN3 cell lines were plated in triplicate and treated with increasing concentrations of the indicated compounds or DMSO control. After 72 hours, the MTT assay was used to assess the potency of each compound against cell viability. Results represent mean of 2-3 independent experiments ± SD.
Evaluating the anti-MM activity of lead compound, BP-4-018, against HMCLs and primary MM patient samples

Selecting the most potent compound from the evaluated series of small molecule STAT3 inhibitors, the anti-MM activity of BP-4-018 was evaluated against an expanded panel of HMCLs by MTT assay. As shown in Figure 3.3.2, BP-4-018 demonstrated dose-dependent inhibition of MM cell viability after 72 hours of treatment. Although the response of each HMCL to BP-4-018 was variable, a plateau of inhibition was reached for all cell lines at the maximal dose of 10 μM. The dose-response effect of BP-4-018 in each HMCL was determined and expressed as an IC$_{50}$ value, representing the concentration of BP-4-018 required to reduce MTT activity by 50%. Based on these calculations, the IC$_{50}$ for BP-4-018 ranged from 1.91 μM to 6.48 μM, suggesting broad and potent anti-MM activity in vitro.
Figure 3.3.2 Extended analysis of BP-4-018-mediated growth inhibition against a panel of HMCL. Cells were plated in triplicate and treated with increasing concentrations of BP-4-018 or DMSO control. After 72 hours, the MTT assay was used to evaluate the effects of BP-4-018 on cell viability. Results are presented as the percent MTT activity relative to DMSO vehicle-treated control (mean of 2-3 independent experiments ± SD. The resulting dose-response effect of BP-4-018 treatment in HMCLs was used to calculate IC₅₀ values for each HMCL (below).

Since aberrant activation of STAT3 is known to promote cell survival and previous results indicated that knockdown of STAT3 induced apoptosis, the ability of BP-4-018 to induce apoptosis was evaluated. The time-dependent apoptotic effects of BP-4-018 on RPMI-8226 cells were first evaluated by flow cytometric analysis of Annexin V/PI staining. Compared to the negligible levels of apoptosis observed in vehicle-treated control cells (4.81% - 6.62%), the percentage of Annexin V⁺ stained cells increased time-dependently in RPMI-8226 cells treated with 6 µM of BP-4-018, from 47.16% at 24 hours to 69.6% at 48 hours (Figure 3.3.3A). Importantly, the vast majority of Annexin V⁺ cells at 24 hours did not stain positive for PI (41.1%), which is indicative of cells undergoing the early phase of apoptosis. Conversely, the majority of cells treated for 48 hours stained positive for both Annexin V and PI (45.2%), indicating that cells were either undergoing the later stages of apoptosis or necrosis.

The ability of BP-4-018 to induce apoptosis dose-dependently was also evaluated in RPMI-8226, as well as three additional HMCLs, including H929, XG6 and JKN3 (Figure 3.3.3B). After 48 hours of treatment, 4 µM of BP-4-018 significantly increased the percentage of Annexin V⁺ cells in RPMI-8226 and H929 compared to vehicle-treated control (p=0.0211 and p=0.0028, respectively). Consistent with its higher IC₅₀ value, XG6 cells required a higher dose of BP-4-
018 (6 μM) to significantly increase the percentage of Annexin V\(^+\) stained cells (p=0.0006). Although the induction of apoptosis in BP-4-018-treated JJN3 cells was also dose-dependent, the percentage of Annexin V\(^+\) cells was not significantly different compared to vehicle-treated control cells (p=0.2766 at 4 μM and p=0.0858 at 6 μM). This may suggest that higher doses of BP-4-018 are required to induce significant levels of apoptosis in this cell line. Nonetheless, these results are again consistent with findings from the preceding MTT assay, as the IC\(_{50}\) value calculated for JJN3 was higher compared to XG6, RPMI-8226 and H929.

![Figure 3.3.3 Flow cytometric analysis of BP-4-018-induced apoptosis.](image)

**Figure 3.3.3 Flow cytometric analysis of BP-4-018-induced apoptosis.** The indicated HMCLs were treated with increasing concentrations of BP-4-018 for 24 and 48 hours and incubated with Annexin V-FITC and PI for analysis of apoptosis by flow cytometry. Representative flow cytometry scatterplots of RPMI-8226 after treatment with indicated doses of BP-4-018 are presented (A), as well as dose-response effects of BP-4-018-induced apoptosis after 48 hours of treatment (B). Results represent mean of 2-3 independent experiments ± SD (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant, compared to vehicle-treated control).

Although BP-4-018 has demonstrated promising activity against a broad range of HMCLs, the use of cell lines for pre-clinical drug testing is often criticized because they fail to fully recapitulate the characteristics of primary BM-derived MM cells. In particular, the vast majority of HMCLs exhibit significantly higher proliferative indexes, and often have additional acquired mutations as a result of long term culture. Furthermore, unlike patient-derived MM tumours that can only be propagated *in vitro* for a few days, HMCLs are able to grow independently of the BM microenvironment. Thus, the activity of BP-4-018 against malignant BM plasma cells
derived from MM patients was also evaluated. MNCs were isolated from primary MM patient BM aspirates, which collectively include both malignant myeloma plasma cells and normal non-malignant BM cells. Samples were treated with the indicated doses of BP-4-018 or DMSO vehicle control. After 24 hours, samples were analyzed for viability using cell surface staining with Annexin V and CD138. Since CD138 is highly expressed on plasma cells, but rapidly shed from MM cells entering into apoptosis,\textsuperscript{116} it can also be used as a marker of MM cell viability. As shown in Figure 3.3.4A, BP-4-018 treatment significantly reduced the percentage of viable malignant BM plasma cells (CD138\textsuperscript{+}/Annexin V\textsuperscript{−}) over vehicle control, albeit with varying sensitivities. Representative scatterplots from two patients are provided in Figure 3.3.4B, which indicates the population of MM tumour cells (CD138\textsuperscript{+}/Annexin V\textsuperscript{−}) in the upper left quadrant, and the significant reduction of this population of cells upon treatment with BP-4-018.

![Figure 3.3.4](image)

**Figure 3.3.4 Activity of BP-4-018 against malignant plasma cells from primary MM patient BM aspirates.** MNCs isolated from myeloma patient BM aspirates were cultured with the indicated concentrations of BP-4-018. After 24 hours, cells were incubated with Annexin V-FITC conjugate and anti-CD138-PE antibodies for analysis by flow cytometry. Results are presented graphically (A) as the percentage of viable MM plasma cells (CD138\textsuperscript{+}/Annexin V\textsuperscript{−}) after treatment with BP-4-018 relative to viable MM cells from DMSO vehicle-treated control samples. Representative scatterplots for patient 2 and 3 are provided (B) with the percentage of viable MM plasma cells (CD138\textsuperscript{+}/Annexin V\textsuperscript{−}) in bold (upper left quadrant).

The activity of BP-4-018 was also evaluated against normal non-malignant cells derived from primary MM patient BM samples. As shown in Figure 3.3.5A, BP-4-018 demonstrated little toxicity against normal MNCs from three of five primary patient BM samples. In the remaining two BM samples, BP-4-018 demonstrated significant toxicity against normal MNCs, reducing
cell viability by as much as 66%. However, the doses demonstrating non-selective toxicities were well above those that demonstrated activity against the MM tumour cell fraction. To further address the potential cytotoxicity of BP-4-018 against primary non-malignant cells, colony formation assays were performed using BM-derived hematopoietic progenitor cells. MNCs from BM aspirates were treated with 15 μM of BP-4-018 or vehicle control and plated in methylcellulose semi-solid medium to allow the formation of colonies. After 2 weeks, no significant differences in the number of formed colonies between vehicle control- or BP-4-018-treated samples were observed (Figure 3.3.5B, p=0.9451), suggesting that BP-4-018 does not inhibit the ability of normal hematopoietic progenitors to proliferate or form distinct colonies. Taken together, our analysis of BP-4-018 in the context of primary MM patient samples has revealed that there is indeed a therapeutic window for BP-4-018, which ultimately contributes to the therapeutic validity of small molecule STAT3 inhibitors in at least a subset of MM tumours.

![Figure 3.3.5 Activity of BP-4-018 against non-malignant BM cells from primary MM patient samples.](image)

Figure 3.3.5 Activity of BP-4-018 against non-malignant BM cells from primary MM patient samples. MNCs isolated from MM patient BM aspirates were cultured with the indicated concentrations of BP-4-018 for 24 hours, and subsequently labeled with Annexin V-FITC conjugate and anti-CD138-PE antibodies for flow cytometric analysis (A). Results represent the percentage of viable MNCs (CD138+/Annexin V−) relative to viable MNCs from DMSO vehicle-treated control samples. Alternatively, MNCs were treated with BP-4-018 or vehicle control and plated in methylcellulose semisolid medium. After two weeks, formed colonies were counted manually (B). Results are presented as the percentage of colonies formed in BP-4-018-treated samples compared to control and represent mean of three independent BM samples ± SD (n=3; ns, not significant).

**Activity of BP-4-018 in recapitulative myeloma BM microenvironment**

While MM tumour cells may have certain patterns of growth and drug response when cultured autonomously, these responses can be markedly divergent in the presence of BM stroma. We
therefore employed *in vitro* co-culture models with HMCLs and patient-derived BM stromal cells to assess whether microenvironment-derived factors conferred resistance to BP-4-018. HMCLs were co-cultured with BM stromal cells isolated from primary MM patient-derived BM aspirates, and treated with BP-4-018 for 72 hours for analysis by MTT assay (Figure 3.3.6). From these experiments, patient derived BM stroma did not appear to substantially affect the activity of BP-4-018 against RPMI-8226 cells when compared to cells cultured autonomously in the absence of BM stroma. In contrast, XG6 cells were considerably, but not completely, protected from the activity of BP-4-018 when cultured together with patient-derived BM stroma. This conferred protection was most evident at 6 μM of BP-4-018, as the growth of co-cultured XG6 cells was over 10-fold higher than that observed in cells cultured in the absence of BM stroma. Although not shown, similar results were also observed in co-culture experiments performed with the BM stromal cell line, HS-5. Finally, it is worth noting that BP-4-018 demonstrated little activity against primary BM stromal cells at doses up to 12 μM, which further supports its utility as an anti-MM agent.

Figure 3.3.6 Activity of BP-4-018 against HMCLs in the presence of a recapitulative BM microenvironment. RPMI-8226 and XG6 were either plated alone in suspension or in combination with MM patient-derived BM stromal cells for two hours prior to the addition of BP-4-018. After 72 hours, samples were evaluated by MTT assay. Results are presented as the percent MTT activity relative to DMSO vehicle-treated control and represent the mean of triplicate replicates ± SD.

**Direct molecular targets of BP-4-018 and modulation of downstream target protein expression**

Although many different strategies have been investigated to inhibit aberrant STAT3 activity, BP-4-018 was rationally designed to block the SH2 domain of STAT3. As such, BP-4-018
mechanistically inhibits STAT3 phosphorylation by abrogating its recruitment and binding to gp130 pTyr motifs, which ultimately prevents de novo JAK-mediated STAT3 phosphorylation. Consistent with this predicted mechanism of action, BP-4-018 demonstrated dose- and time-dependent inhibition of constitutive STAT3 phosphorylation in the HMCLs XG6 and RPMI-8226 (Figure 3.3.7A). Conversely, while BP-4-018 markedly reduced the level of STAT3 phosphorylation in JNJ3 cells, it had no significant effect on the phosphorylation of other proteins involved in MM tumour cell survival, including Tyr1007/1008 phosphorylation of extracellular signal-regulated kinase (ERK) and Ser473 phosphorylation of protein kinase B (PKB/AKT) (Figure 3.3.7B). Although more extensive selectivity profiling is required, including the evaluation of BP-4-018 activity against other STAT family members and SH2 domain-containing proteins, these results support the selectivity of BP-4-018 for STAT3. Of note is the reduction of total STAT3 protein in XG6 cells after 16 hours of treatment with BP-4-018. We speculate that this is likely to be a secondary effect to BP-4-018-induced apoptosis, as results previously demonstrated that BP-4-018 induces a significant level of apoptosis in XG6 cells after 48 hours. In support of this, BP-4-018 did not significantly induce apoptosis in JNJ3 cells, and no reduction in total STAT3 protein was observed after treating cells with BP-4-018 for up to 24 hours.

To confirm that BP-4-018-mediated inhibition of STAT3 phosphorylation was sufficient to abrogate downstream STAT3 signaling and transcriptional activity, the resulting effects of BP-4-018 on STAT3-driven luciferase expression and downstream STAT3 target gene expression were analyzed. After 6 hours of BP-4-018 treatment, STAT3-driven luciferase expression was reduced in both XG6 and RPMI-8226 cells (Figure 3.3.7C). STAT3-driven luciferase expression was reduced in XG6-treated cells, but only at doses of 10 µM BP-4-018 (76.5% reduction), whereas RPMI-8226-treated cells demonstrated a dose-dependent decrease in STAT3-driven luciferase expression (13.6%, 42.2% and 74.5% decrease at 2.5 µM, 5 µM and 10 µM, respectively). These results are congruent with the differential sensitivities of XG6 and RPMI-8226 to BP-4-018, where RPMI-8226 cells are markedly more sensitive. Further, the observed abrogation of STAT3 transcriptional activity by BP-4-018 corresponds with decreased protein expression of STAT3 target genes including c-Myc, Mcl-1 and Bcl-xL, but interestingly not Bcl-2 (Figure 3.3.7D). Nonetheless, these results support that the anti-MM activity of BP-4-018 is mediated, at least in part, through inhibition of STAT3 signaling.
Figure 3.3.7 BP-4-018-mediated inhibition of STAT3 signaling in HMCLs. Cell lines were treated with BP-4-018 under the indicated experimental conditions to evaluate targeted inhibition of STAT3 phosphorylation by immunoblot analysis for Tyr705 pSTAT3 and total STAT3 (A). Whole cell lysates were also collected from JJN3 cells to evaluate the selectivity of BP-4-018 for pSTAT3 inhibition compared to pERK and pAKT (Ser473 pAKT, total AKT, Tyr1007/1008 pERK and total ERK) (B). The downstream effects of BP-4-018 on STAT3 transcriptional activity were evaluated using the Cell Titer-Fluor™ and ONE-Glo Luciferase Assay Reagent™ System (C), and immunoblot analysis of STAT3 target genes c-Myc, Mcl-1, Bcl-xL and Bcl-2 (D). Relative luciferase units (RLU) in (C) were calculated as ratio of luciferase to fluorescence signal intensities and represent the mean RLU from duplicate replicates ± SD.

Evaluating subtherapeutic drug combinations \textit{in vitro}: BP-4-018 plus bortezomib

The \textit{in vitro} evaluation of BP-4-018 thus far has yielded promising results, demonstrating potent anti-MM activity against a range of HMCLs and primary MM patient samples. However, clinical experience with molecular targeted therapy in solid and hematological cancers demonstrates that these therapies may have limited utility as single agents. It is therefore highly probable that in a clinical setting, STAT3 inhibitors will need to be combined with other standard of care anti-myeloma agents to obtain the greatest clinical benefit. Indeed, the addition of novel agents in early clinical development to the treatment backbone of IMiDs or bortezomib is an area of active clinical study in MM. Bortezomib in particular has been shown to provide a platform for combination with other novel agents to increase efficacy, decrease dosage, reduce toxicity and minimize the development of drug resistance. Thus, the activity of BP-4-018 was evaluated in combination with bortezomib, a first in class proteasome inhibitor currently used in the treatment of newly diagnosed and relapsed MM patients.
Combinatorial studies were performed to evaluate whether the activity of BP-4-018 in combination with bortezomib was greater than that which could be obtained when either compound was used alone under identical conditions of treatment. Subtherapeutic doses of these agents were first established in RPMI-8226 cells using a 48 hours MTT assay. As shown in Figure 3.3.8A, although 2 µM of BP-4-018 significantly inhibited the viability of RPMI-8226 cells (p<0.0001), no significant differences in cell viability were observed for cells treated with 1.5 µM compared to vehicle-treated control (p=0.0867). As for bortezomib, we have previously established an IC\textsubscript{50} value of 3.5 nM for RPMI-8226. Accordingly, neither 2 nor 3 nM of bortezomib had significant activity against RPMI-8226 cells (Figure 3.3.6A, 2nM, p=0.6918; 3nM, p=0.1474) and thus, both were utilized as subtherapeutic doses in combination with 1.5 µM of BP-4-018.

Remarkably, although treatment of RPMI-8226 cells with the established subtherapeutic doses of BP-4-018 or bortezomib failed to significantly inhibit the viability of RPMI-8226 cells, marked anti-MM activity was observed when administered in combination (Figure 3.3.8B). RPMI-8226 viability was reduced by over 65% with the combination of 1.5 µM BP-4-018 and 2 nM bortezomib, a significant difference from that observed in cells treated with the same doses of BP-4-018 or bortezomib alone (p=0.0004, p=0.0008, respectively). Similarly, when treated with a combination of 1.5 µM BP-4-018 and 3 nM of bortezomib, the viability of RPMI-8226 cells was decreased by approximately 80%, which is significantly different from cells treated with the same doses of either agent alone (p=0.0002, p=0.0017, respectively). Although the molecular mechanism that underlies the enhanced activity of BP-4-018 and bortezomib is unknown, these data are compelling enough for further studies to determine the efficacy of this combination \textit{in vivo}.
Figure 3.3.8 Subtherapeutic dose combinations of BP-4-018 and bortezomib. RPMI-8226 cells were plated in triplicate and treated with BP-4-018 (1.5 µM or 2 µM) alone or bortezomib (2 nM or 3 nM) alone and after 48 hours, samples were analyzed by MTT assay (A: ***, p < 0.001; ns, not significant, compared to vehicle-treated control). Once subtherapeutic dose concentrations were established, RPMI-8226 cells were plated in triplicate and treated with the indicated doses of BP-4-018/bortezomib alone or in combination. After 48 hours, cells were analyzed by MTT assay (B: ***, p<0.001, relative to 1.5 µM BP-4-018 alone; ††, p<0.01, relative to 2 nM bortezomib alone; †††, p<0.001, relative to 3 nM bortezomib alone). Results are presented as the percent MTT activity relative to vehicle-treated control and represent the mean of 2-3 independent experiments ± SD.
3.4. Summary

The current study was performed to identify and characterize the most potent compound from the screened BP-series of small molecule STAT3 inhibitors. From the 5 evaluated compounds, BP-4-018 demonstrated the most potent anti-MM activity against XG6, RPMI-8226 and JJN3 cell lines, as assessed by its effects on cell viability by MTT assay. Upon inclusion of additional HMCLs, BP-4-018 demonstrated broad anti-MM activity at low micromolar concentrations, with reported IC$_{50}$ values of 1.91 – 6.48 µM. Inhibition of aberrant STAT3 signaling by BP-4-018 was confirmed to occur in a dose- and time-dependent manner, and had profound effects on HMCL viability. This effect was observed to be associated with the induction of apoptosis in highly sensitive cell lines such as XG6, RPMI-8226 and H929, but to a lesser degree in the less sensitive JJN3 cell line. In addition to pSTAT3 inhibition and consistent with the predicted mechanism of action, treatment of HMCLs with BP-4-018 suppressed STAT3 transcriptional activity and reduced the protein expression of STAT3 target genes. Accordingly, BP-4-018 did not demonstrate inhibitory activity against ERK or AKT phosphorylation. Although these findings are consistent with the predicted molecular target of BP-4-018, future experiments will be required to gain more insight into its mechanisms of action and to evaluate its activity against other cellular targets that are involved in MM cell survival and proliferation.

BP-4-018 also demonstrated significant activity against primary malignant plasma cells derived from MM patient BM aspirates, markedly reducing the percentage of malignant CD138$^+$ plasma cells. While higher doses of BP-4-018 demonstrated toxicity against the normal MNC population in some patients, these doses were lower than those that elicited anti-MM against malignant plasma cells. This therefore suggests that there is a therapeutic window for the utility of BP-4-018. In support, BP-4-018 did not inhibit the ability of hematopoietic progenitor cells to form colonies in long-term culture, and did not reduce the viability of patient-derived BM stromal cells. These data are consistent with the notion that transformed cells are more dependent on aberrant STAT3 signaling. In BM stroma co-culture experiments, BP-4-018 remained equally as potent against RPMI-8226 cells treated in the presence of BM stromal cells. However, these results differed for XG6 cells, which were conferred modest, but not complete resistance to the anti-MM activity of BP-4-018 when cultured together with BM stroma.
For the purposes of this investigation, BP-4-018 has served as an informative molecular tool for determining the therapeutic efficacy of small molecule STAT3 inhibitors against MM, and moreover, their potential utility in bortezomib-based combination strategies. The pharmacological relevance of BP-4-018 when used alone and in combination with bortezomib will certainly require further analysis which can be, at least partially, explored in relevant in vivo models of MM.
Chapter 4
In vivo Efficacy Studies of BP-4-018 Alone and in Combination with Bortezomib Using a Xenograft MM Mouse Model

4.1. Objectives

While the use of cell lines and in vitro studies are certainly useful for evaluating the pre-clinical efficacy and molecular mechanism of novel therapeutics, the anti-tumour activity and physiological tolerability of such investigational agents also needs to be investigated in vivo. The whole organism approach can provide realistic insight regarding drug responses and perhaps more importantly, reveal unwanted side effects and toxicities that would otherwise go undetected in vitro. Furthermore, it is possible that the activity of a compound can be positively or negatively modulated in a physiological context that includes both a microenvironment and immune system. And finally, in vivo models are more recapitulative of complex disease features and can therefore answer biological questions relevant to drug discovery that simply cannot be addressed using in vitro cell culture models. Accordingly, our final objective was to corroborate our in vitro findings from Chapter 3 by assessing the efficacy of BP-4-018 alone, and in combination with bortezomib in as xenograft mouse model of MM.

The objectives of this study were to document in vivo inhibition of STAT3 after oral administration of BP-4-018, demonstrate in vivo anti-myeloma activity and assess short-term toxicities. Towards this goal, we established a MM xenograft model using the constitutively STAT3-activated RPMI-8226 HMCL engineered to stably express a STAT3-driven firefly luciferase reporter construct. When used in combination with real-time bioluminescence imaging (BLI), we postulated that this model would allow real-time analysis of STAT3 transcriptional activity in intact animals, and thus be used as a tool to assess initial tumour engraftment, intrinsic STAT3 activation and the effects of BP-4-018 on STAT3 activity in vivo.
4.2. Methods

Establishing a STAT3-driven firefly luciferase xenograft mouse model of MM. The described studies were approved by the Institutional Animal Care Committee (Protocol Number: 958.11) and performed in accordance with the Canadian Council on Animal Care Guidelines. Six to ten week old female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed in sterile filter-top cages at the Ontario Cancer Institute animal facility. The MM tumour cell line RPMI-8226 containing the STAT3-driven luciferase reporter construct was established as described in Section 2.2 and expanded in the weeks prior to establishing the mouse model. On the day of engraftment, cell suspensions were collected, counted and resuspended in growth medium mixed with BD Matrigel Basement Membrane Matrix (BD Biosciences, Mountain View, CA, USA) at a final concentration of 5 x 10^6 cells per 200 µL. Mice were irradiated with 150 Rad shortly before tumour engraftment, and subcutaneously injected with 5 x 10^6 tumour cells (in 200 µL matrigel) into the right flank.

In vivo real-time BLI. STAT3-driven firefly luciferase activity was evaluated in vivo using the D-Luciferin substrate. D-Luciferin firefly potassium salt (Caliper Lifesciences, Hopkinton, MA, USA) was dissolved in sterile PBS to obtain a stock solution of 25 mg/ml, sterilized using a 0.22 µm filter, and stored at -20 °C in 1 ml aliquots. Prior to in vivo use, D-Luciferin was thawed to room temperature and vortexed to obtain a homogenous mixture. Mice harboring RPMI-8226 tumours were anesthetized prior to imaging by injection with a solution of 100 mg/kg Ketamine and 10 mg/kg Xylazine (Ketaset, Ayerst Veterinary Laboratories, Guelph, ON, CAN; Rompun, Bayer Pharmaceuticals, Toronto, ON, CAN) into the intraperitoneal cavity (IP). Anesthetized mice were administered 100 µl of D-Luciferin substrate (150 mg/kg) IP and placed into a sterile, clear-top Plexiglas encasement to ensure sterility during imaging. The encasement was placed into the BLI camera on a heated platform to maintain a physiological body temperature throughout the duration of imaging. Images were acquired using the IVIS® Imaging System and analyzed using LivingImage™ Software by generating an elliptical region of interest (ROI) over the tumour area.
An *in vivo* kinetics study was performed to optimize the imaging parameters by acquiring images at 3-minute intervals for up to 1 hour starting 2 minutes post-substrate administration. The time at which peak signal intensities occurred was used as the expected time point for the remaining *in vivo* BLI studies, where multiple images were acquired surrounding this expected time point and the peak value was used. Results for this study can be found in Appendix C.

**Therapeutic compounds and treatment parameters.** Stock solutions of 50 mM BP-4-018 dissolved in DMSO were diluted in sterile water, and based on the observed poor solubility of BP-4-018, it was highly suggested that the agent be administered by oral gavage (personal communication with Ontario Cancer Institute animal facility). Mice were treated daily or twice daily (BID) by oral gavage (20/22 gauge feeding needle) with escalating doses of BP-4-018 for at least 1 week to determine the maximum tolerated dose. Mice were weighed and monitored each day for signs of toxicity including decreased activity, lethargy or wasting. A significant toxicity response was defined as greater than 10% weight loss from the first day of treatment. Clinical grade bortezomib/PS-341 (Velcade®; Millennium Pharmaceuticals, Cambridge, MA, USA) was obtained from the Princess Margaret Cancer Center Pharmacy, dissolved in 0.9% saline solution and diluted in sterile PBS. The sub-therapeutic dose and treatment parameters for bortezomib-based combinations had been previously established.

**Combinatorial study design.** Mice were injected subcutaneously with 5 x 10⁶ RPMI-8226 cells in 200 µL of matrigel as previously described. Upon establishment of palpable tumours, mice were imaged for STAT3-driven BLI and randomized into the following treatment arms based on palpable tumour size and BLI signal intensity: Vehicle (0.7% DMSO in water), BP-4-018 (15 mg/kg daily) only, bortezomib (0.5 mg/kg twice weekly; IP) only, BP-4-018/bortezomib combination. BP-4-018 was administered for 5 consecutive days per week for 4 weeks. Mice treated with bortezomib were dosed twice a week for two consecutive weeks, rested for 1 week and treated again twice a week for 1 week. Mice were weighed and monitored daily for signs of toxicity. On day 28, all mice were sacrificed by CO₂ inhalation and tumours excised to determine the final tumour weight. A schematic overview of the study design is provided in Figure 4.2.1 below.
Analysis of treatment outcome and endpoints. All mice included in the study were evaluated for tumour volume and endpoint tumour weight. For the duration of the combinatorial study, tumour volumes were measured at least twice per week in two dimensions by caliper measurements from the first day of treatment until sacrifice. Tumour volumes were calculator based on the prolate ellipsoid formula: \[ V = \frac{1}{2}L \times W^2 \] where \( L \) is the long diameter of the tumour and \( W \) is the short diameter of the tumour perpendicular to \( L \).

BLI of short-term BP-4-018 treatment. The effect of short-term BP-4-018 treatment on STAT3 transcriptional activity was evaluated in a small cohort of mice. We found that administering substances by oral gavage in anesthetized mice was challenging and carried risk for inadvertent tracheal administration. Thus, pre-treatment images of mice for this short-term study were acquired the night prior to treatment with BP-4-018. The following morning, mice were administered either vehicle (n=2) or 15 mg/kg of BP-4-018 (n=2) by oral gavage and then re-imaged after 8 hours.

Statistical analysis. Given the highly variable nature of live subjects, all graphs with error bars represent the mean measurement from each treatment arm ± standard error of the mean (SEM). To compare the effects of treatment on tumour volume over time, a two-way repeated
measures analysis of variance (ANOVA) was used with a Bonferroni post-test to determine where significant differences existed. A non-paired two-tailed students’ t test was used to compare the slope of tumour growth curves and mean weight of tumours extracted from sacrificed mice at the end of the study. All statistical analyses were performed using Prism Software version 5.0 (GraphPad) at a 95% confidence interval (α=0.05).
4.3. Results

We first set out to establish a tolerable dose of BP-4-018 to be used in subsequent efficacy studies. To our knowledge, BP-4-018 has never been evaluated in vivo, however its parent compound (BP-1-102) has been studied in mice by our lab, and others. Published data reveals that BP-1-102 has significant anti-tumour activity against human breast tumour xenografts when administered by oral gavage at doses of 1 mg/kg and 3 mg/kg, with no obvious signs of toxicity. Further, pharmacokinetic and pharmacodynamic studies demonstrate that BP-1-102 is detectable at micromolar concentrations in the plasma and accumulates at sufficient levels in tumour tissue to modulate STAT3 activity, collectively confirming this compound’s oral bioavailability. However, in the recently described Vκ*MYC transgenic MM mouse model, 3 mg/kg of BP-1-102 was not sufficient to induce anti-tumour responses, as assessed by serum monoclonal protein (Dr. Marta Chesi, unpublished data). Similarly, in MM xenograft models, once a day oral dosing of BP-1-102 at 3 mg/kg failed to display significant anti-MM activity (Dr. Trudel lab, unpublished data). Pilot dose escalation studies have since established that BP-1-102 is tolerable in the NOD/SCID strain of mice at doses of up to 20 mg/kg. We therefore chose to evaluate BP-4-018 using doses of 10 mg/kg, 12 mg/kg (6 mg/kg BID) and 15 mg/kg in short-term tolerability experiments, since BP-4-018 is a more potent derivative of BP-1-102. Results from this study are provided in Appendix D, and reveal no significant signs of toxicity (weight loss, loss of appetite, decreased activity or lethargy) between vehicle-treated control mice and mice treated with 15 mg/kg of BP-4-018. The 15 mg/kg dose was therefore chosen for continued in vivo efficacy studies.

Evaluating the in vivo activity of BP-4-018 alone and in combination with bortezomib

To evaluate the in vivo efficacy of BP-4-018 as a single agent and in combination with bortezomib, we used a xenograft mouse model of MM. NOD/SCID mice inoculated subcutaneously with RPMI-8226 cells developed palpable tumours within 5 days of engraftment. Whole-body BLI was then employed to measure STAT3-driven luciferase activity in each tumour. As shown in Figure 4.3.1, luciferase activity was detected in all tumour-bearing mice at
the original site of engraftment. Based on these BLI signals, and tumour volume measurements, mice were randomized into four groups and treated in accordance to the dose and schedule described in Figure 4.2.1.

![Figure 4.3.1 BLI analysis of in vivo STAT3 transcriptional activity prior to treatment initiation. Dorsal BLI images were obtained 5 days post-engraftment of RPMI-8226 subcutaneous tumours, and are expressed on a radiance scale (p/sec/cm²/sr) as determined from ROI measurements. Randomization of subjects into the indicated treatment arms was based on ROI-quantified signal intensity from BLI and palpable tumour sizes.](image)

Shown in Figure 4.3.2A are the effects of single agent BP-4-018 and BP-4-018 in combination with bortezomib on tumour growth. For ease of interpretation, the rate of tumour growth in each treatment group was determined from the slope of tumour growth curves using a simple linear regression model. However, although the tumour growth curves appeared reasonably monophasic, inclusion of tumour volume data measured prior to day 9 revealed significant deviation from linearity. Thus, tumour growth curves were derived from tumour volume data measured on day 9 onwards. According to slopes derived from this model, bortezomib alone failed to significantly inhibit tumour growth, confirming that the dose employed was in fact subtherapeutic. The average rate of tumour growth in vehicle control-treated mice was 21.7 mm³/day, compared to 24.5 mm³/day in bortezomib treated mice (p=0.7787). Conversely, the growth of RPMI-8226 tumours was significantly inhibited by treatment with BP-4-018, with tumours growing at an average rate of 7.1 mm³/day which is a 67% decrease compared to
vehicle control treated mice (p=0.0493). Most impressively, the mean growth rate of tumours in mice treated with a combination of BP-4-018 and bortezomib was negative (-0.08 mm³/day), indicating that this therapeutic combination induced the regression of tumours that were palpable prior to treatment (p=0.0033 compared to vehicle-treated control).

For confirmation of this response, mice were sacrificed on day 28 of treatment, and tumours excised for measurement of tumour weight. As shown in Figure 4.3.2B, the mean tumour weight from vehicle-control treated mice was 447.6 mg (± 43.50 mg), compared to 337.6 mg (± 102.2) for bortezomib-treated mice, which did not meet statistical significance (p=0.5182). In comparison, the mean weight of tumours extracted from BP-4-018-treated mice was significantly lower than that of vehicle-treated control mice (113.5 mg ± 15.88 mg, p=0.0032). Most importantly, and consistent with tumour regression, 2 of the 3 mice treated with the combination of BP-4-018 and bortezomib completely lacked tumours, while the tumour extracted from the third animal weighed only 67 mg. Collectively, the average tumour weight from combination treated mice was significantly different from that observed in both vehicle-treated control mice (p=0.0023), as well as mice treated with bortezomib alone (p=0.0497) (Figure 4.3.2B). Finally, although no overt toxicities were observed in mice treated with bortezomib or BP-4-018, as evidenced by the absence of weight loss, mice treated with the combination of BP-4-018 and bortezomib did demonstrate some degree of weight loss towards the end of treatment week four. However, since the net weight loss of this treatment group did not exceed 10%, it is not considered significant (Figure 4.3.2C).
Figure 4.3.2 Effect of BP-4-018 alone or in combination with bortezomib in MM xenograft tumour model. Mice were treated with vehicle (n=3), BP-4-018 (15 mg/kg, n=4), bortezomib (0.5 mg/kg, n=4) or the combination of BP-4-018 plus bortezomib (n=3) for 4 weeks. Tumour volumes were assessed by caliper measurements throughout the duration of treatment, with resulting data presented as the mean tumour volume ± SEM (A). After 4 weeks of treatment, mice were sacrificed and tumours extracted for final tumour weight measurements (B), which are presented as the mean tumour weight ± SE (ns, not significant; **, p < 0.01, compared to vehicle-treated control; †, p < 0.05 compared to bortezomib-treated). Results from mouse weight monitoring are presented (C), with data points representing the mean percentage weight change compared to day 1 of treatment ± SEM, and the broken red line indicative of a significant toxicity response (>10% net weight loss from beginning of treatment).

BP-4-018 inhibits STAT3 transcriptional activity in vivo

Finally, to confirm that the observed anti-tumour responses to BP-4-018 were associated with inhibition of STAT3, BLI studies for STAT3-driven luciferase expression pre-treatment (baseline), and then 8 hours after dosing with 15 mg/kg BP-4-018 (or vehicle control). As shown in Figure 4.3.3, STAT3-driven firefly luciferase expression was detected in all mice at baseline, with radiance ranging from 2.13 – 4.65 x 10^6 in the vehicle-control arm, and 5.66 – 9.89 x 10^6 in BP-4-018 arm (Figure 4.3.3A, upper panel). Whereas luciferase expression remained stable or increased after 8 hours in vehicle-treated mice, a marked decrease in luciferase signal was consistently observed in BP-4-018-treated mice (Figure 4.3.3A, lower panel and Figure 4.3.3B).
Eight hours after administering BP-4-018 at 15 mg/kg, BLI signal intensities were reduced by 87.5% and 88.7% in each animal, respectively (Figure 4.3.3B), and although it is possible that the decrease in luciferase expression may be a result of decreased cell viability, we speculate that it more likely reflects in vivo inhibition of STAT3 transcriptional activity by BP-4-018.

Figure 4.3.3 BLI analysis of short-term BP-4-018 treatment effects on STAT3 transcriptional activity in vivo. Dorsal images of mice harboring subcutaneous RPMI-8226 tumours were acquired prior to (baseline), and 8 hours after treatment with BP-4-018 (oral gavage; 15 mg/kg) or vehicle control (oral gavage; 0.7% DMSO in water) and depict BLI intensity signals (A) expressed in radiance (p/sec/cm²/sr). Acquired BLI images were analyzed by generating elliptical ROIs over the tumour area to quantify the respective BLI intensity for each subject (n=2 each for treatment/vehicle), which is presented graphically in (B) on a scale of BLI radiance (p/sec/cm²/sr).
4.4. Summary

The current study sought to extend the *in vitro* findings from Chapter 3 by continuing the pre-clinical evaluation of BP-4-018 *in vivo*. Using a MM tumour xenograft model, our findings demonstrate that single agent BP-4-018 significantly delays tumour growth, and in combination with subtherapeutic doses of bortezomib, promotes tumour regression. In agreement, the mean weight of tumours extracted from BP-4-018-treated mice at the end of this study was significantly less than vehicle control-treated mice, and tumours were undetectable in 2 of 3 mice treated with the combination of BP-4-018 and bortezomib. It was our original intention to establish a MM xenograft model that enabled the *in vivo* measurement of STAT3 transcriptional activity. Therefore, we generated xenografts using a MM cell line that stably expressed a STAT3-driven firefly luciferase reporter construct, and employed BLI to detect the expression of STAT3-driven firefly luciferase activity. Using this model, we have demonstrated that after 8 hours of treatment, STAT3-driven luciferase expression is consistently and markedly lower in BP-4-018-treated mice compared to vehicle controls, confirming target inhibition at tolerated and effective doses of BP-4-018. Although this model requires further optimization, we have successfully validated its utility for evaluating molecular therapeutics that target STAT3 *in vivo*. 
Chapter 5
Discussion

The oncogenic potential of aberrant STAT3 signaling has been reported in numerous human tumours, where it has been shown to mediate a wide range of malignant processes. Constitutive activation of STAT3 is reported in MM as well, and it is suggested that its aberrant activity directly contributes to the pathogenesis of this disease. Although there are several studies supporting the premise that aberrant STAT3 signaling provides an important survival signal in MM cells, controversy still exists as to whether the persistent state of STAT3 activation directly contributes to the pathogenesis of this disease, or merely reflects a bystander effect with little physiological consequence. This thesis focused on the determining the therapeutic potential of targeting the STAT3 transcription factor in MM, and further aimed to characterize the functional role of STAT3 in MM cell survival. Using both RNAi and pharmacological tools, our results provide strong evidence that STAT3 plays a pivotal role in the survival of MM tumour cells, and ultimately validates this protein as an important therapeutic target in MM.

In the first section of this thesis, we aimed to validate STAT3 as a therapeutic target in MM using RNAi technology, and to characterize the functional role that STAT3 plays in MM cell survival. Our studies were performed using three different HMCLs, one of which has previously been shown to be dependent on STAT3 for survival using siRNA knockdown experiments. To our knowledge, the consequences of direct STAT3 abrogation using RNAi has never been investigated in the other two cell lines, XG6 and JJN3, and therefore should instill further confidence in the tractability of STAT3 as a drug target in MM. Following knockdown of STAT3 in XG6, JJN3 and RPMI-8226 cell lines, which were previously established to harbor constitutively active STAT3, we detected an increase in cell surface staining with the phospholipid-binding protein Annexin V, and the induction of PARP cleavage. Taken together, these observations are indicative of the induction of apoptosis. It is noteworthy that although residual STAT3 transcriptional activity was still detectable following shRNA-mediated knockdown of STAT3, this degree of STAT3 silencing was sufficient to induce the observed functional response of apoptosis.
Our findings strongly support a specific anti-apoptotic function for STAT3 in this set of HMCLs. In one of the earliest reports pertaining to the role of STAT3 in MM, Catlett et al. targeted IL-6-driven STAT3 activation in the U266 MM cell line by expressing a dominant-negative STAT3 mutant, and demonstrated that the proliferation and survival of these cells were strongly diminished following inactivation of STAT3.\textsuperscript{81} A number of published studies have since demonstrated that direct inhibition of STAT3 using an siRNA approach similarly induces apoptosis in KMS11 and RPMI-8226 cells.\textsuperscript{96,97,118} The results we have provided for RPMI-8226 cells are consistent with those reported by Ge et al., which established that silencing STAT3 by stable transfection with siRNA plasmids induces apoptosis in RPMI-8226 cells, as well as cell cycle arrest.\textsuperscript{97} However, this is to our knowledge, the first study to define a specific role for STAT3 in the survival of both XG6 and JJN3 cells by using a direct shRNA-mediated knockdown approach. Although we recognize the importance of evaluating additional independent (i.e. non-sequence overlapping) STAT3-targeting shRNA to reduce the probability that the observed phenotype is due to an off-target effect, there are several observations that strengthen our conclusions. First, the STAT3 shRNA sequence used in this study has also been employed by others,\textsuperscript{119,120} which instills confidence that our results are not due to off-target effects. In addition, the phenotypic readout that is observed following STAT3 silencing from our studies are consistent with those previously reported, even though different experimental approaches were employed (RNAi vs. dominant-negative mutants). Together, these observations ultimately provide strong support for the validity of STAT3 as a therapeutic target in MM.

To gain insight into molecular mechanisms underlying the observed induction of apoptosis, we evaluated the resulting effects of STAT3 knockdown on the expression of known anti-apoptotic and pro-proliferative STAT3 targets, including Mcl-1, Bcl-2, Bcl-xL, c-Myc and Survivin. Following knockdown of STAT3, all three HMCLs exhibited a reduction in the expression of Mcl-1, Bcl-2 and Bcl-xL protein expression, which is consistent with the previously observed induction of apoptosis. These findings corroborate with previous reports that STAT3-driven dysregulation of anti-apoptotic Bcl-2 family of proteins plays a significant role in maintaining the survival MM tumour cells.\textsuperscript{81,90,121} However, it is important to consider that some members of the Bcl-2 protein family have been suggested to be more important than others. By employing an antisense oligonucleotide strategy, Derenne et al. showed that the survival of MM cells is only
dependent on Mcl-1, but not Bcl-xL or Bcl-2. Consistently, RNAi lethality screens in HMCLs have identified Mcl-1 as one of the most important and selective MM survival genes. In the context of STAT3, MM cells have been shown to respond to IL-6 stimulation by upregulating the expression of Mcl-1, but not Bcl-xL or Bcl-2. Thus, although we observed a decrease in Mcl-1, Bcl-xL and Bcl-2 following knockdown of STAT3 in this study, we can speculate that mechanistically, the induction of apoptosis following knockdown of STAT3 may be mediated by the resulting reduction in Mcl-1 expression. Conversely, although we observed a reduction in Bcl-2 and Bcl-xL expression following knockdown of STAT3, these proteins may be more dispensable in the context of MM cell survival. Unexpectedly, the protein level of Survivin was observed to increase following knockdown of STAT3 in all three HMCLs, which contradicts the observations of other studies that report Survivin as a target gene regulated by STAT3. However, in other cell types such as lymphoma and leukemia, the transcriptional regulation of Survivin is alternatively mediated by transcription factors, such as NF-κB, or other members of the STAT protein family, including STAT5 and STAT1. Thus, it is plausible that in the absence of STAT3 in MM, other transcription factors may attempt to compensate for the suppression of anti-apoptotic protein expression by increasing the expression of Survivin.

In validating STAT3 as a therapeutic target in MM, the results provided the rationale for Chapter 3 and 4 of this thesis, where the pre-clinical anti-MM activity of novel small molecule STAT3 inhibitors were evaluated. We first screened a selection of salicylic acid-based small molecule STAT3 inhibitors to identify a candidate compound with potent anti-MM activity. This BP-series of compounds were derived from first generation tolyl-N-alkyl and perfluorobenzene-N-alkyl analogues. Definitive conclusions about which class of analogues are most cytotoxic to MM cells could not be drawn for our analysis, but these structure-activity relationships are beyond the scope of this thesis. In the current study however, BP-4-018 consistently demonstrated the most potent anti-MM activity, which served as the primary basis for selecting this compound over the others for further evaluation. Nevertheless, it should also be noted that the remaining compounds from this screen continue to be evaluated and characterized for their potential therapeutic utility in MM. In retrospect, it is interesting that of the 5 compounds evaluated, the cytotoxic effects of BP-4-018 against each individual cell line most closely resembled that achieved by shRNA-mediated silencing of STAT3 in Chapter 2, with RPMI-8226 being the most sensitive, followed by XG6 and JJN3. We speculate that this could potentially reflect the favorable selectivity of BP-
4-018 for targeting STAT3 compared to the other compounds evaluated.

The findings presented in Chapter 3 and 4 are the first to characterize the anti-tumour activity of BP-4-018. In cell-based assays, we observed dose- and time-dependent inhibition of STAT3 phosphorylation by BP-4-018 that correlated with induction of apoptosis in MM cells. Consistent with targeted inhibition of STAT3 phosphorylation, BP-4-018-treated cells demonstrated a decrease in STAT3 transcriptional activity and reduction in protein expression of STAT3 target genes that are known to regulate cell proliferation and survival, including Bcl-xL, Mcl-1 and c-Myc. The phenotype induced by STAT3 inhibition and the molecular mechanism of action of BP-4-018 are consistent with that published for other small molecule STAT3 inhibitors.\textsuperscript{115,127} Thus, collectively, the pro-apoptotic effects of BP-4-018 in MM are mechanistically consistent with targeted inhibition of STAT3 signaling.

In addition to demonstrating the anti-MM activity of BP-4-018 in a genetically diverse panel of HMCLs, we also present results that address some of the limitations surrounding the use of established HMCLs for pre-clinical drug testing. For example, although MM is characterized as a slowly proliferating malignancy, the majority of HMCLs have been described as having a much higher proliferative capacity, and as a result, have been criticized for poorly recapitulating the disease and molecular features of MM.\textsuperscript{128} Furthermore, since HMCLs are generally cultured alone, without the influence of non-malignant accessory cells, they are not exposed to the same microenvironmental pressures as primary tumour cells. Here we demonstrate that BP-4-018 retains anti-MM activity in the presence of BM stromal cells, albeit with modest protection conferred by BM stroma for certain HMCLs. Most noteworthy was the potent cytotoxic effects observed against CD138\textsuperscript{+} MM tumour cells from primary patient specimens. Taken together, these observations instill further confidence in BP-4-018 as a promising therapeutic candidate for MM.

An important consideration for the development of any novel therapeutic agent is ensuring an appropriate therapeutic window, that is, the differential potency comparing tumour cells to normal, non-transformed cells. We found that in one patient sample, BP-4-018 demonstrated cytotoxicity against normal primary MNCs, but at higher doses than those required to inhibit viability of the myeloma tumour cell fraction. Furthermore, BP-4-018 failed to inhibit normal hematopoietic progenitor cell growth and differentiation in colony formation assays and lacked
cytotoxic activity against normal BM stromal cells. These results are indeed consistent with the observations that, although intact STAT3 signaling has been shown to be critical during early embryogenesis, it is not required to maintain basal cell survival in relatively differentiated cell types.\textsuperscript{103,104,129,130} Thus, there does appear to be a therapeutic window for BP-4-018, supporting its utility for the treatment of MM.

We further demonstrated that BP-4-018 had broad anti-MM activity against a panel of genetically heterogeneous HMCLs, with IC\textsubscript{50} values ranging from 1.9 – 6.5 \(\mu\)M. Despite confirming the inhibitory effect of BP-4-018 on STAT3 activity, we could not correlate the responsiveness of HMCLs to BP-4-018 to their basal levels of STAT3 phosphorylation. It is possible that these results reflect a universal dependence of plasma cells on intact STAT3 signaling, which would be consistent with the role of STAT3 in regulating the balance of gene expression necessary for the terminal differentiation of plasma cells, such as BCL-6 and BLIMP1.\textsuperscript{98,99} In further support of universal STAT3-depedence in MM, mice in which STAT3 has been selectively deleted from the B-cell lineage display normal germinal center formation and baseline serum antibody levels, but have markedly diminished long-lived antibodies.\textsuperscript{131} Similarly, naïve B-cells from patients with heterozygous inactivating mutations in the STAT3 gene fail to differentiate into plasma cells upon stimulation with IL-21.\textsuperscript{132} Alternatively, the lack of correlation between basal STAT3 activation and sensitivity to BP-4-018 may be the result of off-target activities that are an invariable feature of small molecule inhibitors. Experiments aimed at evaluating the activity of BP-4-018 against additional signaling molecules such as ERK and AKT indeed demonstrated its selectivity for STAT3 inhibition. However, future work will certainly be required to further address the specificity of BP-4-018, including more extensive activity profiling, large-scale kinase screens, and experiments to determine the activity of BP-4-018 in STAT3C mutant cell lines. Although we cannot exclude the possibility that BP-4-018 has additional cellular targets, we would like to highlight that the findings presented from Chapter 3 using pharmacological inhibition of STAT3 are consistent with those from Chapter 2, where silencing of STAT3 was accomplished with RNAi-mediated knockdown. Taken together, our findings up to this point would therefore suggest that the activity of BP-4-018 against MM cells is, at least in part, mediated by its targeted inhibition of STAT3 signaling.

While cell lines and \textit{in vitro} studies are useful for establishing the anti-tumour activity of novel therapeutics against MM tumour cells, they are by no means predictive of \textit{in vivo} responses,
which can be impacted by factors such as drug distribution, metabolism, and microenvironmental influences. Therefore, in addition to in vitro cell-based studies, we evaluated the in vivo inhibitory effect of BP-4-018 on STAT3 activity and its therapeutic effect against MM xenograft tumors. For the purposes of these experiments, we established a xenograft mouse model using MM tumor cells that express a STAT3-driven luciferase reporter construct, thus enabling the real-time in vivo imaging of STAT3 transcriptional activity. Using this model, we confirmed targeted inhibition of STAT3-driven luciferase expression upon treatment of mice with BP-4-018. Although these experiments were only performed using a small cohort of mice, the model itself represents a promising tool for real-time monitoring of molecular specific-pharmacodynamics in vivo. Indeed, coupling in vivo promoter-driven luciferase activity and BLI has been previously described for ubiquitous promoters, as well as for transcription factor-specific promoters, such as NF-kB. However, it is to our knowledge, the first application of this model to specifically evaluate STAT3 activity. Thus, with further optimization, this model could be extremely useful for evaluating the mechanism-based activity of STAT3 targeted therapeutics under highly relevant physiologic conditions.

Based on our cellular and in vivo studies, BP-4-018 represents a promising therapeutic candidate for the treatment of MM. However, within the landscape of MM treatment, it is clear that combinatorial therapeutic strategies demonstrate significant clinical benefit over those that employ single agent regimens. Innovative drug combination strategies are continually being explored in MM to augment the efficacy of current monotherapies, circumvent the development of drug resistance, and/or reduce the severity of unwanted toxicities. With this in mind, one of the most striking findings from the in vitro and in vivo studies was the ability of BP-4-018 to markedly enhance the sensitivity of MM tumours to bortezomib. Although more comprehensive studies are required to determine the true nature of the interaction between these two agents, we speculate that the combination of BP-4-018 and bortezomib is synergistic, as the in vitro anti-MM activity of BP-4-018 in combination with bortezomib was clearly greater than the summed activity of each drug alone. Nonetheless, our results are encouragingly consistent with that reported by Voorhees et al., who demonstrated enhanced cytotoxicity of bortezomib when combined with CNTO-328, a chimeric monoclonal antibody that neutralizes IL-6 function. Interestingly however, others have reported that while bortezomib inhibits IL-6-mediated activation of pERK, it has no affect on pSTAT3. It is therefore possible that the benefit of
combining bortezomib with an agent that targets IL-6/STAT3 signaling may result from the inhibition of alternative and non-overlapping signaling pathways that are critical for MM cell survival.

As the first-in-class proteasome inhibitor, bortezomib represents a major advance in the treatment of MM, demonstrating significant clinical activity as a single agent and when incorporated into combinatorial therapeutic regimens. Through inhibition of the ubiquitin-proteasome pathway, bortezomib has been shown to have multiple direct and indirect mechanisms of anti-tumour activity including disruption of the cell cycle, induction of apoptosis, and inhibition of proliferation and angiogenesis.\textsuperscript{12} The results from numerous preclinical investigations have also provided the rationale for bortezomib-based combinatorial therapies. Most notably, bortezomib has demonstrated additive activity with dexamethasone,\textsuperscript{139} enhanced activity with lenalidomide,\textsuperscript{140} and synergistic activity when combined with melphalan or doxorubicin.\textsuperscript{141} Yet despite the unequivocal benefit of combining these agents with bortezomib, the molecular mechanisms that underlie this potentiation are, for the most part, unclear. Considering the possibility that BP-4-018 may inhibit additional targets beyond STAT3, it is difficult to decipher the direct mechanisms responsible for the enhanced activity of bortezomib that was observed in this study. Nonetheless, there are several observations that offer mechanistic insight into a synergistic relationship between bortezomib and inhibitors of STAT3. For example, small molecule-mediated inhibition of the proteasome is reported to induce Mcl-1 protein accumulation in several pre-clinical cancer models,\textsuperscript{142} including RPMI-8226 cells.\textsuperscript{143} Further, reducing Mcl-1 accumulation by siRNA knockdown has been shown to enhance proteasome inhibitor induced-cell death in solid tumours.\textsuperscript{144} Thus it is possible that the observed synergy between BP-4-018 and bortezomib could be due to the augmentation or complementation of apoptotic mechanisms, specifically as it pertains to BP-4-018-mediated inhibition of pSTAT3 and the resulting downregulation of Mcl-1.

Despite the significant impact that bortezomib has had for the treatment of MM, its use is often associated with dose-limiting toxicities such as thrombocytopenia and peripheral neuropathy.\textsuperscript{145–147} It is therefore significant that in our \textit{in vivo} efficacy study, the dose of bortezomib employed was subtherapeutic, well tolerated and demonstrated markedly enhance anti-tumor responses when combined with BP-4-018. Thus, the simultaneous enhancement of therapeutic actions and
dose tolerability provides strong rationale for the further investigation of this combination and its molecular mechanism of action.
Chapter 6
Conclusions and Future Directions

The findings from this thesis have demonstrated the importance of STAT3 signaling for the survival of MM tumour cells. The concordance between the biological readout of silencing STAT3 signaling, regardless of the inhibitory approach, provides confidence that this transcription factor represents a significant vulnerability in MM. Furthermore, given the issues surrounding small molecule promiscuity, the alignment of this phenotype supports the predicted mechanisms of BP-4-018 activity. Although we acknowledge that our studies have only peripherally examined the underlying role of STAT3 in MM pathogenesis, we believe that further elucidation of STAT3’s role in MM biology has the potential to uncover more biologically and therapeutically relevant information.

Molecular tools such as gene expression profiling and chromatin immunoprecipitation (CHIP) have become invaluable for uncovering direct gene targets of transcription factors. It would therefore be desirable to employ these STAT3 knockdown experiments in conjunction with gene expression profiling and/or CHIP analysis to identify additional tissue specific STAT3 target genes that have relevance to the underlying molecular pathology of MM. It is anticipated that identification of aberrantly activated STAT3 response genes will provide insight into oncogenic processes and biological readouts that will supplement our findings of the pro-survival effects of constitutive STAT3 activation in MM cells.

MM is a heterogeneous disease that lacks a demonstrable hallmark or fundamental molecular aberration driving its pathogenesis. As such, the approach taken in the described studies involved evaluating the therapeutic potential of abrogating aberrant STAT3 signaling in the context of broad clinical utility. However, we acknowledge the importance of tailoring therapies to patient subgroups or even individual patients, which should, without a doubt, be explored in the future for BP-4-018, as well as other STAT3 inhibitors. These future studies should involve characterizing primary MM patient samples for aberrant STAT3 activation by methods such as gene expression profiling for known STAT3-regulate target genes or immunofluorescent staining of nuclear STAT3. Comparing these data to corresponding patient data could also serve to
identify whether STAT3 activation status represents a therapeutically informative biomarker. Moreover, primary MM tumour cells should be assessed for their response to STAT3 inhibition to determine whether STAT3 inhibitors are more efficacious in certain patient subsets. Collectively, the translational focus of these studies will highlight the biological relevance of STAT3 as a molecular target in MM to ultimately guide the clinical development and implementation of small molecule STAT3 inhibitors.

Our data support the therapeutic potential of BP-4-018 against MM tumors and provide rationale for the further development of novel STAT3 inhibitors. We acknowledge however that subcutaneous xenograft tumour models do not truly recapitulate the BM microenvironment and the critical molecular influence that this niche has on MM cells. Further, the drug responses observed in subcutaneous model systems do not often correlate with their clinical activity in patients, and so it would be desirable for future experiments to additionally evaluate the activity of BP-4-018 in alternative in vivo model systems. Although IV injection of HMCLs can be used to establish orthotopic xenograft models, homing of IV transplanted primary plasma cells to BM is relatively low and there is a lack of cross-reactivity of some murine BM cytokines with human plasma cells. For this reason, BP-4-018 could in the future be evaluated using the hu-SCID model of MM, which involves injecting HMCLs or primary MM plasma cells directly into implanted human fetal bone fragments where they grow in, disseminate to, and interact with a human BM microenvironment. As an alternative to these xenograft models, there are several transgenic models that should also be considered for in vivo efficacy studies of BP-4-018, including the Eμ-xbp-1s or VK*MYC MM mice, which, to date, have been described to most closely model the pathogenesis and clinical features of MM. Furthermore, given that constitutive STAT3 activation has been implicated in restraining anti-tumour immune responses, these immune-competent transgenic models would provide additional insight into the activity of BP-4-018 and other STAT3 inhibitors in the context of an immunological microenvironment.

Data from our pre-clinical studies of BP-4-018 support its development and that of any future derivative analogues for the clinic, and as such, studies to better define the targeted mechanism of action of this small molecule are warranted, including gene expression profiling or large-scale kinase screens. Although the clinical utility of BP-4-018 may be limited by its potential promiscuity, its use as an informative molecular tool has potentially uncovered a molecular
mechanism by which combinatorial synergism with bortezomib can be achieved. Thus, future work aiming to uncover its mechanism of action could serendipitously guide the development of more efficacious proteosome inhibitor-based combinatorial strategies for the treatment of MM.

Taken collectively, these future studies will further build upon the existing knowledge as it pertains to the role of STAT3 in MM biology, and the therapeutic utility of novel agents that inhibit its aberrant activation. Such studies will provide scientists and clinicians with a deeper understanding of how constitutive STAT3 activation drives the molecular pathophysiology of MM, and ultimately guide decisions on how STAT3 inhibitors should be implemented for the treatment of this incurable disease.
References


63. Stephanou A, Isenberg DA, Akira S, Kishimoto T, Latchman DS. The nuclear factor interleukin-6 (nf-il6) and signal transducer and activator of transcription-3 (stat-3) signalling pathways co-operate to mediate the activation of the hsp90beta gene by interleukin-6 but have opposite effects on its inducibility by heat. *The Biochemical journal*. 1998;330 (Pt 1):189–95.


100. Leticia Quintanilla-Martinez MK. Analysis of signal transducer and activator of transcription 3 (stat 3) pathway in multiple myeloma: stat 3 activation and cyclin d1 dysregulation are mutually exclusive events. *The American Journal of Pathology*. 2003;162(5):1449.


Appendices

Appendix A. STAT3 inhibitory activity of small molecule analogues as assessed by FP assay

Figure A1. FP dose-response curves. Polarized fluorescence was plotted against concentration of inhibitor and IC$_{50}$ values determined by fitting to a dose response curve. The FP assay for measuring phosphopeptide:STAT3-SH2 domain disruption was performed as previously reported.$^{154}$ Briefly, fluorescently labeled peptide probe (5-FAM-GpYLPQTV-NH$_2$) was incubated with recombinant STAT3 protein and inhibitor for 30 minutes, then analyzed on a Tecan M1000 fluorimeter. (Experiments performed by Dr. Patrick Gunning and Brent Page).

Table A1. IC$_{50}$ values of small molecule STAT3 inhibitors derived from FP dose-response curves.

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
<th>IC$_{50}$ (µM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolyl-N-alkyl</td>
<td>SF-1-066</td>
<td>30.93 ± 9.46</td>
</tr>
<tr>
<td></td>
<td>BP-2-047</td>
<td>8.76 ± 2.92</td>
</tr>
<tr>
<td></td>
<td>BP-2-061</td>
<td>2.78 ± 4.33</td>
</tr>
<tr>
<td>Perfluorobenzene-N-alkyl</td>
<td>BP-1-102</td>
<td>25.0 ± 1.88</td>
</tr>
<tr>
<td></td>
<td>BP-3-163</td>
<td>12.66 ± 2.77</td>
</tr>
<tr>
<td></td>
<td>BP-4-018</td>
<td>10.96 ± 1.62</td>
</tr>
</tbody>
</table>
Appendix B. Inhibiting Activated STAT3 Proteins with Tetrapodal, Small Molecule SH2 domain binders: Promising Agents Against Multiple Myeloma

The following manuscript has been submitted to the *Journal of Medicinal Chemistry* (American Chemical Society: http://pubs.acs.org/journal/jmcmar)

**Contributions:** The author of this thesis and Brent Page will be first co-authors of this work when it is published (currently pending acceptance to the Journal of Medicinal Chemistry). Additional contributions were made by the following: Zhi Hua Li, Yoong Lim Wong, Sina Haftchenary, Victor H. Jimenez-Zepeda, Jennifer Atkinson, Paul A. Spagnuolo, Robert Colagouri, and Aaron D. Schimmer. Dr. Patrick Gunning and Dr. Suzanne Trudel provided supervisory support.

**Note:** Compound codes are as followed:

<table>
<thead>
<tr>
<th>Manuscript Code</th>
<th>Compound Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S31-201</td>
</tr>
<tr>
<td>7</td>
<td>SF-1-066</td>
</tr>
<tr>
<td>9</td>
<td>BP-1-102</td>
</tr>
<tr>
<td>16i</td>
<td>BP-2-061</td>
</tr>
<tr>
<td>16f</td>
<td>BP-2-047</td>
</tr>
<tr>
<td>21h</td>
<td>BP-3-163</td>
</tr>
<tr>
<td>21z</td>
<td>BP-4-018</td>
</tr>
</tbody>
</table>

**Abstract.** The signal transducer and activator of transcription (STAT) proteins represent a family of cytoplasmic transcription factors that regulate a pleiotropic range of biological processes in response to extracellular signals. Of the seven mammalian members described to date, STAT3 has received particular attention, as it regulates the expression of genes involved in a variety of malignant processes including proliferation, survival, migration and drug resistance. Indeed, aberrant STAT3 activation has been observed in a number of human cancers, and its inhibition has shown promising anti-tumour activity in cancer cells with elevated STAT3
activity. Multiple myeloma (MM) is an incurable haematological malignancy that often exhibits abnormally high levels of STAT3 activity. Although aggressive treatment with currently available anti-cancer agents can improve the clinical management of MM, it remains uniformly incurable with a dismal median survival time post-treatment of 3-4 years. It is therefore clear that novel targeted therapeutics are critically needed to improve MM patient outcomes. We herein report the development of a series of small molecule STAT3 inhibitors with potent anti-MM activity in vitro. These compounds showed high-affinity binding to STAT3’s SH2 domain, inhibited intracellular STAT3 phosphorylation, and induced apoptosis in MM cell lines at low micromolar concentrations. Lead compounds identified represent the most potent small molecule STAT3 inhibitors to emerge from our salicylic acid-based series of inhibitors.

**Introduction.** As a master regulator of cell signaling and tumourigenesis, signal transducer and activator of transcription (Stat) 3 has emerged at the forefront of cancer drug discovery programs.\(^1\)\(^-\)\(^3\) Aberrant activation of STAT3 has been observed in a number of solid and hematological malignancies and is correlated with a variety of hallmark oncogenic processes including cell growth and survival, angiogenesis, inflammation, and metastatic potential.\(^1\)\(^-\)\(^7\) Multiple myeloma (MM) is the second most common hematological malignancy and is responsible for approximately 13 % of blood cancers and 1 % of all cancers.\(^8\) Although MM is generally regarded as incurable, traditional high-dose chemotherapeutics and currently available targeted therapies can improve the prognosis of MM patients when used as part of an aggressive treatment regimen.\(^8\)\(^-\)\(^11\) Despite this, the median survival time after conventional treatment remains disappointingly low (3-4 years).\(^8\) In the search for novel molecular targets in MM, STAT3 has emerged as a driving force behind the maintenance and progression of the disease and it is anticipated that STAT3 inhibitors will provide a novel and effective weapon in the fight against MM.\(^12\)\(^-\)\(^14\)

The STAT3 signaling cascade is initiated by binding of extracellular ligands such as cytokines and growth factors to their respective cell surface receptors.\(^1\)\(^,\)\(^4\)\(^,\)\(^15\) A subsequent conformational change in the receptor results in intrinsic or kinase-mediated receptor activation via the phosphorylation of key tyrosine residue within the receptor’s cytoplasmic domain. These phosphorylated tyrosine residues (pTyr) serve as docking sites for a number of Src-homology 2
(SH2)-domain containing proteins such as STAT3. Thus, the phosphorylated receptor complex recruits unphosphorylated STAT3 via its SH2 domain and in turn STAT3 is activated by phosphorylation of a C-terminal tyrosine residue (Tyr705). Following dissociation from the receptor complex, phosphorylated STAT3 (pSTAT3) proteins form transcriptionally active pSTAT3–pSTAT3 homo-dimers via reciprocal interactions between the SH2 domain of one activated STAT3 monomer and the pTyr705 of another. Activated pSTAT3 dimers subsequently translocate to the nucleus, where they bind specific DNA response elements to induce target gene expression. In contrast to the transient nature of STAT3 activation in normal cells, constitutive STAT3 activity, such as that observed in MM, drives the expression of target genes with known oncogenic roles, contributing to the maintenance and progression of tumorigenic processes.

The SH2 domain of STAT3 plays a critical role in the STAT3 signaling cascade, facilitating recruitment of STAT3 to activating cell surface receptors, and playing a key role in the formation of STAT3 homo-dimers.\(^5,6,16\) Thus, there has been significant effort to silence aberrant STAT3 signaling by targeting the SH2 domain.\(^1-4\) As a therapeutic target, the SH2 domain of STAT3, like many protein–protein interaction interfaces, is devoid of a classical binding pocket and is relatively flat and hydrophobic.\(^17,18\) However, the SH2 domain possesses a hydrophilic region (containing residues Lys591, Arg609 and Glu612) responsible for binding to pTyr residues.\(^18\) Many groups, including our own, have targeted this sub-pocket for developing STAT3 inhibitors, employing polar groups such as, phosphate esters,\(^17,19-23\) catechols,\(^24\) carboxylates\(^25\) and salicylic acids\(^15,26-29\) that mimic the native pTyr705 substrate. To design STAT3 selective SH2 domain binders, two proximal sub-pockets (one hydrophobic and one amphipathic) have been simultaneously targeted using tripodal STAT3 inhibitors.\(^25\)

Our research group has approached the development of STAT3 inhibitors by performing extensive structure activity relationship (SAR) analysis of a known STAT3-SH2 domain binder, S3I-201 (1). This compound was identified through an in silico high-throughput screen,\(^29\) and led to the generation of two lead compounds, 7 and 9 (BP-1-102), that are now entering advanced pre-clinical trials as cancer therapeutic agents (Table 1).\(^15,26,30\)
Table 1: Preliminary SAR leading to the development of lead compounds 7 and 9.\textsuperscript{15, 27} Compound activity was assessed using an electrophoretic mobility shift assay (EMSA) as previously reported.\textsuperscript{26, 30}

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>X</th>
<th>R'</th>
<th>IC\textsubscript{50} (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>O</td>
<td>p-Tolyl</td>
<td>84 ± 33</td>
</tr>
<tr>
<td>2</td>
<td>H</td>
<td>NH</td>
<td>p-Tolyl</td>
<td>&gt; 300</td>
</tr>
<tr>
<td>3</td>
<td>H</td>
<td>NCH\textsubscript{3}</td>
<td>p-Tolyl</td>
<td>&gt; 300</td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td>NBOc</td>
<td>p-Tolyl</td>
<td>&gt; 300</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>O</td>
<td>p-Tolyl</td>
<td>43 ± 13</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>NH</td>
<td>p-Tolyl</td>
<td>95 ± 35</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>NCH\textsubscript{3}</td>
<td>p-Tolyl</td>
<td>35 ± 9</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>NBOc</td>
<td>p-Tolyl</td>
<td>115 ± 35</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>NCH\textsubscript{3}</td>
<td>C\textsubscript{8}F\textsubscript{5}</td>
<td>6.8 ± 0.8</td>
</tr>
</tbody>
</table>

In previous work, we discovered several tripodal inhibitors of STAT3 that have demonstrated promising anti-cancer activity.\textsuperscript{26, 30} Using the parent compound S3I-201 (1), we generated two lead compounds 7 and 9 that have demonstrated more potent STAT3 SH2 domain binding, improved inhibition of cellular STAT3 activity, and promising activity against cancer cell lines and xenograft models.\textsuperscript{15, 27, 30, 31} Interestingly, substitution of the N-methyl group with an oxygen atom, NH or N-Boc resulted in significant changes in inhibitor activity. This prompted further investigation into the sulphonamide nitrogen substituent.
Materials and Methods

**GOLD Docking Simulations.** Inhibitors were docked using GOLD docking software to STAT3 crystal structure, pdb 1BG1. Compounds were first optimized into a low energy geometry. The compound binding site was set to an area with a 12 Å radius surrounding Ser636. Best solutions were visualized using Pymol, which was utilized to create the images shown in Figures 2 and 3 and in supplementary Figure S.1.

**Fluorescence Polarization Assay.** The fluorescence polarization assay was performed as previously reported. Briefly, fluorescently labelled peptide probe (5-FAM-GpYLPQTV-NH2, CanPeptide, Pointe-Claire (Montreal), Quebec, Canada) was incubated with STAT3 protein (SignalChem, Richmond, British Columbia, Canada), and inhibitor for 30 minutes then analyzed on a Tecan M1000 fluorimeter (Tecan, Mannedorf, Switzerland). Polarized fluorescence was plotted against concentration of inhibitor and IC\textsubscript{50} values were determined by fitting to a dose response curve. Representative curves of the top compounds are shown in the supplementary information.

**Cell Viability Assays.** The MTS and MTT assays were used to measure cellular metabolic activity, which reflects the number of viable cells. Cells were seeded in 96 well plates at 1-3 x 10^4 cells/well in 90 µL of fresh culture medium. Prior to the addition of cell suspensions, 10 µL of test compound (or vehicle control) was added to wells in triplicate. Cultures were then incubated for 72 hours at 37 °C, 5% CO\textsubscript{2}. Following treatment, cell viability was assessed by MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium for AML2, DU145 and MDA468 cell lines, or MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for MM cell lines. Relative cell viability (to DMSO control) was determined colorometrically and EC\textsubscript{50} values determined by fitting to a standard dose response curve when applicable.

**Immunoblot Analysis.** Following treatment, target cells were harvested and washed twice in ice cold PBS. The resulting cell pellets were lysed in lysis buffer ((50mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetra acetic acid (EDTA), and 1% NP-40) supplemented with 1 mM phenylmthanesulfonyl fluoride (PMSF), 2 mM sodium vanidate (Na\textsubscript{3}VO\textsubscript{4}) and protease inhibitor cocktail (Roche Applied Science) for 30 minutes on ice. Protein lysates were collected
by centrifugation at 14,000 g for 15 minutes. Protein concentration was determined by Bradford Assay (Thermo Scientific, Rockford, IL) and normalized with lysis buffer before the addition of β-Mercaptoethanol-supplemented Lamelli sample buffer (Bio-Rad Laboratories, Hercules, CA). Proteins (10-30 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7-10% gels, and transferred to polyvinylidene fluoride (PVDF) membranes using wet transfer at 70 V for 1 hour. Membranes were rinsed in Tris-buffered Saline with 0.01% Tween-20 (TBST) and blocked for 1 hour at room temperature in TBST containing 5% bovine serum albumin (BSA) powder, followed by overnight incubation with primary antibodies at 4 °C. Primary antibodies against the indicated proteins were diluted in TBST with either 5% BSA or 5% milk, as specified by manufacturer. Following three 15-minute washes in TBST, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Thermo Scientific Pierce, Rockford, IL) diluted 1:4000 in TBST for 1 hour at room temperature. Membranes were developed using the enhanced chemiluminescence kit (Perkin Elmer, Waltham, MA) according to the manufacturer’s instructions and visualized by autoradiography. Resulting autoradiographs were analyzed by densitometry using the Gel Doc XR station and Quantity One Software (Bio-Rad, Hercules, CA).

**Luciferase Reporter Assay.** Target cells were transduced with replication incompetent, VSV-g pseudotyped lentiviral particles containing the STAT3-driven *Firefly* luciferase reporter constructs (pCignal Lenti-STAT3\(_{\text{TRE}}\)-FLuc). Transductions were performed with polybrene (8 µg/µl) in accordance with the Cignal Lenti Reporter Assay Kit (SA Biosciences, Frederick, MD). The pCignal Lenti-STAT3\(_{\text{TRE}}\)-FLuc reporter construct is under the control of a basal promoter element (TATA box) joined to tandem repeats of a specific STAT3 transcriptional response element (TRE), and regulates the expression of the mammalian codon-optimized, non-secreted form of the *Firefly* luciferase gene. Stably transduced cells were selected using puromycin (2 µg/µl) for 2 weeks. As an internal control, STAT3FLuc-expressing cells were stably transfected with the pCignal Lenti-CMV-RLuc reporter construct, which contains a CMV immediately early enhancer/promoter that constitutively drives *Renilla* luciferase expression. Transductions were performed as previously described and stable cells selected with hygromycin (50 µg/µl) for 2 weeks. *In vitro* reporter construct activity of drug treated cells was measured using the Stop & Glo® Dual Luciferase Assay System (Promega, Madison, WI), with data presented as relative luciferase units (RLU = *Firefly* luciferase/*Renilla* luciferase).
**Results.** We first utilized docking simulations to explore potential binding interactions of compounds 8 which possessed the hydrophobic Boc group appended to the sulfonamide nitrogen. Comparing 8 to parent compounds S3I-201 (1), 7 and 9, we observed interesting differences. Compounds S3I-201 (1), 7 and 9 were found to dock to the SH2 domain of STAT3 with similar conformations as previously reported. We presume the salicylic acid group mimics the pTyr motif and facilitates docking with the polar, phosphate binding region. The N-cyclohexylbenzyl substituent, common to both 7 and 9, was found to interact *in silico* with the hydrophobic residues, Val637 and Trp623. The N-methyltoluenesulphonamide group interacted with the amphipathic region which contained Ile634 and Glu594 as well as the hydrophobic side-chain of Lys591.

When the N-Boc derivative (8) was docked *in silico*, it was found to orient similarly to 7 and 9. However, the bulky, hydrophobic t-butyl group was found to disfavorably orientate away from the protein surface (Figure 2, A and B). However, this seemingly unfavorable docking position is not reflected in the *in vitro* EMSA disruption assay, with only a slight decrease in potency, and an improved binding affinity observed in the fluorescence polarization (FP) binding assay (Compound 8 IC$_{50}$ = 15.8 ± 0.2 µM *c.f.* compound 7 IC$_{50}$ = 31.0 ± 9.4 µM).

![Figure 1. GOLD $^{32, 33}$ docking images of compounds bound to STAT3’s SH2 domain (STAT3 pdb 1BG1$^{18}$). A: S3I-201 (1); B: 7; C: 9; D: compound 8.](image)
Further docking studies revealed an alternative binding mode for 8 where the Boc group contributed to protein surface binding (Figure 2, C and D). However, unlike previous studies, the N-cyclohexylbenzyl moiety was positioned within the amphipathic binding pocket containing residues Ile634, Glu594 and the side chain of Lys591 (Figure 2). As a result, the substituted sulphonamide group projects into the hydrophobic cleft composed of residues including, Trp623 and Val637. The substituent on the sulphonamide nitrogen then interacts with Trp623 and Phe716 and places the sulfonamide S-substituent in closer proximity to Cys712. This orientation allows for improved interaction between the protein and larger hydrophobic substituents appended to the sulphonamide nitrogen.

Figure 2. Popular binding modalities of 8 bound to STAT3’s SH2 domain (pdb 1BG1). Images A and B show the previously predicted docking poses of 8. Images C and D show an alternative binding mode, where the N-cyclohexylbenzyl and sulphonamide substituents are orientated differently.

To further explore the in vitro STAT3 binding potency and whole cell biological effects of N-alkylated analogs of both 7 and 9, we herein report the synthesis of a novel library of salicylic acid-based inhibitors. Inhibitors were functionalized with select substituents stemming from the sulphonamide nitrogen to furnish a series of tolyl-N-alkyl and perfluorobenzene-N-alkyl derivatives of 7 and 9, respectively.
The tolyl-N-alkyl derivatives were prepared from doubly O-benzyl protected 4-aminosalicylic acid (10) which was coupled to 4-cyclohexylbenzaldehyde under standard reductive amination conditions using NaCNBH₃. The resultant secondary aniline, 11, was then coupled to functionalized carboxylic acid, 13. Carboxylate 13 was prepared from glycine methyl ester hydrochloride which was tosyalted using tosyl chloride and then Boc protected using Boc-anhydride. Saponification of the methyl ester gave the acid (13) which was coupled to the aniline using triphenylphosphine dichloride. The peptide coupling also cleaved the Boc-protecting group as two equivalents of HCl are generated during this reaction. The sulphonamide nitrogen was then functionalized with a variety of alkyl bromides or alkyl chlorides and then deprotected using hydrogenolysis or a step-wise saponification of the benzyl ester followed by treatment with TFA to cleave the benzyl ether. This protocol was used to produce a library of 45 compounds in a divergent fashion. All compounds were subjected to analysis in a fluorescence polarization (FP) assay and MTS proliferation assay against DU145, MDA-468 and AML2 cells.
To make the perfluorobenzene-N-alkyl derivatives, Fmoc-glycine was coupled to secondary aniline, 11, using triphenylphosphine dichloride. The Fmoc group was removed using piperidine in DMF to afford the free amine. Sulfonamide 19 was prepared by treating amine 18 with pentafluorobenzenesulfonyl chloride. A variety of different alkyl bromides were then used to furnish the sulphonamide nitrogen then treatment with hydrogen and 10% Pd/C gave the deprotected final molecules. Of note, a step-wise deprotection procedure could not be used for the synthesis of the perfluorobenzene-N-alkyl derivatives as treatment with LiOH.H₂O led to nucleophilic aromatic substitution of the perfluorobenzene ring, placing a hydroxyl group para to the sulphonamide. Again, all compounds were subjected to analysis in an FP assay and MTS cell proliferation assay against DU145, MDA-468 and AML2 cells. Selected compounds were further evaluated via Western blot analysis for pSTAT3 and in MM cell lines for cytotoxicity.
Table 2: Tolyl-N-alkyl Derivatives IC$_{50}$ reported for FP assay.

<table>
<thead>
<tr>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>&gt; 50</td>
<td>16g</td>
<td>5.2 ± 0.4</td>
<td>16p</td>
<td>6.0 ± 2.2</td>
<td>16y</td>
<td>12.4 ± 3.2</td>
<td>16ah</td>
<td>15.4 ± 2.0</td>
</tr>
<tr>
<td>7</td>
<td>&gt; 50</td>
<td>16h</td>
<td>&gt; 50</td>
<td>16q</td>
<td>6.2 ± 0.2</td>
<td>16z</td>
<td>20.8 ± 6.6</td>
<td>16al</td>
<td>22.8 ± 0.8</td>
</tr>
<tr>
<td>8</td>
<td>15.8 ± 0.2</td>
<td>16i</td>
<td>2.8 ± 4.3</td>
<td>16r</td>
<td>7.2 ± 1.0</td>
<td>16aa</td>
<td>45.2 ± 5.0</td>
<td>16aj</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td>16a</td>
<td>17.8 ± 2.4</td>
<td>16j</td>
<td>10.2 ± 0.8</td>
<td>16s</td>
<td>8.0 ± 2.2</td>
<td>16ab</td>
<td>&gt; 50</td>
<td>16ak</td>
<td>5.0 ± 2.0</td>
</tr>
<tr>
<td>16b</td>
<td>17.0 ± 0.6</td>
<td>16k</td>
<td>9.2 ± 3.2</td>
<td>16t</td>
<td>11.8 ± 1.2</td>
<td>16ac</td>
<td>46.6 ± 1.4</td>
<td>16bl</td>
<td>11.0 ± 0.8</td>
</tr>
<tr>
<td>16c</td>
<td>&gt; 50</td>
<td>16l</td>
<td>10.6 ± 0.6</td>
<td>16u</td>
<td>10.0 ± 3.0</td>
<td>16ad</td>
<td>22.6 ± 1.6</td>
<td>16am</td>
<td>9.6 ± 1.2</td>
</tr>
<tr>
<td>16d</td>
<td>&gt; 50</td>
<td>16m</td>
<td>9.0 ± 2.8</td>
<td>16v</td>
<td>5.0 ± 4.4</td>
<td>16ae</td>
<td>12.4 ± 1.2</td>
<td>16an</td>
<td>7.6 ± 3.0</td>
</tr>
<tr>
<td>16e</td>
<td>14.4 ± 4.4</td>
<td>16n</td>
<td>7.6 ± 3.2</td>
<td>16w</td>
<td>11.0 ± 7.4</td>
<td>16af</td>
<td>8.6 ± 1.4</td>
<td>16ao</td>
<td>18.4 ± 1.6</td>
</tr>
<tr>
<td>16f</td>
<td>8.8 ± 2.9</td>
<td>16o</td>
<td>15.8 ± 0.6</td>
<td>16x</td>
<td>24.0 ± 1.0</td>
<td>16ag</td>
<td>22.2 ± 1.2</td>
<td>16ap</td>
<td>11.6 ± 1.2</td>
</tr>
</tbody>
</table>
The FP assay for measuring phosphopeptide:STAT3-SH2 domain disruption was performed as previously reported. Relative to parent compounds, 7 and 9, many of the N-alkyl derivatives showed improved activity. Moderate improvements were observed through addition of simple alkyl groups however, it appears that substituted benzyl moieties provided the greatest enhancement of inhibitory activity. Substitution with the polar pyridine or aminobenzyl appendages led to a marked loss in protein binding affinity. Compounds that incorporated an N-(arylsulfonyl)amide exhibited lower binding affinities, with the exception of the N-Boc analogs.

As a preliminary screen, inhibitors were subjected to an MTS assay to assess the in vitro antitumour activity of compounds. Most promisingly, a number of these compounds potently inhibited the viability of a range of human cancer cell lines including prostate cancer (DU145), breast cancer (MDA-468) and leukemia (AML2). EC\textsubscript{50} data and corresponding structures for the leading five compounds from each family and their corresponding parent structures are summarized in Figure 3. Dose response curves can be found in the supplementary information.

Table 3: Perfluorobenzene-N-alkyl derivatives.

<table>
<thead>
<tr>
<th>R</th>
<th>IC\textsubscript{50} (\textmu M)</th>
<th>R</th>
<th>IC\textsubscript{50} (\textmu M)</th>
<th>R</th>
<th>IC\textsubscript{50} (\textmu M)</th>
<th>R</th>
<th>IC\textsubscript{50} (\textmu M)</th>
<th>R</th>
<th>IC\textsubscript{50} (\textmu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21a</td>
<td>CH\textsubscript{3} 25.6 ± 0.6</td>
<td>91f</td>
<td>14.8 ± 3.4</td>
<td>21i</td>
<td>17.8 ± 0.6</td>
<td>21r</td>
<td>17.8 ± 3.6</td>
<td>21x</td>
<td>17.6 ± 1.2</td>
</tr>
<tr>
<td>21b</td>
<td>H               32.4 ± 4.6</td>
<td>21g</td>
<td>14.4 ± 1.4</td>
<td>21m</td>
<td>17.6 ± 1.0</td>
<td>21s</td>
<td>21.6 ± 1.6</td>
<td>21y</td>
<td>16.6 ± 3.2</td>
</tr>
<tr>
<td>21c</td>
<td>CH\textsubscript{3} 20.6 ± 5.2</td>
<td>21h</td>
<td>12.8 ± 2.8</td>
<td>21n</td>
<td>11.0 ± 1.6</td>
<td>21t</td>
<td>11.4 ± 2.2</td>
<td>21z</td>
<td>11.0 ± 1.6</td>
</tr>
<tr>
<td>21d</td>
<td>27.4 ± 0.8</td>
<td>21i</td>
<td>9.6 ± 2.8</td>
<td>21o</td>
<td>18.2 ± 5.4</td>
<td>21u</td>
<td>19.8 ± 1.4</td>
<td>21aa</td>
<td>21.8 ± 2.6</td>
</tr>
<tr>
<td>21e</td>
<td>14.4 ± 1.6</td>
<td>21j</td>
<td>22.0 ± 4.8</td>
<td>21p</td>
<td>13.8 ± 3.4</td>
<td>21v</td>
<td>9.4 ± 3.8</td>
<td>21ab</td>
<td>17.4 ± 1.0</td>
</tr>
<tr>
<td>21f</td>
<td>11.8 ± 2.0</td>
<td>21k</td>
<td>28.0 ± 3.2</td>
<td>21q</td>
<td>10.2 ± 2.8</td>
<td>21w</td>
<td>12.4 ± 3.6</td>
<td>21ac</td>
<td>30.8 ± 7.0</td>
</tr>
</tbody>
</table>
Based on protein binding affinity and inhibition of cell proliferation, lead compounds 16i and 21h demonstrated very promising activity within their respective libraries and were therefore chosen for further analysis against a panel of human MM cell lines. We reasoned that lead compounds selected should demonstrate more activity against MM cell lines that harbor high levels of pSTAT3 as they would presumably be more reliant on aberrant STAT3 activity. Therefore, prior to testing lead compound potency, baseline STAT3 activation was first examined in a panel of genetically heterogeneous MM cell lines using Tyr705 phosphorylation as a surrogate marker of STAT3 activation. Whole cell lysates prepared from MM cell lines in log growth conditions were subject to immunoblot analysis and probed with antibodies against pSTAT3 (Tyr705) and total STAT3 protein. Although STAT3 protein was expressed in all MM cell lines, albeit to varying degrees, constitutively pSTAT3 was only detected in 6 of 8 MM cell lines, with two cell lines, MM1.S and SKMM2, lacking detectable pSTAT3 (Figure 4A). Densitometric analysis performed on immunoblots to quantitate the ratio of pSTAT3 to total STAT3 protein confirmed variability in baseline pSTAT3. In the interests of examining the effects of our STAT3 inhibitors, we selected MM cell lines possessing a variety of pSTAT3 levels for screening lead compounds, predicting that cell lines with high pSTAT3 should be more sensitive to STAT3 inhibition.

As shown in Figure 4B, both 16i and 21h demonstrated dose-dependent inhibition of MM cell viability after 72 hours of treatment, as assessed by MTT assay. Compared with immunoblot
analysis of relative baseline pSTAT3 levels in tested MM cell lines, we noted that 21h, the pentafluorophenyl-containing analog, showed activity against non-pSTAT3 containing MM cell lines such as SKMM2 and MMM1s, whereas 16i exhibited lower biological activity against the cell lines. Moreover, JNJ3, which contains high constitutive STAT3 activation, was more resistant to 21h than 16i. Notably, these new lead compounds were generally around two-fold more potent than parent compounds 7 and 9 (data not shown). Taken together, these findings suggest that the potent anti-MM activity of 21h may be a result of off-target effects, whereas 16i delivered a more desirable activity profile and will likely provide a larger therapeutic window. Alternatively, the broad activity of 21h against this panel of MM cell lines, regardless of baseline STAT3 phosphorylation status, may reflect a universal dependence of MM tumour cells on non-canonical STAT3 signaling pathways that are dependent on a functional SH2 domain, but not STAT3 phosphorylation.

Figure 4. A: Western blot analysis of basal pSTAT3 activation in a panel of human MM cell lines. Quantitative analysis by densitometry shown below reveals relative levels of pSTAT3 to total STAT3 protein, and relative total STAT3 protein to GAPDH; B: MTT cell viability assay with 16i (upper) and 21h (lower) against panel of MM cell lines.
To further characterize the cellular activity of 16i and 21h, and the mechanisms of their effect on MM cell viability, we evaluated whether treatment with 16i and 21h induced apoptosis using flow cytometric analysis of Annexin V and PI staining. As indicated in Figure 5A, both 16i and 21h induced apoptosis dose- and time-dependently in 8226 cells as represented by a shift of cells from the lower left quadrant (viable cells), to the lower right quadrant (early apoptotic cells) at 24 hours, and migration to the upper right quadrant (late apoptosis) at 48 hours. Most promisingly, analysis of apoptosis in MM cell lines with varying degrees of sensitivity to 16i and 21h revealed similar results to those observed in the MTT assay, with a greater induction of apoptosis in 8226 cells compared to XG6 and JJN3 cells (supplementary results).

To further confirm the induction of apoptosis following treatment with 16i and 21h, whole cell lysates drug treated JJN3 cells were collected and subject to immunoblot analysis for the detection of an additional marker of apoptosis, cleaved Poly ADP-ribose polymerase (cPARP). During the process of programmed cell death, PARP proteins are cleaved by proteases such as caspase-3, with the resulting cleavage fragment facilitating cellular disassembly, and thus serving as a marker of cells undergoing apoptosis. As shown in Figure 5B, both 16i and 21h induced cleavage of PARP, results which are consistent with flow cytometry experiments and the induction of the apoptotic program.
Figure 5. A: Flow cytometric analysis of 16i- and 21h- mediated apoptosis as measured by Annexin V and PI staining. Representative scatterplots for 8226 cells showing increased population of cells in the lower right quadrant after 24 hours of treatment, which migrate to the upper right quadrant after 48 hours. This pattern is indicative of time-dependent apoptotic responses, and consistent with cells respectively undergoing the early and late stages of apoptosis. B: Whole cell lysates from 16i- or 21h-treated JJN3 cells were subject to immunoblot analysis, and consistently, results reveal a dose-dependent increase in the apoptotic marker, cPARP.

Given the activity profiles of 16i and 21h in MM cell lines, we next evaluated the ability of these compounds to inhibit STAT3 phosphorylation. Exposure to 16i and 21h for 6 hours lead to dose-dependent inhibition of pSTAT3, and as expected, no inhibition of total STAT3 protein levels (Figure 6A). As STAT3 is a master transcriptional regulator, we also employed a STAT3-driven luciferase reporter construct to evaluate STAT3 transcriptional activity. In agreement with inhibition of STAT3 phosphorylation, treatment with 16i and 21h potently inhibited the transcriptional activity of STAT3 in 8226 and XG6 cell lines, with reductions in relative luciferase ranging from approximately 50-80% after 6 hours (Figure 6B). For 16i, inhibition of transcriptional activity correlated well our initial MTT results. Conversely, treatment with 7.5 μM of 21h had little effect on luciferase production, however this concentration had drastic effects in our MTT assay. These results suggest that while both 16i and 21h inhibit STAT3 phosphorylation and transcriptional activity, it is proposed that some of the increased cellular activity of 21h may be due to off-target effects.

To evaluate whether 16i-/21h-mediated inhibition of STAT3 phosphorylation and transcriptional activity was sufficient to abrogate downstream STAT3-induced gene expression, we evaluated the effect of these compounds on a known STAT3 target gene, c-Myc. Since this particular protein is known to have a very short half-life (20-30 minutes), we evaluated the resulting effects of drug treatment on c-Myc protein expression after 6 hours using immunoblot analysis. Consistent with the previously observed decrease in STAT3 transcriptional activity, both 16i and 21h dose-dependently reduced c-Myc protein expression (Figure 6C), however, in a separate analysis, negligible decreases were observed in other known STAT3 targets such as Bcl-xL and survivin (data not shown), which we speculate to be a result of differences in protein-specific kinetics.
Figure 6. Analysis of 16i and 21h targeted inhibition of STAT3 signaling. A: Western blot analysis of 16i- and 21h-mediated effects on pSTAT3 inhibition in JJN3 tumour cells, revealing dose-dependent inhibition of STAT3 phosphorylation. B: Luciferase assay demonstrating that after 6 hours, both 16i and 21h dose dependently inhibit STAT3-driven luciferase expression, with consistent reduction in protein expression of STAT3 target gene c-Myc, as assessed by immunoblot analysis (C).

To address the selectivity of these compounds for inhibiting STAT3 over other Stat proteins, we performed FP assays to look at compound binding to Stat1 and Stat5. In this assay we found that both 16i and 21h show little selectivity for the STAT3 isoform over Stat1 and Stat5 (16i, Stat1 IC$_{50}$ = 5.8 ± 0.6 µM, Stat5 IC$_{50}$ = 8.5 ± 1.1 µM cf. STAT3 IC$_{50}$ = 2.8 ± 4.3 µM; 21h Stat1 IC$_{50}$ = 10.9 ± 0.8 µM, Stat5 IC$_{50}$ = 13.3 ± 0.9 µM cf. STAT3 IC$_{50}$ = 12.8 ± 2.8 µM, supplementary results). This was also reflected using phospho-flow cytometry to investigate the effects of these compounds on cytokine-induced Stat1/3/5 phosphorylation. Although both 16i and 21h were shown to inhibit IL-6-induced STAT3 phosphorylation in these experiments, similar levels of inhibition were also observed for GM-CSF-induced Stat5 phosphorylation and IFNλ-induced Stat1 phosphorylation (supplementary results). Thus, improving STAT isoform selectivity remains a goal for future compound libraries.
Nonetheless, given the preferred activity profile observed of 16i in the initial MTT assay, we continued analysis of this compound in primary MM patient samples. Most promisingly, 16i demonstrated significant cytotoxic activity against malignant plasma cells (CD138+) from primary MM patient samples (Figure 7A), with 20 µM treatment reducing MM tumour cell viability by over 50% in 3 patient samples. Most importantly, at doses exceeding 20µM, 16i demonstrated little activity against non-MM (CD138-) cells (Figure 7B). Furthermore, at doses of 30 µM, 16i had little effect on haematopoietic progenitor colony formation, suggesting that this compound does not inhibit the ability of normal haematopoietic progenitors to proliferate or form distinct colonies (Figure 7C). Taken together, our analysis of 16i in the context of primary MM patient samples has revealed that there is indeed a therapeutic window for this compound, which ultimately contributes its therapeutic validity, and that of other small molecule STAT3 inhibitors in at least a subset of MM tumours. Furthermore, although our data suggests that 21h may be a less selective inhibitor compared to 16i, it remains an intriguing anti-cancer compound, displaying potent in vitro cytotoxic effects in MM cell lines at low µM concentrations.

Figure 7. Activity of 16i against primary MM patient samples. Mononuclear cells (MNC) from MM patients were obtained by Ficoll-Paque separation of 6 patient derived bone marrow aspirates. Samples were cultured and treated with 16i followed by staining with antibodies against CD138 (MM cell surface marker) or Annexin V (apoptosis). Results are presented as the decrease in CD138+ cell population, representing MM cells (A), and decrease in CD138- cell population, representing non-MM cells (B left). Alternatively, isolated MNCs were cultured in MethoCult (StemCell Technologies), and treated with 16i to evaluate the activity of this agent on healthy hematopoietic progenitor colony formation (B right).
Discussion and Conclusions. We have presented a novel library of salicylic acid-based small molecule STAT3 inhibitors that offer promising STAT3-SH2 binding affinity and potent anti-MM activity. Lead compounds, 16i and 21h, offer improved in vitro binding activity over precursors, 7 and 9, respectively, and improved anti-cancer whole cell activity. We have presented in silico binding evidence that suggests that these compounds offer improved binding potency to the STAT3 SH2 domain by docking in a different orientation than parent compounds (supplementary information). While we have yet to verify this through NMR or X-ray structural data, what we can deduce from the SAR presented is that STAT3’s SH2 domain can accommodate larger, tetrapodal analogs of the 7 and 9 scaffolds.

Both 16i and 21h are among the most potent small molecule STAT3 inhibitors to emerge from our laboratory. Although 21h was among the most potent inhibitor, further evaluation of its potential off-target effects are needed, and are currently underway. Both 16i and 21h were shown to disrupt phosphopeptide:STAT3 protein complexes, inhibit STAT3 phosphorylation and STAT3 transcriptional activity, with concurrent downregulation of STAT3 target genes. Moreover, both compounds have significant anti-MM activity, potently reducing MM cell line viability and promoting the induction of apoptosis. Although not as potent, 16i is predicted to have a more favorable selectivity profile, with no observed cytotoxicity in healthy hematopoietic cells or in MM cell lines that harbor minimal pSTAT3. Current biological experiments are underway to further characterize the therapeutic utility of both 16i and 21h for MM, as well as in vivo studies to delineate their drug-like potential and in vivo efficacy in the hopes of identifying a STAT3 inhibitor candidate suitable for advanced preclinical trials in MM.

References


Appendix C. BLI kinetics study for *in vivo* STAT3-driven luciferase expression in MM xenograft mouse model

Prior to evaluating the effects of BP-4-018 on STAT3 signaling *in vivo*, the BLI kinetics of D-Luciferin were established in RPMI-8226 xenograft mice. A time course study was performed to determine the time at which peak signal intensities were obtained. Peak signal intensities were measured approximately 25 minutes post-D-Luciferin injection in mouse 1, 2 and 4, and approximately 30 minutes post-D-Luciferin injection in mouse 3 (Figure 4.3.1). The BLI signals remained relatively stable from the time at which these peak signal intensities were recorded to when the final images were acquired at 60 minutes. Thus for the remaining *in vivo* imaging studies, images were acquired at approximately 20, 25 and 30 minutes post-D-Luciferin injection, and the peak BLI signal intensity reported.

![Figure C1. Assessment of in vivo STAT3-driven luciferase BLI kinetics in RPMI-8226 xenograft mouse model of MM. Following IP administration of D-Luciferin substrate, mice were imaged every 3 minutes for the first 30 minutes, and at 40 and 60 minutes post-substrate injection. Data obtained from four independent experiments are presented as the normalized BLI intensity (% of peak signal) generated from the ROI for each tumour.](image-url)
Appendix D. Pilot study to establish tolerable dose of BP-4-018 in MM xenograft mouse model

**Figure D1.** Pilot study to establish a tolerable dose of BP-4-018 *in vivo*. Mice were administered BP-4-018 by oral gavage at the indicated doses either BID (A) or daily (B). Mice were weighed at least every three days to identify the dose-associated toxicity response, with a significant response defined as greater than 10% weight loss from beginning of treatment (broken red line). Data points represent mean percentage weight change compared to day 1 of treatment ± SEM (n=2-5 mice per treatment cohort).