Assessing the Combined Effect of Targeting ILK Signaling and Chemotherapy in Rhabdomyosarcoma

by

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

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2011

Abstract

The pediatric sarcoma alveolar rhabdomyosarcoma (ARMS) is highly aggressive with a poor prognosis for diagnosed patients. Here, we demonstrate that targeting the unique oncogene integrin-linked kinase (ILK) in ARMS cells in conjunction with the common chemotherapy agent vincristine, a synergistic effect is found in the reduction of cell viability in vitro. This result was achieved by both RNAi-mediated depletion of ILK and using a small molecule kinase inhibitor specific for ILK. Both techniques were found to disrupt important protein interactions at the site of the centrosome. Combination ILK disruption and vincristine treatment of cells induced the expression of apoptotic markers and arrested cells in the G2/M stage of the cell cycle. Interestingly, protein levels of JNK and its target c-Jun were regulated with combined treatment. Altogether, these findings indicate that the use of molecular targets like ILK may further improve the clinical treatment of ARMS.
Acknowledgments

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<tr>
<td>α-NAC</td>
<td>nascent polypeptide-associated complex and coactivator α</td>
</tr>
<tr>
<td>AIR-1</td>
<td>Aurora/Ipl1 related</td>
</tr>
<tr>
<td>ARMS</td>
<td>alveolar rhabdomyosarcoma</td>
</tr>
<tr>
<td>ALK</td>
<td>anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>Cdds</td>
<td>cyclin-dependent kinases</td>
</tr>
<tr>
<td>ch-TOG</td>
<td>colonic and hepatic tumour over-expressed protein</td>
</tr>
<tr>
<td>DISC</td>
<td>death-inducing signaling complex</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>ERMS</td>
<td>embryonal rhabdomyosarcoma</td>
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<tr>
<td>FGFR4</td>
<td>fibroblast growth factor receptor 4</td>
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<td>FKHR</td>
<td>forkhead homolog in rhabdomyosarcoma</td>
</tr>
<tr>
<td>FOXO1A</td>
<td>forkhead box O1A</td>
</tr>
<tr>
<td>GSK3β</td>
<td>glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>IGF1R</td>
<td>insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
</tr>
<tr>
<td>ILKAP</td>
<td>integrin-linked kinase-associated serine/threonine phosphatase 2C</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MIA</td>
<td>microtubule-interfering agent</td>
</tr>
<tr>
<td>MLC</td>
<td>myosin regulatory light chain</td>
</tr>
<tr>
<td>NEK</td>
<td>NIMA-related kinase</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small-cell lung carcinoma</td>
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<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
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<td>PAX3</td>
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<tr>
<td>PI3K</td>
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<td>particularly interesting new cys-his protein</td>
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<td>PH</td>
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<td>rhabdomyosarcoma</td>
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<tr>
<td>TACC3</td>
<td>transforming acidic coiled-coil-containing protein 3</td>
</tr>
<tr>
<td>VAC</td>
<td>vincristine, dactinomycin, cyclophosphamide</td>
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<tr>
<td>VIE</td>
<td>vincristine, ifosfamide, etoposide</td>
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<td>VCR</td>
<td>vincristine</td>
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Chapter 1
Introduction and Background

1.1 Rhabdomyosarcoma

1.1.1 Overview

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children with annual incidences of approximately 350 and 40 cases per year in the United States\(^1\) and Canada\(^2\), respectively. Most RMS tumours are diagnosed either under the age of 6 years or during adolescence; it is rarely found in adults\(^3\). Multi-modal therapy has improved treatment of RMS, but disease-free survival is only 50% and patients with metastatic disease have less than 20% chance of cure\(^3-5\). Furthermore, patients with more than three metastases have a 3-year overall survival of less than 5%\(^3\). Even for patients that respond to therapy, toxicities of those methods result in long-term disabilities or disease\(^6-9\).

RMS tumours are thought to arise from genetic damage incurred by committed myogenic or mesenchymal progenitor cells resulting in a failure to terminally differentiate\(^6\). While RMS tumours are usually found in areas of the body that have skeletal muscle tissue\(^10\), they can also be found where skeletal muscle is absent\(^11-13\). More than two-thirds of all RMS are found in the head and neck region or genitourinary tract\(^10\). Tumours in the extremities are less common, representing about 15% of RMS\(^10\).

Clinically, RMS is staged using a complex three-step process that involves assigning a stage, a surgico-pathologic group, and a risk group\(^14\). Staging considers the site (favourable versus unfavourable) and size of tumours, while also considering the presence of metastatic disease in lymph nodes or elsewhere\(^14-16\). Stages for RMS range from I through IV, with IV being the most
serious (tumour at favourable or unfavourable sites, tumour of any size, spread into lymph nodes beyond the primary site, and metastasis)\textsuperscript{15,16}. Assigning a group is based on whether the cancer has spread and how much cancer remains after surgery\textsuperscript{17,18}. There are four groupings (I-IV) with Group IV meaning distant metastases found at diagnosis\textsuperscript{17,18}. Lastly, risk groups are assigned by considering the stage, group and histology\textsuperscript{3,19}. There are three risk groups (low, intermediate, high) with the high risk group having tumours of any histology, Stage 4 and Group 4\textsuperscript{3,19}.

1.1.2 Alveolar Rhabdomyosarcoma

As discussed earlier, part of the risk group assignment for RMS is based on the histology of the tumour. RMS can present in a number of histological subtypes, most commonly in the forms of embryonal RMS (ERMS) and alveolar RMS (ARMS) accounting for 63\% and 20\% of all RMS tumours respectively\textsuperscript{9}. These subtypes differ in morphology, clinical profiles and molecular characteristics. ARMS is named based on its histological resemblance to pulmonary alveoli\textsuperscript{9,10}. As opposed to ERMS which tends to be found in younger children, ARMS is more evenly distributed between birth and 19 years of age and is usually found in the extremities of the body\textsuperscript{10}. The clinical outlook for ARMS patients is relatively poor compared to those with ERMS. ARMS tumours are more aggressive with a greater number of metastases and higher mortality rate\textsuperscript{10}. While the 10-year survival rate for ERMS is 75\%, the corresponding survival for ARMS is less than 20\%\textsuperscript{3,6}. Clearly, there is a strong rationale for pursuing novel treatment options to improve the prognosis of ARMS patients which will be the focus of this thesis.

There are two non-random chromosomal translocations, both of which result in a fusion gene, that are unique to ARMS. The first translocation, t(2;13)(q35;q14), is found in roughly 70\% of ARMS cases and encodes a protein that is generated by fusion of the 5’ sequence of the paired-box 3 (\textit{PAX3}) gene and the 3’ sequence of the forkhead box O1A/forkhead homolog in
rhabdomyosarcoma (FOXO1A, FKHR) gene. The other is a similar translocation, t(1;13)(p36;q14), that fuses the paired-box 7 (PAX3) gene to the FKHR gene instead of PAX3. Both the paired-box and forkhead genes are families of transcription factors with a wide range of targets. The PAX3-FKHR fusion protein maintains the DNA binding motif of PAX3 while replacing the transactivation domain with part of the FKHR DNA binding motif and all of its transactivation domain. The fusion product’s oncogenic potential is attributed to the greater transcriptional activity than PAX3 has alone. Some of the transcriptional targets for PAX3-FKHR in ARMS include the myogenic determination genes, MYF5 and MYOD, the signaling receptors fibroblast growth factor receptor 4 (FGFR4) and insulin-like growth factor 1 receptor (IGF1R), and the oncogenes MYCN and anaplastic lymphoma kinase (ALK). Altogether, fusion-positive ARMS represent up to 80% of all ARMS cases, with PAX3-FKHR having a frequency of up to 10 times that of PAX7-FKHR. Although there is some debate, PAX3-FKHR is generally associated with more clinically aggressive ARMS than the PAX7-FKHR fusion. A smaller proportion of ARMS cases can be classified as translocation-negative – this subset of ARMS would include translocations that require high sensitivity RT-PCR to detect, PAX3 fusions with other genes, and ARMS variants that are truly negative. This minority may represent an intermediate with respect to biology between the two more common translocations described above. Interestingly, in RMS, the introduction of PAX3-FKHR into ERMS cells (translocation-negative) can convert intracellular signaling to resemble ARMS cells.

1.1.3 Treatment of Rhabdomyosarcoma

Treatment of RMS is based on the staging of the cancer. Multimodality therapy for RMS can involve surgery, chemotherapy and radiotherapy and more than 70% of patients can be cured if they have non-metastatic disease. However, as RMS is a childhood cancer that can arise in areas of the body that cannot be easily accessed, there are important considerations to be made in
the treatment of the disease including the timing, intensity and use of each modality. Particularly in the genitourinary tract or head and neck regions of children, resection or radiation of tumours may not be in the best interest of the patient in terms of short and long-term morbidity. Thus, chemotherapy is needed by all patients with RMS. The current strategy for treating RMS patients is with different combinations of multi-agent therapy. The most commonly used regimen of drugs is vincristine (a microtubule polymerization inhibitor), dactinomycin (a DNA intercalator) and cyclophosphamide (an alkylating agent) or altogether referred to as “VAC”. Ifosfamide (an alternative alkylating agent) can take the place of cyclophosphamide to make the “VAI” regimen and etoposide (a topoisomerase II inhibitor) for dactinomycin in “VIE”. Other drugs used in the treatment of RMS include cisplatin (a DNA crosslinker), and doxorubicin (a DNA intercalator). It is important to note that doxorubicin is commonly used for adult malignancies and there have been studies that both support and question its efficacy in childhood RMS. Nonetheless, the use of chemotherapy in these young patients can have both negative short- (such as leucopenia and neutropenia) and long-term side effects (for example infertility, neurotoxicity or development of secondary malignancies like leukemia). Importantly, the costs incurred by these sequela arise from successfully treating the RMS tumours to begin with. Perhaps the identification of specific molecular therapeutic targets would allow for greater overall success in treatment of RMS while reducing chemotherapy usage and the corresponding secondary effects.

1.2 Integrin-Linked Kinase

1.2.1 Overview

Integrins are receptor proteins found at the cell surface that mediate the interaction between cells and the extracellular matrix. This important role for integrins allows cells to respond to extracellular stimuli with intracellular changes. As a consequence, integrins play a role in a
number of signaling pathways and cellular processes such as motility, adhesion, and survival. Therefore, in the context of cancer, where cell-cell and cell-matrix communication is crucial, proteins involved in integrin signaling are worthy candidates for further investigation. Integrin-linked kinase (ILK) is one important example of these signaling proteins. ILK is a Ser/Thr protein kinase that was identified as a β1-integrin subunit binding protein. ILK’s genetic locus is chromosome 11p15.4/5, a region that commonly exhibits loss of heterozygosity (LOH) in ERMS cells. ILK contains several ankyrin repeats at the N-terminus, a central plekstrin homology (PH) domain, and a kinase domain at the C-terminus. Like other proteins with a PH domain, ILK is regulated by phosphatidylinositol 3-kinase (PI3K). Constitutively activated PI3K was linked to ILK activity and inhibition of PI3K inhibited cell adhesion-induced ILK activation. Two downstream signaling proteins of PI3K are protein kinase B/Akt and GSK3β. It was found that only normal, functional ILK and not kinase-deficient ILK, was able to phosphorylate Akt on Ser473. Furthermore, GSK3β was directly phosphorylated and inhibited by ILK. The in vitro studies also showed that kinase-deficient ILK was unable to perform the same function. Therefore, ILK was found to be an important mediator of PI3K signaling. Both Akt and GSK3β themselves are involved in a wide range of signaling pathways and downstream cellular processes. Akt is implicated in metabolism, proliferation, apoptosis and migration while GSK3β is involved in metabolism, cell survival and as a mediator of transcription. Interestingly, Akt can regulate GSK3β and inhibit its signaling. Unlike the activation of ILK by PI3K, phosphatase and tensin homology (PTEN) has been shown to regulate ILK signaling and activity. Mutated or absent PTEN in cells can both lead to increased ILK activity and Akt phosphorylation. Conversely, transfection of PTEN can suppress the same ILK functions. Interestingly, the PAX3-FKHR fusion that is found in ARMS promotes the downregulation of PTEN. In addition to GSK3β and Akt, ILK has other kinase substrates, including the β1
integrin tail and myosin light chain (MLC). Therefore, changes in the activity of ILK can have a wide range of effects on the cell. Among reports suggesting that ILK lacks a putative kinase function, more evidence clearly demonstrates the importance of the kinase domain in human cancer cells.

Figure 1.2.1. ILK participates in a number of signaling pathways. ILK is regulated in a PI3K-dependent manner and is implicated in a number of cellular processes relevant to cancer. Akt and GSK3 have both been shown to be direct targets of ILK. JNK activation has been associated with ILK in RMS but how this is mediated remains unclear.

The intracellular localization of ILK is varied and not restricted to specific areas of the cell. In fact, ILK has been implicated in a number of intracellular locations. In model organisms like mice, Caenorhabditis elegans, and Drosophila melanogaster, ILK has been shown to be...
crucial in mediating the interactions between the integrins and the cytoskeleton\textsuperscript{57}. For example, at focal adhesions proximal to the cell surface membrane, ILK has been shown to bind other proteins like α- and β-parvin, PINCH, and paxillin, where it can regulate processes like cell adhesion, spreading and migration\textsuperscript{58}. Both Akt and GSK3β are cytoplasmic proteins which demonstrate that ILK signaling does not necessarily occur at the cell surface. Furthermore, there have been reports that ILK plays an important role in the regulation of the mitotic apparatus\textsuperscript{59-61}. At the centrosome, ILK has been shown to regulate mitotic spindle organization while inhibition of ILK and integrin signaling can inhibit microtubule growth and lead to cell death\textsuperscript{60, 62}. This is supported by other studies in HeLa\textsuperscript{60}, prostate and breast cancer cells\textsuperscript{61} that show that ILK’s involvement in these processes is in part due to the regulation of the interaction between the proteins Aurora A kinase, transforming acidic coiled-coil-containing protein 3 (TACC3), and colonic and hepatic tumour over-expressed protein (ch-TOG).

Disruption of ILK can also alter the function of other kinases, including JNK\textsuperscript{36, 63-64}, ERK1/2\textsuperscript{63}, 65, and p38\textsuperscript{64-66} in addition to its classical targets GSK3β and Akt\textsuperscript{43-45, 67}. Altogether, whether through mediating protein interactions via scaffolding or direct signaling, ILK involvement is crucial for a wide range of cellular processes. It is for this reason that ILK has been widely investigated for its role in numerous cancers including RMS.

1.2.2 Oncogenic Function of ILK

ILK has been found to be overexpressed in a number of cancers and ILK expression has been found to correlate with tumour grade and poorer prognosis\textsuperscript{68-73}. Sustained high levels of ILK can therefore be linked to a number of cellular processes that are relevant to cancer including cell migration, motility, invasion, survival and proliferation\textsuperscript{51}. 
There are a number of studies, both \textit{in vitro} and \textit{in vivo}, that have established that ILK acts as an oncogene. In rat epithelial cells, overexpression of ILK promoted cell cycle progression\textsuperscript{74} while overexpression of ILK in mouse mammary epithelial cells suppressed anoikis\textsuperscript{75}. Furthermore, in two anoikis-resistant human breast cancer cell lines, ILK inhibition induced anoikis\textsuperscript{75}. Using siRNA targeted ILK, growth of human bladder cancer cells was inhibited and apoptosis was induced\textsuperscript{76}. In addition, ILK seems to play a role in altering tumour microenvironments by inducing angiogenesis\textsuperscript{77-78}. There has also been a growing body of work using QLT0267, a second-generation ILK inhibitor compound identified as a result of high-throughput screening\textsuperscript{79}. QLT0267 is able to inhibit ILK kinase function in cell-free assays and is approximately 1000-fold more selective for ILK than other kinases such as Akt, PKC, CK2, CSK, DNA-PK and exhibits 100-fold selectivity over GSK3\(\beta\), LCK, PKA, and RSK1\textsuperscript{79}. It has been used in a number of settings exhibiting its efficacy in the regulation of growth in glioblastoma\textsuperscript{80}, head and neck squamous cell carcinoma\textsuperscript{81}, thyroid\textsuperscript{79}, breast\textsuperscript{61,82}, and prostate\textsuperscript{61} cancer systems.

It is important to note that ILK is a member of a small subset of proteins that seem to play contradictory roles in different contexts. Although there is evidence for ILK’s oncogenic function as shown above, there are also studies which support the notion ILK has growth suppressive properties. For example, in breast and lung carcinoma cell lines, ILK overexpression suppressed xenograft growth and heightened sensitivity to radiation, respectively\textsuperscript{83-84}. Importantly, if ILK is to be used as a therapeutic target in RMS or cancer therapy, the context of ILK manipulation must be clearly determined.

1.2.3 ILK in Rhabdomyosarcoma

Interestingly, ILK plays contradictory roles in ARMS and ERMS. Specifically, \textit{in vitro} and \textit{in vivo} studies have shown that ILK acts as an oncogene in ARMS and as a tumour suppressor in
ERMS\textsuperscript{36}. These findings were determined by regulating ILK expression with ILK-targeted siRNA and overexpression of ILK protein using an adenoviral vector\textsuperscript{36}. Normally, ILK expression in ARMS and ERMS differ, with lower levels of both mRNA and protein in ARMS\textsuperscript{36}. In RMS, ILK expression negatively correlates with patient outcome\textsuperscript{36}. Interestingly, the expression of the ARMS-specific PAX3-FKHR fusion was able to alter the role of ILK in certain contexts. The introduction of PAX3-FKHR into ERMS cells changed their response in the setting of ILK siRNA to resemble that of the ARMS phenotype\textsuperscript{36}. This supports the notion that ILK behaviour in RMS may be dependent on background genetic events. Altogether, the information gained in these studies suggests that ILK may be a worthwhile molecular target in the therapy of RMS. With respect to ARMS, regulating ILK protein expression or inhibiting ILK kinase function may be a strategy that potentially synergizes with current therapeutic protocols in controlling growth and proliferation.

1.3 JNK/c-Jun Signaling Axis

1.3.1 Overview

The c-Jun N-terminal kinase (JNK) is one of several mitogen-activated protein kinases (MAPK) which are a family of Ser/Thr kinases that play a role in a number of signaling pathways by mediating extracellular signals to intracellular responses. Two other commonly investigated MAPKs are extracellular signal-regulated kinase (ERK) and p38. There are three JNK genes (JNK 1-3) with different isoforms and tissue-specific expression and function\textsuperscript{85}. JNK3 is restricted to the brain, heart and testes, while JNK1 and JNK2 are found in all tissues\textsuperscript{86}. JNK has a number of phosphorylation substrates which include non-transcription factors like the members of the Bcl-2 family of proteins\textsuperscript{87}, and transcription factors such as c-Jun, p53, and c-Myc\textsuperscript{85,88-90}. The activator protein 1 (AP-1) transcription factor is made up of several proteins which include c-Jun and the FOS, MAF, and ATF subfamilies of transcription factors\textsuperscript{91}. The JNK1β isoforms
are involved in the phosphorylation of c-Jun, while the JNK2β isoforms have been implicated in ATF-2 phosphorylation\textsuperscript{92}. Of all of those proteins, c-Jun is the most potent as a transcription factor\textsuperscript{93}. AP-1 is involved in the regulation of a wide range of cellular processes that range from transformation and proliferation to cell survival and death\textsuperscript{91}. Therefore, JNK-mediated phosphorylation of c-Jun is associated with pro-survival effects such as the induction of cell cycle progression, upregulation of proliferative gene expression, and downregulation of death signaling\textsuperscript{91}. Interestingly, AP-1 activity can be stimulated by ILK activity in a GSK3β-dependent manner\textsuperscript{94}.

As a MAPK, activation of JNK is downstream of the mitogen-activated protein kinase kinase kinase (MAPKKK) apoptosis signal-regulating kinase 1 (ASK1)\textsuperscript{95}. Interestingly, ILK and JNK signaling crosstalk has been found to exist. The integrin-linked kinase-associated serine/threonine phosphatase 2C (ILKAP) which interacts with and inhibits ILK kinase activity positively regulates ASK1\textsuperscript{96}. Furthermore, overexpression of ILK stimulates c-Jun dependent transcription by activating the c-Jun coactivator nascent polypeptide-associated complex and coactivator α (α-NAC)\textsuperscript{97}. Phosphorylation of the ILK substrate α-NAC leads to its entry into the nucleus and potentiation of c-Jun effects\textsuperscript{97}.
Figure 1.3.1 The JNK/c-Jun signaling axis. JNK is a stress-response kinase that can respond to a number of stimuli. Activation of JNK leads to subsequent phosphorylation and activation of transcription factors including c-Jun which lead to a number of cellular processes relevant to cancer.

1.3.2 JNK/c-Jun in Rhabdomyosarcoma

There are limited reports related to the function of this signaling axis in RMS. It has been shown that inactivation of JNK is associated with reduced migration\textsuperscript{98,99}, proliferation and invasiveness \textit{in vitro}\textsuperscript{98}. Recent work in our lab found that the unique competing roles of ILK in ERMS and ARMS were dependent on the c-Jun amino terminal kinase-1 (JNK1)\textsuperscript{36}. JNK1β isoform expression is elevated in ERMS compared to ARMS\textsuperscript{100}. Endogenous ILK in ERMS inhibits
signaling of the JNK/c-Jun cascade while loss of ILK removes this inhibition leading to increased cell growth. In ARMS, ILK induces JNK phosphorylation and JNK/c-Jun pathway activation leading to increased growth. The PAX3-FKHR fusion introduced into ERMS cells changed the response to ILK siRNA as described earlier by decreasing JNK1 expression and converting the cells to exhibit ARMS-like signaling. Restoring JNK1 expression in ARMS caused ERMS-like signaling of increased growth in the setting of ILK depletion. Further work investigating the interplay between ILK and JNK showed that JNK expression levels correlated strongly with ILK function. Specifically, in RMS, breast carcinoma, non-small-cell lung carcinoma (NSCLC), hepatoblastoma, neuroblastoma, adrenal cortical carcinoma, glioblastoma and prostatic carcinoma cells, high JNK expression was associated with a growth-suppressive ILK effect while low JNK expression levels was associated with a growth-promoting role for ILK. Altogether, this shows that JNK may act as an important biomarker for ILK function in both RMS and other cancers.

1.4 Rationale and Hypothesis

ARMS is a less common, yet more aggressive subtype of RMS. The generally poor outcomes for ARMS patients receiving current therapies including combination chemotherapy, surgery and radiation therapy indicate that new methods are needed to improve treatment success. The identification of molecular targets, of which ILK is a candidate, is a logical step to achieve those goals. Using the knowledge of ILK oncogenic function in ARMS with a related role for the JNK/c-Jun signaling axis, I will discuss in this thesis my aim to discover novel ways to control ARMS growth. I hypothesize that the targeting of ILK signaling will cooperate with chemotherapy agents in the treatment of ARMS.
Chapter 2
Materials and Methods

2.1 Cell Culture

Rh4, Rh18 and Rh30 cell lines were a gift from Dr. T. Look (Dana-Farber Cancer Institute, Boston, MA) and were derived from alveolar RMS tumours. Cells were grown in Dulbecco’s Modified Eagle Medium (Wisent) supplemented with 10% Fetal Bovine Serum (Wisent).

2.2 Reagents

$ILK$-targeted oligonucleotide siRNA (sequence, 5`-GGGCAAMGACAGUGCGM3`) or nontargeted oligonucleotide siRNA (control siRNA no.1) were purchased from Ambion. To achieve similar transfection efficiency in each of the cell lines, siRNAs were used at concentrations of 20nM (Rh18, Rh30) and 40nM (Rh4). Transfections were completed with Lipofectamine 2000 (Invitrogen) as per the manufacturer’s protocol.

Vincristine sulfate salt (V8879), Actinomycin D (A1410), Doxorubicin hydrochloride (44583) and Etoposide (E1383) were purchased from Sigma and resuspended in ethanol or DMSO. For synergy studies, vincristine concentrations of 5nM (Rh4) and 100nM (Rh18 and Rh30) were used. QLT0267 was a gift from Dr. S. Dedhar (BC Cancer Agency, Vancouver, BC) and was diluted in DMSO. For synergy studies, QLT0267 concentrations of 2.5μM, 5μM and 7.5μM were used for Rh4, Rh18 and Rh30, respectively. All compounds were added to culture media directly.

2.3 Proliferation and Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega) was used as per manufacturer’s protocol. Cells were plated
at $10^4$ cells/well in a 96-well plate in triplicate, quadruplicate or quintuplicate in 100μL/well of medium containing MTS reagent and incubated for 2 hours at 37°C. Absorbance was measured at 490nm. Raw values were normalized to vehicle or negative control averages as appropriate to determine percent cell proliferation and viability.

2.4 Western Blots

Protein samples were prepared in EBC lysis buffer (120mM NaCl, 50nM Tris base, and 0.5% NP-40) supplemented with protease and phosphatase inhibitors (Roche Applied Science). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore). Membranes were blocked with Odyssey Blocking Buffer (Li-Cor Biosciences) and probed with primary antibodies (in 10mM TBS-T) overnight at 4°C. The following primary antibodies were used: anti-ILK, phospho-Ser63-c-Jun, phospho-Thr183/Thr185-JNK, SAPK/JNK, phospho-Ser9-GSK3β, cleaved caspase-3, cleaved PARP (Cell Signaling); Vinculin (Millipore). Membranes were probed with infrared dye-tagged secondary antibodies (Li-Cor Biosciences) for 30 minutes and visualized on the Li-Cor Odyssey System (Li-Cor Biosciences).

2.5 Immunoprecipitation

Protein was precleared from cell lysates with protein G-plus agarose beads and mouse IgG (Santa Cruz Biotechnology Inc.) for 4 hours. Supernatants were immunoprecipitated overnight with protein G-plus agarose beads plus either control mouse IgG or mouse anti-Aurora A antibody (Sigma). Following washes in EBC buffer, pellet was resuspended in 6x SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE and blotted with rabbit anti-Aurora A kinase (Millipore) and anti-TACC3 (Santa Cruz) antibodies.
2.6 Kinase Assay

Protein was precleared from cell lysates with protein G-plus agarose beads and rabbit IgG (Santa Cruz Biotechnology Inc.) for 4 hours. Supernatants were immunoprecipitated overnight with protein G-plus agarose beads plus either control rabbit IgG or rabbit monoclonal anti-ILK antibody (Cell Signaling). Following washes in EBC buffer, the pellet was incubated in 40μL of 1x kinase assay buffer (Cell Signaling, 25mM Tris-HCl (pH 7.5), 5mM beta-glycerophosphate, 2mM dithiothreitol (DTT), 0.1mM Na3VO4, 10mM MgCl2) supplemented with 0.25mM ATP (Fermentas Inc), vehicle or QLT0267, with or without 2.5µg GST-tagged GSK3β peptide (Cell Signaling Technology) for 30 minutes at 30°C. The kinase reaction was terminated by boiling in 6x SDS-PAGE sample buffer. Half of the sample volume was resolved by SDS-PAGE and blotted with anti-ILK clone 65.1.9 (Upstate Biotechnology). The other half was incubated with Glutathione Sepharose 4B beads (GE Healthcare) overnight at 4 degrees before resolving by SDS-PAGE and blotted with anti-GST (Millipore) and phospho-Ser9-GSK3β (Cell Signaling) antibodies.

2.7 Cell Cycle Analysis

DNA content of cells were measured with propidium iodide (Sigma). Approximately 10^5 cells per treatment were collected by trypsinization and fixed with 70% ethanol for at least 24 hours at 4°. Fixed cells were washed with PBS three times before being resuspended with 20µg/mL RNase (Fermentas) and 100µg/mL Propidium Iodide (Sigma-Aldrich) for 30 minutes, in the dark, at room temperature. Stained cells were analyzed using a LSRII (Becton Dickinson) flow cytometry instrument with BD Diva software (Becton Dickinson). Data analysis was performed using FloJo software (Tree Star Inc).
2.8 Statistics

Data were analyzed using Microsoft Excel. Results are presented as mean ± SEM unless otherwise indicated. Statistical analysis was performed with 2-tailed Student’s t test. A P value less than 0.05 was considered to be statistically significant.

To determine synergy in combined treatments of ILKsi and vincristine or QLT0267 and vincristine, the Webb equation was used. Normalized percentage cell viability was determined for individual treatments (A% and B%), and if the normalized percentage cell viability of the combination therapy (C%) was less than 70% of the product of the individual therapies, the combination therapy was deemed synergistic (if C% < 0.70 [A% x B%] then combination treatment is synergistic).
Chapter 3
Results

3.1 Effect of chemotherapy on ARMS cell viability

3.1.1 ARMS cells are sensitive to a number of chemotherapy agents

Previous studies from our lab have revealed an important role for ILK biology in ARMS\textsuperscript{100}. As our hypothesis was to investigate whether ILK manipulation potentiates the known effects of chemotherapy agents, I first determined the sensitivity of ARMS cells to a number of common chemotherapy agents. The agents used in this study were vincristine, actinomycin D, doxorubicin and etoposide, all of which are used clinically in the treatment of RMS, and given to cells for 24, 36 or 48 hours. It was determined that the Rh4 ARMS cell line was sensitive to each of the above agents to varying degrees (Figure 3.1.1). The dose response study found that after 24 hours, the IC\textsubscript{25} values for vincristine, dactinomycin, doxorubicin and etoposide were around 5\text{nM}, 100\text{nM}, 1.5\text{µM}, and greater than 25\text{µM} respectively. After 24 hours, Rh4 cells were most sensitive to vincristine and actinomycin D. As expected, cells treated for longer periods exhibited a greater reduction in viability. The data collected guided the dosage of subsequent experiments.
Figure 3.1.1 Chemotherapy agent treatments lead to reduction in Rh4 cell viability. Cells were plated at a density of $10^4$ per well in a 96-well plate and treated with vincristine, actinomycin D, doxorubicin and etoposide for the time indicated after which cell viability was measured using the MTS assay. Values represent the mean of three treatments normalized to vehicle control.

3.2 Effect of combined ILK protein regulation and vincristine treatment on ARMS cells

3.2.1 Combined treatment leads to synergistic reduction in viability

As our lab had previously shown that regulation of ILK protein expression using siRNA reduced ARMS cell viability, the next aim was to determine if the combined treatment of ILKsi with the above four chemotherapy agents would exhibit synergy. An IC 25 for each of the drugs was used in combination with ILKsi. The choice of not using a higher concentration of the agents was to allow for the observation of any synergistic effects. Interestingly, after reducing ILK protein levels, vincristine was the only agent to demonstrate synergy (Figure 3.2.1). In other words, the reduction in viability of Rh4 cells with combined treatment was greater than the sum of either treatment alone after 24 hours. Furthermore, synergy was found when treating Rh18 and Rh30...
(both ARMS) cells in the same manner. Interestingly, Rh18 had the lowest reduction in viability with combined treatment compared to Rh4 and Rh30.

Figure 3.2.1. Combined ILKsi and vincristine treatment leads to a synergistic effect on viability reduction in ARMS cells. (A) ARMS cells were transfected with ILK (ILKsi) or control (Ctrlsi) siRNA and cultured for 3 days. Cells were subsequently treated with vincristine (VCR) or vehicle for 24 hours after which cell viability was measured using MTS assay. Values normalized to Ctrlsi treatment and represent mean ± SEM. n=3 (Rh4, Rh30), n=7 (Rh18). ** p <0.01, *** p<0.001. (B) Cell lysates for western blotting were collected 3 days after siRNA transfection and before vincristine treatment. Data are representative of three independent blots.
3.2.2 Combined treatment leads to an increase in expression of apoptotic markers in ARMS cells

To further investigate the effect of the combined treatment on the ARMS cells, markers of apoptotic signaling were examined using Western blot. Using the same treatment conditions in 3.2.1, expression of cleaved caspase-3 and poly (ADP-ribose) polymerase (PARP) protein were found to be at highest levels under combined treatments in each of the three cell lines (Figure 3.2.2).

**Figure 3.2.2. Combined ILKsi and vincristine treatment in ARMS cells leads to induction of apoptotic signaling.** ARMS cells were first transfected with ILKsi and treated with vincristine followed by collection of cell lysates. Western blots were run to analyze cleaved PARP and cleaved caspase-3 expression. (A and B) Background-adjusted densitometric values were normalized to Ctrlsi treatment and represent mean ± SEM. n=3. * p<0.05, ** p<0.01, ***p<0.001. (C) Data are representative of three independent blots.
3.2.3 ILKsi and vincristine lead to alteration in ARMS cell cycle distribution

Vincristine is known to inhibit microtubule dynamics by binding tubulin monomers. As expected, ARMS cells treated with vincristine were found to arrest in the G2/M phase of the cell cycle in greater proportions than vehicle or ILKsi treatments (Figure 3.2.3) in all cell lines. Interestingly, a smaller proportion of cells were found to arrest in G2/M for combined treatments in Rh4 and Rh30 cells. Upon further investigation, the highest proportion of cells found in the sub-G1 gate was under combined treatment with a smaller proportion with vincristine treatment alone. As an indirect measure of cells undergoing apoptosis, this finding was consistent with the data from 3.2.1 and 3.2.2 as apoptotic markers were induced in each of the cell lines.

![Figure 3.2.3. Vincristine treatment leads to ARMS cells arresting in G2/M.](image)

After transfecting with siRNA and treating with vincristine, ARMS cells (10^4 events per treatment per cell line) were measured for DNA content using propidium iodide staining. Cells were distributed into gates corresponding to the different phases of the cell cycle. The percentage of all cells gated in G2/M (A) and sub-G1 (B) are shown. Values represent mean ± SEM. n=3. * p<0.05, ** p< 0.01, ***p<0.001.
3.2.4 ILKsi and vincristine treatments disrupt centrosome organization

As discussed earlier, regulation of ILK signaling has been shown to disrupt important protein-protein interactions at the site of the centrosome\textsuperscript{61}. To further elucidate what effect the combined treatment had on ARMS cells, the interaction between TACC3 and Aurora A kinase was investigated. As expected, reducing ILK expression by siRNA resulted in the absence of the TACC3-Aurora A interaction (Figure 3.2.4). Vincristine and combined treatment alone had the same result with the two proteins failing to co-immunoprecipitate.

![Figure 3.2.4. ILKsi and vincristine treatments disrupt the TACC3-Aurora A interaction.](image)

Whole cell lysates (WCL) were collected from Rh4 cells and the interaction between Aurora A kinase and TACC3 was determined under ILKsi, vincristine and combined treatment. IgG lane represents negative control. All lanes shown above were taken from the same blot. Data is representative of three independent blots.

3.2.5 Combined treatment affects JNK/c-Jun phosphorylation

Previous work in our lab has shown that the JNK/c-Jun signaling axis plays an important role in ILK function in RMS\textsuperscript{36}. To determine whether this signaling axis played a role in the effects I found with combined ILKsi and vincristine treatment, I investigated the phosphorylation status of both JNK and c-Jun as a measure of activity. ILKsi and vincristine each exhibited lower phosphorylated levels of JNK and c-Jun while combined treatment displayed an even lower expression of phosphorylated proteins (Figure 3.2.5) in each of the three ARMS cell lines.
Figure 3.2.5. Combined ILKsi and vincristine treatment is associated with a reduction in JNK/c-Jun phosphorylation. Rh4 cells were treated with ILKsi and vincristine followed by collection of cell lysates. Western blots were run to analyze relative phospho- to total JNK and c-Jun expression. Background-adjusted densitometric values of the phospho- to total ratio, normalized to loading control (vincristine), represent mean ± SEM (A). n=3. * p<0.05, ** p<0.01, ***p<0.001. (B) Data are representative of three independent blots.
3.3 Effect of QLT0267 on RMS cells

3.3.1 QLT0267 inhibits ILK kinase activity

To confirm the efficacy of QLT0267 on ILK, kinase activity was measured using an *in vitro* kinase assay and it was determined that kinase function was reduced in Rh4 cells when treating with QLT0267 over a range of concentrations (Figure 3.3.1).

![Image](image)

**Figure 3.3.1. QLT0267 treatment reduces ILK kinase activity in Rh4 cells.** ILK in vitro kinase assay were run with increasing concentrations of QLT0267. Briefly, cell lysates were immunoprecipitated with anti-ILK antibody followed by incubation in kinase assay buffer supplemented with ATP. Half of the sample was blotted for ILK while the other half was incubated with Glutathione Sepharose 4B beads before resolving for GST and phospho-GSK3. Data is representative of three independent blots.

3.3.2 ARMS cell are sensitive to QLT0267 treatment

To determine whether ILK’s kinase function played a role in the synergistic effect on ARMS cell viability, I first treated ARMS cells with QLT0267 to determine if it had an anti-RMS cell effect. After 24 hour treatment with QLT0267, each of the ARMS cells had a reduction in viability. Rh4
cells had the greatest reduction in viability over a range of QLT0267 concentrations compared to Rh18 and Rh30 cells (Figure 3.3.2).

![Figure 3.3.2](image)

**Figure 3.3.2.** QLT0267 treatment of ARMS cells leads to reduction in viability. Cells were plated at a density of $10^4$ per well in a 96-well plate and treated with QLT0267 for 24 hours after which cell viability was measured using the MTS assay. QLT0267 concentrations of 2.5µM, 5µM and 7.5µM were used for Rh4, Rh18 and Rh30, respectively. Values represent the mean ± SEM normalized to vehicle control. n=3.

### 3.4 Effect of combined QLT0267 and vincristine treatment on ARMS cells

#### 3.4.1 Combined treatment leads to synergistic reduction in viability

Similar to the effect found when regulating ILK protein expression, the treatment of ARMS cells with QLT0267 and vincristine led to a synergistic reduction in viability in each of the cell lines (Figure 3.4.1). The combined reduction in viability differed when treating with ILKsi or QLT0267 in Rh4 and Rh30.
Figure 3.4.1. Combined QLT0267 and vincristine treatment leads to a synergistic effect on viability reduction in ARMS cells. ARMS cells were treated with QLT0267 and/or vincristine for 24 hours after which cell viability was measured using MTS assay. QLT0267 concentrations of 2.5μM, 5μM and 7.5μM were used for Rh4, Rh18 and Rh30, respectively. Values normalized to vehicle treatment and represent mean ± SEM. n=3. * p<0.05, ** p <0.01, *** p<0.001.

3.4.2 Combined treatments lead to an increase in expression of apoptotic markers in ARMS cells

The greatest induction of the cleaved caspase-3 and PARP apoptotic markers was also found when treating with combined QLT0267 and vincristine (Figure 3.4.2). In contrast to ILKsi, levels of these apoptotic markers was lower in each of the cell lines.
Figure 3.4.2. Combined QLT0267 and vincristine treatment in ARMS cells leads to induction of apoptotic signaling. ARMS cells were first treated with QLT0267 and vincristine followed by collection of cell lysates. QLT0267 concentrations of 2.5μM, 5μM and 7.5μM were used for Rh4, Rh18 and Rh30, respectively. Western blots were run to analyze cleaved PARP and cleaved caspase-3 expression. (A and B) Background-adjusted densitometric values were normalized to vehicle treatment and represent mean ± SEM. n=3. * p<0.05, ** p<0.01, ***p<0.001. (C) Data are representative of three independent blots.
3.4.3 Vincristine and QLT0267 lead to alteration in ARMS cell cycle distribution

In each of the ARMS cell lines, treatment with vincristine alone led to the highest proportion of cells in the G2/M phase of the cell cycle (Figure 3.4.3). However, the combined treatment of vincristine and QLT0267 had the highest proportion of cells in the sub-G1 gate which were consistent with the findings in 3.2.3.
Figure 3.4.3. Vincristine and QLT0267 treatment leads to changes in ARMS cell cycle distribution. After treating with QLT0267 and vincristine, ARMS cells (10^4 events per treatment per cell line) were measured for DNA content using propidium iodide staining. Cells were distributed into gates corresponding to the different phases of the cell cycle. QLT0267 concentrations of 2.5μM, 5μM and 7.5μM were used for Rh4, Rh18 and Rh30, respectively. The percentage of all cells gated in G2/M (A) and sub-G1 (B) are shown. Values represent mean ± SEM. n=3. ** p< 0.01, ***p<0.001.
3.4.4 QLT0267 and vincristine treatments disrupt centrosome organization

Similar to ILKsi treatment, QLT0267 treatment also disrupted the interaction between Aurora A kinase and TACC3 (Figure 3.4.4).

![Figure 3.4.4. QLT0267 and vincristine treatments disrupt the TACC3-Aurora A interaction. Whole cell lysates (WCL) were collected from Rh4 cells and the interaction between Aurora A kinase and TACC3 was determined under QLT0267, vincristine and combined treatment. Data is representative of three independent blots.]

3.4.5 Combined QLT0267 and vincristine treatment affects JNK/c-Jun phosphorylation

Combined treatment of QLT0267 and vincristine led to a decrease in phosphorylated JNK and c-Jun compared to vehicle or individual treatments alone (Figure 3.4.5). These results were consistent with those found in 3.2.5 using ILKsi. In Rh4 cells, the disruption of JNK phosphorylation was similar when comparing the use of ILKsi to QLT0267 individually or in combination with vincristine. However, the inhibitor resulted in a greater reduction in baseline c-Jun phosphorylation compared to ILKsi, but when used in combination with vincristine, the combined effect was approximately equal with a 60% reduction in c-Jun phosphorylation.
Combined QLT0267 and vincristine treatment is associated with a reduction in JNK/c-Jun phosphorylation. ARMS cells were treated with QLT0267 and vincristine (VCR) followed by collection of cell lysates. QLT0267 concentrations of 2.5μM, 5μM and 7.5μM were used for Rh4, Rh18 and Rh30, respectively. Western blots were run to analyze phospho- and total JNK and c-Jun expression. (A) Background-adjusted densitometric values of the phospho-to-total ratio, normalized to vehicle treatment, represent mean ± SEM. n=3. * p<0.05, ** p<0.01, ***p<0.001. (B) Data are representative of three independent blots.
Our understanding of ILK’s role as an oncogene in ARMS led to the hypothesis that inhibiting its function would present a novel therapeutic mechanism which could be combined with current therapy options. In this study, I explored the possibility that ILK manipulation synergized with chemotherapy in the treatment of ARMS cells. The main finding of this thesis is that regulating ILK protein expression or inhibiting its kinase function potently synergizes with the use of the drug vincristine in reducing ARMS cell proliferation (Figure 3.2.1 and 3.4.1). Interestingly, with the use of ILKsi, greater synergy was established in the Rh4 and Rh30 cell lines compared to Rh18. As discussed earlier, the function of ILK seems to be dependent on the genetic background found in RMS. Importantly, the Rh4 and Rh30 cell lines are both PAX3-FKHR positive while Rh18 cells are translocation negative. It would be worthwhile to determine what effect combined treatment would have on PAX3-FKHR stably-transfected Rh18 cells compared to normal Rh18 cells. Furthermore, it would be interesting to observe the effect on proliferation of ERMS cells that are fusion positive (which our lab has previously established in the RD cell line) compared to those that are fusion negative. The fusion-positive/negative dichotomy is not as prevalent in the studies performed with QLT0267 instead of the ILKsi. It is possible that the potentiation of vincristine is not solely due to ILK kinase function. As ILK plays a critical role in mediating cellular responses, it may be that important protein-protein interactions that are altered by ILKsi are not affected by the inhibitor. The additional finding described in this thesis attempt to elucidate the mechanisms in which the overall synergy arises. Specifically, the involvement of the following will be discussed below: caspases and apoptosis; cell cycle arrest and the centrosome; and the JNK/c-Jun signaling axis.
4.1 Caspases and apoptosis

Firstly, I have shown that the induction of cleaved caspase-3 and cleaved PARP expression follows combined treatment of vincristine and ILKsi or the ILK inhibitor QLT0267 (Figure 3.2.2. and 3.4.2.). Caspases are considered the central components of the apoptotic response and can irreversibly commit a cell to undergo programmed cell death\textsuperscript{101}. Initially produced as inactive zymogens, the proteolytic cleavage of caspases is required for their activation. Usually classified as either initiators or effectors (of which caspase-3 is an example), a conserved cascade of caspase activation is fairly similar amongst fruitflies, nematodes and mammals\textsuperscript{101}. Generally, initiator caspases are auto-activated, which in turn activate effector caspases, which are then able to cleave a broad range of targets within the cell – with the ultimate result being cell death\textsuperscript{101}. PARP is one of several cellular targets for caspase-3\textsuperscript{102}. Furthermore, in mammalian cells, initiation of apoptosis can occur through the intrinsic or extrinsic pathway, or both. The intrinsic pathway is triggered in response to stimuli from within the cell, examples of which include DNA damage or oncogene activation\textsuperscript{103} and involves the release of proteins such as cytochrome c from the mitochondria\textsuperscript{104}. The intrinsic pathway leads to the development of the apoptosome complex which mediates caspase activation\textsuperscript{105}. The extrinsic pathway involves signaling of extracellular ligands such as FasL binding to its receptor Fas which triggers the formation of the death-inducing signaling complex (DISC) that, similarly to the apoptosome, activates caspases\textsuperscript{106}. The results of my studies do not make it clear which particular pathway is triggered. Although vincristine has been used for decades, the exact mechanism by which it induces apoptosis remains unclear. The intrinsic pathway seems to be the early consensus as it is implicated in fibroblast\textsuperscript{107}, cervix carcinoma\textsuperscript{108}, and acute lymphoblastic leukemia cells\textsuperscript{109}. Furthermore, studies in other cell lines found an absence of extrinsic pathway involvement\textsuperscript{110-111}. Interestingly, in a Ewing sarcoma context, vincristine was shown to activate both the intrinsic and extrinsic
pathways. With respect to ILK, *ex vivo* deletion of ILK from adult mice endothelial cells and colitis-associated tumours in ILK knockout mice are associated with activation of the intrinsic pathway. The above evidence suggests that treatment of ARMS cells with vincristine is in part potentiated by ILK regulation causing an additional activation of apoptotic players in the intrinsic pathway. Further study is warranted to determine if ILK is able to trigger events in the extrinsic pathway. This can be done by evaluating the effect of ARMS cells after ILK treatment by subsequently inhibiting the extrinsic pathway.

### 4.2 Cell cycle arrest and the centrosome

Secondly, I have shown that the combined treatment of vincristine and ILKsi or QLT0267 leads to changes in the cell cycle of ARMS cells (Figure 3.2.3 and 3.4.3). In each of the cell lines, vincristine caused a greater fraction of cells to be found in the G2/M phase – greater than any other stage of the cell cycle. Vincristine has been shown to interfere with microtubule dynamics by binding tubulin monomers and can properly be classified as a microtubule-interfering agent (MIA). As microtubules play many important intracellular roles including mitosis, the result of ARMS cells arresting in the G2/M phase is consistent with other studies involving MIAs.

The effect on ARMS cells with ILKsi or QLT0267 is not as consistent. With ILKsi treatment alone, ARMS cells display a slightly greater proportion within the G2/M phase compared to vehicle control. However, with QLT0267, only in Rh4 cells is this increase observed. In fact, in Rh18 and Rh30 cells, the fraction of total cells in the G2/M phase is less than the vehicle treatment. This may be attributed again to the different modes of action between ILKsi and QLT0267. Perhaps the regulation of the physical interactions of ILK with other proteins via ILKsi is what causes the increase in the G2/M phase. Conversely, inhibition of the kinase signaling of ILK using QLT0267 may be the cause of the opposite effect. Interestingly, the proportion of cells in the G2/M phase under combined treatment (regardless of ILKsi or
QLT0267) is less than vincristine treatment alone. Further cell cycle analysis reveals that the discrepancy is in part due to an increased proportion of cells in the sub-G1 gate. Cells counted in this manner represent those with less than the normal diploid DNA content and can be used as an indirect measure of cells undergoing DNA fragmentation and apoptosis. With combined treatment, the proportion of cells in the sub-G1 gate was higher than individual treatments or vehicle and supports the finding that the highest levels of cleaved caspase-3 and PARP protein expression were found with the same combined treatments.

Thirdly, in this study it was found that both vincristine treatment and ILKsi or QLT0267 were able to disrupt an important protein interaction of the centrosome. This is supported by the literature which suggests that ILK not only plays a role in organizing signaling at focal adhesions but also at the site of centrosomes\textsuperscript{59}. Using ARMS cells in my study, I found that the association between Aurora A kinase and transforming acidic coiled coil 3 (TACC3) was disrupted when treated with ILKsi or QLT0267. There is evidence to show that the regulation of mitotic spindle organization begins with a phosphorylation event by Aurora A kinase on TACC3\textsuperscript{121-122}. Phosphorylated TACC3 recruits ch-TOG, which is crucial for proper microtubule polymerization and organization\textsuperscript{121,123}. Without this interaction, cells can arrest in mitosis which leads to death\textsuperscript{61}. Overexpression of Aurora A allows cells to progress more rapidly through the cell cycle and has been found to be overexpressed in a number of malignancies including colorectal, breast, ovarian, prostate and cervical cancer\textsuperscript{124-126}. Although the absence of the Aurora A kinase and TACC3 interaction was found with ILKsi and QLT0267 treatment in ARMS cells, a corresponding robust arrest in G2/M was not. One explanation is that the experimental timing at which the cell cycle distribution was queried was not conducive for detecting this arrest. The loss of interaction between Aurora A and TACC3 was found 24 hours after ILK protein knockdown or the use of QLT0267 but cell cycle analysis occurred immediately after. Perhaps, the
accumulation of cells in G2/M due to this absence required a longer duration. There is also early work that suggests the phosphorylation of TACC3 by Aurora A is not as essential as first thought. Studies assessing Aurora/Ipl1 related (AIR-1), the C. elegans Aurora A orthologue\(^{127}\), have revealed that there is a kinase-independent involvement of AIR-1 in microtubule recruitment, spindle assembly and centrosome maturation\(^{128-129}\). Furthermore, a careful balance between kinase-active and kinase-inactive AIR-1 was important to develop a functional mitotic spindle\(^{128-129}\). Whether these findings will be mirrored in mammalian cells remains to be seen and requires further study. Another explanation for the lack of G2/M arrest in ARMS cells is that ILK’s effect on Aurora A kinase is only one of many pathways that contribute to mitotic spindle regulation and organization at the centrosome. ARMS cells treated by ILKsi or QLT0267 may compensate for the disruption by recruiting other players and signaling pathways. For example, the centrosome is regulated by several families of kinases in addition to the Aurora family\(^{130-131}\), which include the cyclin-dependent kinases (Cdks)\(^{132-133}\), Polo and Polo-like kinases\(^{134-136}\), and the NIMA-related kinases (NEK)\(^{137-138}\). Although it is not apparent why vincristine as a tubulin binding molecule had an effect on Aurora A-TACC3 interaction in ARMS cells, it is interesting to note that the silencing of Aurora A expression by siRNA in colon cancer cells\(^{139}\) and the use of a selective inhibitors of Aurora kinases in leukemic cells\(^{140}\) have both been found to synergize with vincristine in reducing cell growth. With further study, it will be interesting to determine if the inhibition of microtubule dynamics using MIAs is able to feedback on the proteins that regulate them in the first place.

### 4.3 JNK/c-Jun signaling axis

Lastly, the potentiation of vincristine by ILKsi or QLT0267 revealed interesting patterns in the activation of JNK and its downstream target c-Jun. Consistent with previous work in our lab\(^{36}\), regulation of ILK protein in Rh4 cells was associated with a decrease in the ratio of
phosphorylated to total JNK and c-Jun protein levels detected by Western blot (Figure 3.2.5). This relationship was also found when using the ILK inhibitor QLT0267 in a panel of ARMS cells which suggests that the interaction between ILK and JNK is at least partly due to ILK kinase function. Surprisingly, vincristine treatment alone in ARMS cells led to a decrease in JNK and c-Jun activation. There have been some reports of vincristine leading to an induction of phosphorylated JNK in breast\textsuperscript{141}, cervix\textsuperscript{142} and ovarian\textsuperscript{115} cell lines but the exact mechanism remains unclear. Furthermore, downstream signaling events after treatment with MIAs are not restricted to the JNK signaling pathway. Both the ERK and p38 MAPKs have been implicated and reports on their activation are at times contradictory and may depend on cellular context\textsuperscript{142-143}. My findings suggest that activation of JNK/c-Jun axis may be governed by the particular setting as well. With combined treatment, activated JNK and c-Jun were found at levels lower than vincristine, ILKsi or QLT0267 treatments alone. This would indicate that the decreased levels of phosphorylated JNK and c-Jun observed after vincristine or ILK signaling regulation are caused by different means.

It is also intriguing to note that JNK activation was found to be negatively correlated with apoptosis induction in the ARMS system. There is ongoing debate as to what JNK’s role in apoptosis truly is. JNK has been thought of as a pro-apoptotic kinase after using genetic disruption experiments to inhibit JNK signaling\textsuperscript{144-146}. Yet it remains unclear as to whether JNK induces or promotes apoptosis, with evidence to support both hypotheses\textsuperscript{147}. Additionally, other studies hint that JNK activation is associated with anti-apoptosis and cell survival. For example, inhibition of JNK activation in lung carcinoma and glioblastoma cells resulted in reduced growth due to apoptosis\textsuperscript{148-149}. Furthermore, it may be that the p53 status of cancer cells is important in guiding JNK’s role in cell survival as studies have shown that p53-deficient cells have this phenomena as opposed to p53-normal ones\textsuperscript{150}. Interestingly, p53 mutations can be found in up to
50% of all RMS cases\textsuperscript{151-153}, and almost all cancer types display some alteration or modification of the p53 pathway\textsuperscript{154-156}. Therefore, future studies carefully investigating JNK’s involvement in apoptosis in cancer systems will prove important. In this study, the decrease in JNK activation corresponding to an increase in apoptosis suggests JNK is acting in an anti-apoptotic manner. It is important to note that although there is a unique interplay between ILK and JNK in RMS as discussed earlier, it is still unclear how they signal between each other. ILK and JNK are unlikely to complex together in RMS as previous attempts to co-immunoprecipitate the two proteins have failed\textsuperscript{157}. Future investigation involving mass spectrometric analysis of ILK binding partners and/or microarray studies of ILK signaling may potentially yield important mediators between ILK and JNK.

4.4 Conclusion

In this study, I have found that the regulation of ILK protein expression or inhibition of ILK’s kinase function potentiated the effect of vincristine on ARMS cell viability. This led to an induction of apoptotic markers and arrest in the G2/M phase of the cell cycle. Furthermore, disruptions of centrosomal protein interactions and decreased activation of the JNK/c-Jun signaling axis were found. To gain a better understanding of the mechanisms which explain the combinatorial synergy in our system, it will be worth pursuing the following studies. Firstly, the role of the important PAX3-FKHR translocation in ARMS drug-sensitivity should be determined. Secondly, whether the intrinsic or extrinsic pathways of apoptosis are involved can be investigated. Thirdly, determining the importance of Aurora A kinase function in centrosome regulation in ARMS cells can be explored. Fourthly, the role of JNK activation in ARMS apoptosis can also be elucidated. Lastly, and more generally, the discovery of mediators that link ILK and JNK signaling would be extremely valuable in devising new targets for therapy.
There are major efforts to study and understand the molecular mechanisms underlying cancer pathogenesis. As the outlook for RMS patients remains poor with current models of treatment that include chemotherapy, radiotherapy and surgery, it is imperative that specific and targeted therapies are investigated. ILK is a particularly interesting molecular candidate for RMS therapy due to its unique oncogenic and tumour-suppressive roles in ARMS and ERMS respectively. Building on these recent findings in ILK biology, we have identified a potential novel method for therapy in ARMS that regulates ILK signaling and is used in combination with vincristine. However, the *in vitro* experiments described in this thesis would benefit from corresponding *in vivo* studies before being considered for patient therapy. Ultimately, the aim of future studies should be to develop effective *in vivo* strategies that can be readily applied in a clinical setting to reduce the morbidity and mortality of RMS patients.
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