UNIVERSITY OF CALIFORNIA, SAN DIEGO

Inactivation of SHIP1 in Acute Lymphoblastic Leukemia

A Thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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2009

The Thesis of Tony Chung Tung Lo is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2009

Dedication

To my family,

my friends,

and

to those affected.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I would like to thank Dr. Diccianni for giving me the opportunity to learn and train under him. His guidance has shaped me into a better researcher; his persistence has helped me excel; his trust has allowed me freedom to test my own ideas; and his expertise has fostered in me enthusiasm and passion for research.

I would also like to acknowledge Lisa Barnhill for all her advice and support as much of my progress would not have been possible without her.

ABSTRACT OF THE THESIS

Inactivation of SHIP1 in Acute Lymphoblastic Leukemia

by

Tony Chung Tung Lo

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Michael David, Chair

Despite the success of treatment of acute lymphoblastic leukemia (ALL), the most common childhood cancer, 20% of cases continue to relapse and others suffer from secondary malignancies. The PI3K/Akt pathway is central to cell survival. Deregulation of this pathway, through PI3K and PTEN mutations, is often found in many cancers. In ALL, though Akt is deregulated, PI3K and PTEN mutations are infrequent, suggesting that other factors are responsible. The phosphatase, SHIP1, acts similarly to PTEN in regulation of Akt activity. In this study, SHIP1 in B- and T-cell ALL is found to be

translationally inactivated through alternative splicing and mutations. In primaries, wild-type SHIP1 protein is undetectable, and instead, truncated forms are found. Mutational analysis of the *SHIP1* reveals alterations that include deletions and insertions resulting in silent and missense mutations in functional regions of the gene, which explain the loss of full-length protein and render the variants dysfunctional. Analysis of PTEN expression shows either deletion or inactivation in ALL. The deregulation of both PTEN and SHIP1 in ALL substantiates the Akt pathway as an important target for therapeutics. Limited success in treatment is likely due to poor understanding of how oncogenic pathways are deregulated. To elucidate the role of SHIP1 in chemosensitivity, LY294002 and doxorubicin were studied in SHIP1-expressing and non-expressing cell lines. The presence of SHIP1 is shown to potentially elicit greater responses to drugs. This supports the importance of SHIP1 in determining drug sensitivity and potentiates its use as a tool for determining future therapeutic protocols.

Chapter 1: Introduction

1.1 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a malignant clonal disorder characterized by the lymphoid precursor proliferation. At an early stage of development, the lymphoid cells arrest, which can be a consequence of abnormal gene expression. The cells crowd out normal hematopoietic cells in the bone marrow, resulting in a severely diminished production of normal blood cells. Depending on the origin of the lymphoid cells, ALL can be primarily characterized as B-cell ALL (B-ALL) or T-cell ALL (T-ALL) [Esparza and Sakamoto, 2005]. Historically, a T-cell immunophenotype of lymphoid malignancies is often associated with a poorer prognosis than other types of childhood ALL [Carroll et al., 2003; Goldberg et al., 2003].

Although ALL affects both children and adults, it has a peak incidence between the ages of 2 and 5, and is the most common type of childhood leukemia. Current modalities have achieved a cure rate of over 80% in children, but others continue to relapse despite the success. Those that survive often suffer from secondary malignancies due to nonspecific effects of present therapeutics causing risk to normal cells, which can give rise to other complications such as neuropsychological problems [Janzen and Spiegler, 2008]. Thus, one of the major obstacles in ALL is to design better therapeutics to treat those who are resistant to current protocols and also to address the toxicity of present drugs. In order to reach these goals, it is important to characterize and understand the function of the defects responsible for these malignancies in order to exploit them for therapeutic purposes.

1.2 The Akt Pathway

The serine-threonine Akt kinase, also known as protein kinase B (PKB), regulates a myriad of cellular processes involved in cell survivial, cell proliferation, and cellular growth. Akt is deregulated, leading to abnormal activation levels, in a large range of cancers including ALL [Maser et al., 2007; Silva et al., 2008].

Functioning in an anti-apoptotic fashion, Akt acts by directly phosphorylating several substrates involved in the process of cell-death, such as the pro-apoptotic factor BAD and pro-death protease caspase-9 [Cardone et al., 1998; Brunet et al., 1999]. Akt can also have negative regulatory effects on CDK inhibitors (CKIs) $p27^{KIP1}$ and $p21^{WAF1}$ [Graff et al., 2000]. Thus, Akt is crucial in facilitating cell proliferation through numerous factors.

1.2.1 Regulators of Akt

The activation of Akt occurs through the presence of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3] and 3-phosphoinositide dependent protein kinase (PDK). PtdIns(3,4,5)P_3 is created from phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] by phosphatidylinositol-3 kinase (PIK3CA or PI3K) and acts at the plasma membrane as a docking site. By interacting with PtdIns(3,4,5)P_3, Akt is recruited to the plasma membrane where it becomes phosphorylated and activated by PDK [Fresno Vara et al., 2004].

As illustrated in Figure 1, two PIP_3 phosphatases, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and SH2 (Src homology 2)-containing inositol phosphatase-1 (SHIP1), oppose the effects of PI3K. PTEN and SHIP1

dephosphorylate PtdIns(3,4,5)P_3, the product of PI3K, to PtdIns(4,5)P_2 and phosphatidylinositol 4,5-bisphosphate [PtdIns(3,4)P_2], respectively [Stambolic et al., 1998; Rohrschneider et al., 2000]. Thus, by countering the effects of PI3K, PTEN and SHIP1 play a significant role as regulators of Akt activity.

As mentioned earlier, activated Akt levels are often high in cancer. Although Akt can be amplified and overexpressed, it is rare and not often the source of Akt deregulation [Vivanco and Sawyers, 2002]. Instead, Akt deregulation is usually caused by activating and inactivating mutations in PI3K and PTEN, respectively.

1.3 Signaling of PI3K and its Deregulation in Disease

The lipid kinase PI3K is important in regulating signaling pathways involved in cell proliferation and regulation of gene expression [Cantley, 2002]. Activation of PI3K occurs via recruitment to the cell membrane by tyrosine kinases or Ras (Figure 1). PI3K activating mutations are found in many tumor types. Contrary to the frequency of mutations observed in most cancers, a limited study of acute leukemia primary samples and cell lines show PI3K is mutated in only 1% of cases, suggesting that PI3K mutations is not the cause for abnormal Akt activation in ALL [Müller et al., 2007; Lee et al., 2005]. This finding is supported by our analyses of T-ALL, in which no significant mutations are found, reinforcing the idea that the source for Akt deregulation is due to factors other than PI3K.

1.4 PTEN Function and its Implication in Cancer

Known as a tumor suppressor, PTEN is a critical regulator of the PI3K/Akt pathway. Studies overexpressing PTEN show a correlating decrease in Akt phosphorylation and subsequent inhibition in cell growth caused by G1 cell cycle arrest and apoptosis [Weng et al., 1999]. On the other hand, loss of PTEN can lead to increased Akt phosphorylation as well as prolonged duration of Akt activation [Lu et al., 1999]. Homozygous deletion of PTEN in mice is embryonic lethal and heterozygous PTEN knockout mice develop neoplasms in various organs, resulting in diseases such as T-lymphoma [Di Cristofano et al., 1998; Stambolic et al., 1998]. By negatively regulating Akt phosphorylation through PtdIns(3,4,5)P_3, PTEN is an important player in controlling activation of Akt signaling.

Because of PTEN's significance in the regulation of cell growth, dysfunction of PTEN can cause malignancy. PTEN deletions and mutations are seen in a wide range of cancers [Sansal and Sellers, 2004; Simpson and Parsons, 2001]. In a large number of T-ALL cell lines, PTEN is deregulated due to deletions or mutations [Sakai et al., 1998; Takeuchi et al., 2003]. Also, Notch activating mutations are commonly found in T-ALL and are shown to negatively regulate PTEN (Fig. 1) [Weng et al., 2004; Palomero et al., 2007]. However, relative to cell lines, PTEN inactivating mutations occur at lower frequencies in primary T-ALL samples [Palomero et al., 2007; Maser et al., 2007]. Thus, the cause of abnormal Akt activation in ALL remains questionable as both PI3K and PTEN are infrequently mutated. SHIP1, a PIP_3 phosphatase like PTEN, is a possible candidate.

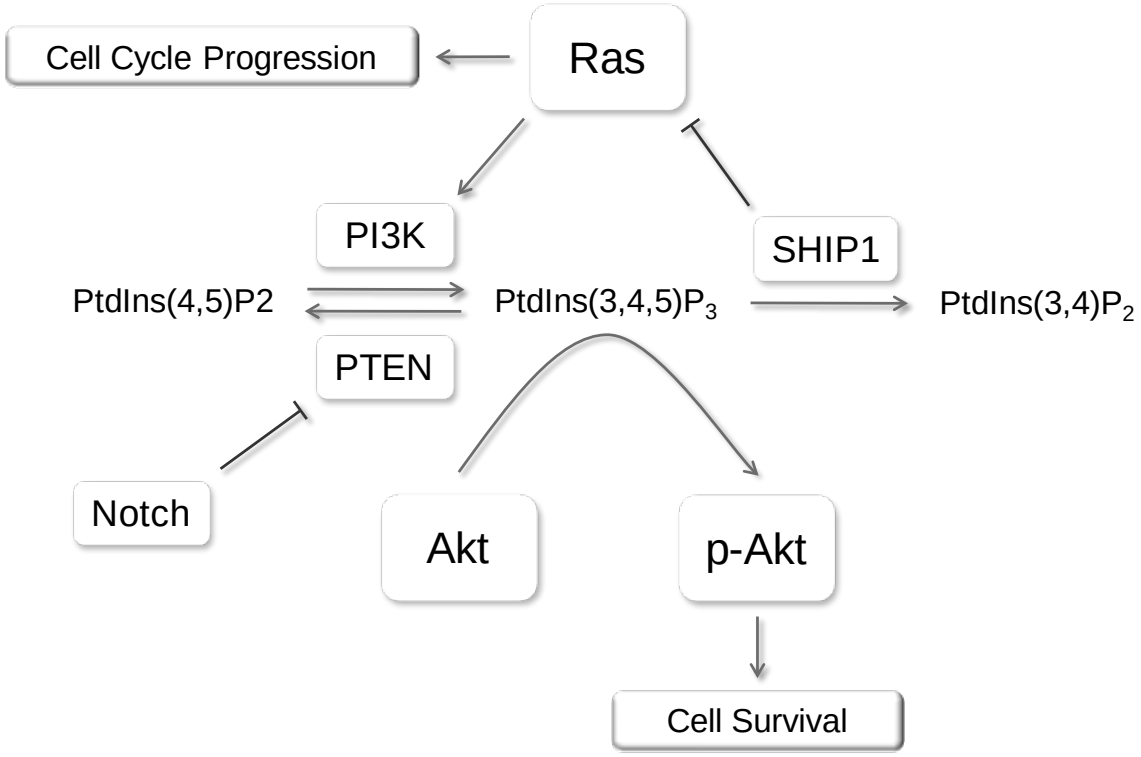

Figure 1: The Ras/Akt pathway. PTEN and SHIP1 dephosphorylate PtdIns(3,4,5)P_3, opposing PI3K activity and subsequently decreasing Akt activation. SHIP1 can also function to indirectly inhibit the Ras pathway. Notch can indirectly downregulate PTEN activity.

1.5 The Structure and Function of SHIP1

Less studied of Akt regulators is SHIP1. SHIP1 acts to hydrolyze the 5'-phosphate of the inositol ring of 3'-phosphorylated phosphoinositides. The 145 kDA SHIP1 protein is made up of several domains that allow for additional functions through the interaction with a large number of signaling proteins (Figure 2). It includes an amino-terminal Src homology 2 (SH2) domain, a central catalytic domain with phosphatase activity, and a carboxy-terminal proline rich domain that contains two NPXY motifs [Damen et al., 1996].

The N-terminal SH2 domain of SHIP1 is capable of binding the immunoreceptor tyrosine inhibitor motif (ITIM) [Unkeless and Jin, 1997.]. The interaction of the ITIM motif of FcγRIIB with SHIP1 plays an important role in SHIP1 recruitment to the cell membrane and FcγRIIB receptor-mediated negative signaling in B-cells [Tridandapani et al., 1997].

The central region of SHIP1 has enzymatic activity that hydrolyzes PtdIns(3,4,5)P_3. By decreasing levels of PtdIns(3,4,5)P_3, SHIP1 can suppress Akt signaling [Aman et al., 1998], affecting Akt activity on cell growth and proliferation. In addition, dephosphorylation of PtdIns(3,4,5)P_3 can affect B-cell receptor (BCR)-mediated calcium flux through regulation of Bruton's tyrosine kinase (BTK) activity [Bolland et al., 1998].

The C-terminal NPXY motifs are capable of being tyrosine phosphorylated and interact with proteins containing a SH2 domain or a phosphotyrosine-binding domain (PTB) [van der Geer and Pawson, 1995]. The proline rich region of the C-terminal interacts with SH3 domain-containing proteins. The interaction of the adaptor protein Shc

with the NPXY motifs of SHIP1 is important for growth factor receptor signaling in the Ras pathway and is further discussed below [Rozakis-Adcock et al., 1992].

SHIP1 belongs to a family of inositolpolyphosphate phosphatases and has close relatives such as SHIP2. SHIP2 has high identity to motifs and domains of SHIP1, but important differences exist between the two proteins. Unlike its homolog SHIP2, SHIP1 expression is restricted to blood cells, suggesting an important role for SHIP1 in hematopoietic signaling [Pesesse et al., 1997]. Also, the two proteins act through different substrates; SHIP1 interacts with both PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4, whereas SHIP2 only utilizes the former of the two lipids [Wisniewski et al., 1999].

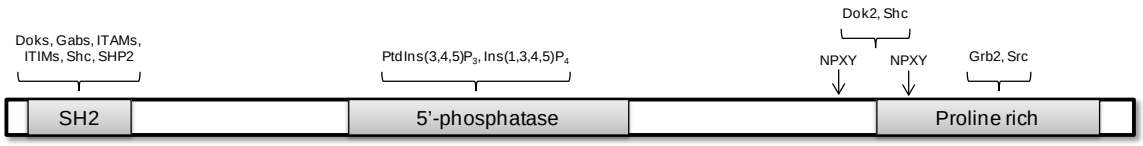

Figure 2: The domains of SHIP1 and its various interactions. The amino-terminal SH2 domain binds to phosphotyrosine residues and interacts with a large number of signaling proteins. The central region encodes for catalytic activity that removes phosphate from the 5' position of inositol phosphates. When tyrosine phosphorylated, the two NPXY motifs in the carboxy-terminal are capable of binding PTB and SH2 domains. The proline rich region has potential to interact with SH3 domains.

1.5.1 SHIP1 Isoforms

SHIP1 is predominately detected as a 145 kDa species, but other sizes of 135 and 110 kDa are also documented. Identified in human myeloid cells, the 135 kDa form of SHIP1 encompasses an in-frame 282 nucleotide deletion. This deletion encompasses the first NPXY motif and also part of the proline rich region [Lucas and Rohrschneider, 1999]. The 110 kDa species is proposed to be caused by a putative start site near exon 6, bypassing the first 214 amino acids including the SH2 domain [Kavanaugh et al., 1996]. Despite its lack of an SH2 domain, the SHIP1 isoform is still able to interact with other proteins. Additional forms of *SHIP1* splice variants are reported, most of which are in murine cells.

1.5.2 Significance of SHIP1 in B- and T-cell Development

The restricted expression of SHIP1 to hematopoietic cells suggests its significance in the development of blood cells. In support of this, SHIP1 is found in normal peripheral blood lymphocytes and bone marrow; immature and mature T-cells; $CD2^+$, $CD4^+$ and $CD8^+$ T-cells; and $CD19^+$ and $CD20^+$ B-cells [Geier et al., 1997; Liu et al., 1998].

In B lymphocytes, SHIP1 is involved in attenuating B-cell growth. Cross-linking of the FcγRIIB receptor to the B-cell receptor (BCR) results in recruitment of SHIP1 [Aman et al., 1998]. Subsequent tyrosine phosphorylation of SHIP1 by FcγRIIB causes dephosphorylation of PtdIns(3,4,5)P_3 and SHIP-dependent inhibition of PLCγ, Akt, and Btk activation, resulting in decrease in proliferation and calcium mobilization [Aman et al., 1998; Bolland et al., 1998]. Less well-documented is the mechanism seen in T-cell signaling. Although similar to B-cell signaling in the respect that FcγRIIB in T-cell co-

aggregates with the T-cell receptor (TCR), leading to tyrosine phosphorylation of SHIP1 and subsequent inhibition in calcium mobilization [Jensen et al., 2001], other effects of SHIP1 phosphorylation are less understood.

1.5.3 The Role of SHIP1 in Pathobiology

Numerous studies suggest SHIP1 association with cell proliferation as well as the pathogenesis of leukemia. Mutations in the SHIP1 gene affecting catalytic activity and the proline rich domain are found in both ALL and acute myelogenous leukemia (AML) [Luo et al., 2003, 2004]. In K562 chronic myelogenous leukemia (CML) cells, transfection of SHIP1 with a mutation in the catalytic region results in cell proliferation and resistance to etoposide, suggesting SHIP1 may play a role in the development of leukemia and resistance to chemotherapy through the deregulation of the PI3K/Akt signaling pathway [Luo et al., 2003].

Studies involving the T-ALL cell line Jurkat reveal that the expression of SHIP1 reduces PtdIns(3,4,5)P_3 levels, leading to a decrease in the localization and phosphorylation of Akt, and ultimately, decrease cell growth [Horn et al., 2004, Freeburn et al., 2002]. Additionally, SHIP1 is able to increase the G1 phase transit time in Jurkat cells [Horn et al., 2004]. SHIP1 deficient mice develop myeloproliferative syndrome, characterized by an increase in number of granulocyte-macrophage progenitor cells in the bone marrow and spleen. The hyperplasia of cells in these mice results in diseases that ultimately shorten their lifespan [Liu et al., 1999; Helgason et al., 1998]. Studies involving the BCR/ABL, which causes CML, show that the oncogene inhibits SHIP1 expression, implicating SHIP1 in the pathogenesis of CML [Sattler et al., 1999; Jiang et

al., 2003]. Additionally, expression of KLF2 (Krüppel-like factor 2), a negative regulator of T-cell proliferation, can be up-regulated by SHIP1, suggesting that SHIP1 may mediate growth inhibition through KLF2 [Garcia-Palma et al., 2005].

In mice with T-cell-specific deletion of SHIP1, the numbers and activation state of T-cells, including Akt activation levels, are no different from wild-type [Tarasenko et al., 2007]. However, this mice model harbors functional PTEN, which may mask effects on T-cell regulation. PTEN is described as a sentinel phosphatase responsible for regulating constitutive/ low basal levels of receptor-stimulated PtdIns(3,4,5)P_3, whereas SHIP1 controls receptor-activated levels of PtdIns(3,4,5)P_3 by acting as a gatekeeper, redirecting PI3K-dependent signaling rather than stopping it [Harris et al., 2008]. Thus, it is possible that the sole inactivation of SHIP1 is insufficient for neoplasia and may require the co-inactivation of PTEN to push cellular signaling past the threshold of normal activity towards malignancy.

1.6 The Interactions between the Ras Pathway and SHIP1

Mutations in Ras are commonly seen in many types of human cancers, with a prevalence of 20% to 30% of all cases. The Ras genes code for GTP-binding proteins which affect various processes such as cell proliferation, differentiation and cell migration. Activating point mutations in Ras cause constitutive signal transduction, leading to uncontrolled proliferation and tumor development [Bos, 1989]. In T-ALL, activated Ras is frequently found [von Lintig et al., 2000].

Aside from being able to regulate the Akt pathway, SHIP1 can affect the Ras pathway independent of its phosphatase activity. Specifically, SHIP1 interacts with

adaptor proteins Shc and Grb2, which play a large role in the Ras pathway [Rozakis-Adcock et al., 1992]. The N- and C-terminal of SHIP1 bind Shc through a bidentate interaction (Figure 2), preventing the Shc engagement with Grb2 needed for Ras activation [Tridandapani et al., 1998]. Alternatively, SHIP1 is capable of inhibiting Ras activity through the adaptor molecule Dok1, which recruits Ras GTPase activating protein (RasGAP) [Isnardi et al., 2006]. Thus, as an alternative to activating Ras mutations, SHIP1 deregulation may also result in high levels of Ras activity. Having a dual association with the oncogenic signaling pathways Akt and Ras, SHIP1 is an important player in pathogenesis.

Chapter 2: Materials and Methods

2.1 Sample Accrual and Preparation

Cell lines were obtained through the American Type Culture Collection with two exceptions: Moltl6 was a generous gift from Dr. M.O. Diaz (University of Chicago, Chicago, IL) and HEK293T was a considerate donation from Dr. J.D. Bui (University of California, San Diego, La Jolla, CA). Cell lines were cultured and maintained in minimum essential medium (MEM) or RPMI supplemented with 10% dialyzed horse serum, 2 mmol/L glutamine, and 1% penicillidstreptomycin.

Primary T-ALL samples were obtained from children treated in Children's Oncology Group (COG) ALL Biology protocols #8862, #9673, #9000 or #9400. B-precursor samples were obtained from children treated at University of California, San Diego (UCSD). Mononuclear cells were isolated from the samples by Ficoll-Hypaque density gradient centrifugation. RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), DNA using the Puregene DNA Purification System Cell & Tissue Kit (Gentra Systems, Minneapolis, MN), and protein as a cell lysate in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) with protease inhibitors. Samples, utilized under a UCSD-approved IRB protocol (#041429), are fully encoded to protect patient confidentiality and conform to HIPAA standards.

2.2 PCR Amplification and Sequencing

The generation of cDNA was conducted by reverse transcribing 2μg RNA using Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). SHIP1 gene analysis was amplified using 10 pmol of either SHIP1-5F or SHIP1-2409F with SHIP1-3863R in a 50 μl volume containing 1 μl cDNA, 2 mM $MgSO_4$, 140 μM dNTPs, and 3.75 U Platinum Taq DNA Polymerase High Fidelity in 1X High Fidelity buffer (Invitrogen). The initial denaturing step at 94°C for 2 min was followed by 35 cycles of 30 sec at 94°C, 30 sec at 62°C, and 120 sec at 68°C. The final extension step at 72°C was 10 min. PCR was performed in a GeneAmp 2720 PCR System (Applied Biosystems, Foster City, CA). For sequence analysis, PCR products were purified with a Qiagen QiaQuick column (Qiagen, Valencia, CA) and sequenced directly using the shared sequencing resource at Moores UCSD Cancer Center. Sequencing primers included internal primers SHIP1-1156R, SHIP1-925F, SHIP1-1695F, SHIP1-2409F, SHIP1-3106F and SHIP1-3836R. Some PCR products were also cloned using the TA cloning kit (Invitrogen) before sequencing.

SHIP1 exons, introns, and splice sites were determined by blasting the 5274 bp *SHIP1* cDNA (GI:64085176) against the chromosome 2 genomic sequence (GI:157724517). Codon numbering was based on variant 2 (GI:64085176), which lacks a valine at codon 116 present in variant 1 (GI:64085166).

Primers			
SHIP1-5F	AGGAAGTCAGTCAGTTAAGCTGGT	SHIP1-2409F	CTCGAGCTGCTTGGAGAGTT
SHIP1-925F	CATCAACATGGTGTCCAAGC	SHIP1-1156R	CCTCAGAACCATCCTTGGAC
SHIP1-1695F	AAACACACTGGGGAACAAGG	SHIP1-3836R	CAAAGCCTTGCATAGGAAGC
SHIP1-3106F	CCCAGGACACAGGAGTCAAG	SHIP1-3863R	CAGAAGCTAGGCCCTTTCCT

2.3 Western Blot of SHIP1, PTEN, Akt and p-Akt

For Western blotting, approximately 50 μg of cell lysate was resolved on a 10% Bis-Tris or 3-8% Tris-Acetate NuPAGE gels (Invitrogen), transferred to an Immobilon-P membrane (Millipore, Billerica, MA), blocked in 5% BSA in 1X TBST and incubated overnight at 4^{o}C with the following antibodies: phospho-Akt^{Ser473} (#9271, 1:1000), Akt (#9272, 1:1000), phosphor-PTEN$^{Ser380/Thr382/383}$ (44A7, #9549, 1:500), and PTEN (138G6, #9559, 1:1000) (Cell Signaling Technologies, Inc., Beverly, MA); SHIP1 (epitope: amino acids 866-1020) (P1C1, #sc-8425, 1 μg/ml) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); SHIP1 (epitope: amino acids 6-21) (SHIP-01, #ab65807, 1:2000) (Abcam Inc., Cambridge, MA); and β-actin (#AC15, 1:250,000) (Sigma-Aldrich, St. Louis, MO). After washing and incubation with an alkaline-phosphatase conjugated anti-mouse or anti-rabbit secondary antibody (1:1000) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), the blot was visualized with ECF substrate and quantified using a Molecular Dynamics STORM 860 Imager (Amersham Biosciences Inc., Buckinghamshire, UK).

2.4 Expression Constructs and Transfection

Entire *SHIP1* gene was amplified with primers modified with restriction sites and ligated into pcDNA 3.1 vector (Invitrogen). The PCR product was purified with a QiaQuick column (Qiagen) and digested with restriction enzymes (NEB) for an hour. The ligation product was transformed into DH5α Extreme Efficiency competent cells (Allele Biotechnology & Pharmaceuticals, Inc., San Diego, CA) and the inserted sequence was verified using various primers mentioned above.

HEK293T cells were cultured as described above and plated 24 hrs before transfection in order to reach 85% confluency at the time of transfection. 3.2µg of plasmid and 8 µl of Lipofectamine 2000 reagent (Invitrogen) were each diluted in 100 µl of serum-free Opti-MEM I Reduced Serum Medium (Invitrogen) and incubated at 25°C for 10 min. Media-diluted plasmid and Lipofectamine 2000 reagent were combined and incubated at 25°C for 30 min. The combined product was directly added to well and incubated for 48 hrs. The cells were harvested as described above.

2.5 Cell Viability

Cells were centrifuged and resuspended in 1 mL of PBS. 0.4% trypan blue (Sigma) was mixed with the cell suspension and allowed to incubate for 3 min at 25°C. A drop of the trypan blue/cell mixture was applied to a hemacytometer and cells were counted. Unstained (viable) and stained (nonviable) cells were compared to determine cell viability.

2.6 Cell Proliferation

Normal peripheral mononuclear cells (MNCs) and T-ALL cell lines were plated in 96 well plates in triplicate. Cell proliferation was assessed using a TACS XTT Cell Proliferation/Viability Assay (R&D Systems, Minneapolis, MN). Briefly, cells were allowed to incubate for 4 hours in the appropriate concentration of LY294002 (Cayman Chemial, Ann Arbor, MI) before the addition of doxorubicin (Bedford Laboratories, Bedford, OH). After 72h in the presence or absence of drugs, 50 µl of XTT (sodium 3´-[1-(phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic

acid hydrate) was added to each well and incubated 37^{o}C for 6 hr. Absorbance was read using a microplate reader at 490 nm with a reference wavelength of 650 nm.

2.7 Drug Synergism

Drug interaction was characterized using two different models: the effect addition model and the Bliss independence model (also called effect multiplication or the fractional product). The effect addition model is as follows: $E(d_1, d_2) = E(d_1) + E(d_2)$, where (d_1) and (d_2) represent the dose of drug 1 and 2, respectively; $E(d_1, d_2)$ is the effect of (d_1) and (d_2) in combination; and $E(d_1)$and $E(d_2)$ are the effects of (d_1) and (d_2) alone, respectively (Lee et al., 2007). Combination doses with effects greater than predicted signify synergism, and the opposite, antagonism. The Bliss independence model has the form: $f_{12} = f_1 f_2$, where f_1, f_2, and f_{12} are fractions of the maximal effects of drug 1, drug 2, and in combination, respectively (Bliss, 1939). The maximal effect is defined as the effect detected with an infinite amount of drug. When the combination dose is less than predicted, it is synergistic, and vice versa.

Chapter 3: SHIP1 in B- and T-cell Acute Lymphoblastic Leukemia

3.1 Background: Determining the Source of Akt Deregulation

Through the Akt signaling pathway, both PI3K and PTEN play important roles in tumor development and have a high frequency of mutations in a variety of cancers. In ALL, however, PI3K mutations are infrequent in cell lines and primary samples. Our analyses of PI3K showed no mutations in 9 T-ALL cell lines, and only one heterozygous conserved mutation and one heterozygous silent mutation in 81 primary T-ALL (data not shown). Although PTEN deregulation is often found in T-ALL cell lines, inactivating mutations are not common in primary ALL. Because of the infrequent mutations of both PI3K and PTEN, the cause of Akt deregulation in ALL remains a question. Not well-documented is SHIP1, which has demonstrated regulation of Akt activity and an important factor in the development of leukemia. Dysfunction of SHIP1 could potentially be the source of Akt deregulation.

3.2 Expression of SHIP1 in Normals and Leukemia Cell Lines

In order to study the status of SHIP1 in ALL, leukemia cell lines were compared to normal thymocytes and peripheral mononuclear cells (MNCs). *SHIP1* transcript was assessed using PCR with primers encompassing the entire gene and SHIP1 protein was measured using Western blot.

Both normals and cell lines express full length *SHIP1* transcript (Fig. 3). A 145 kDa band is detected in both normal thymocytes and MNCs using either an N- or C-terminal-specific antibody, suggesting the presence of full length SHIP1 (Fig. 4, lane 4;

data not shown). When probed with an N-terminal-specific SHIP1 antibody, thymocytes express an additional band of lower molecular weight, which may represent a natural occurring isoform of SHIP1 (see section 1.5.1). In cell lines, SHIP1 is found in 3 of 3 B-cell leukemia and in 5 of 6 T-ALL samples (Fig. 4; Fig. 6B; data not shown). A comparison between normals and leukemia samples reveal low to moderate expression of SHIP1 in normals and T-ALL cell lines, but high expression in B-ALL cell lines.

Some cell lines do not have a clear link between transcript and protein expression. Even with the presence of full length *SHIP1* transcript, CEM cells express low levels of SHIP1 and possess a secondary band of slightly lower molecular weight (Fig. 3; data not shown). Jurkat T-ALL and K562 CML cells, known to lack SHIP1 protein [Luo et al., 2003; Horn et al., 2004], express full *SHIP1* transcript (Fig. 3, Fig. 4).

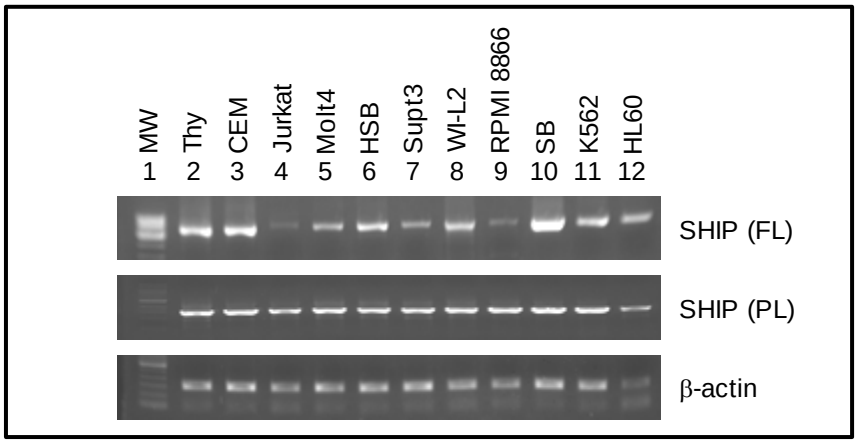

Figure 3: Expression of *SHIP1* transcript in leukemia cell lines. Lanes 3-6 represent T-ALL cells and lanes 7-10 are B-ALL samples. K562 (lane 11) and HL60 (lane 12) are CML and AML cell lines, respectively. Full-length (FL) covers the entire *SHIP1* coding region and is amplified using primers 5F and 3863R. Partial-length (PL) uses primers 2409F and 3863R, amplifying *SHIP1* starting at the enzymatic region. β-actin serves as a loading control.

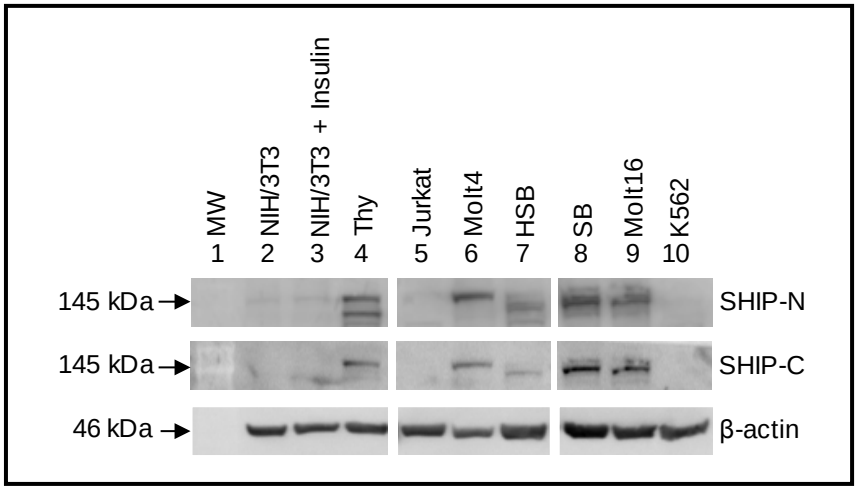

Figure 4: Expression of SHIP1 protein in ALL cell lines. SHIP1 protein, utilizing both an N- and C-terminal antibody, was assessed in B- and T-ALL cell lines. Lanes 5-7 and 9 represent T-ALL lines and lane 8 is from B-ALL. K562 (lane 10) is a CML cell line.

3.3 *SHIP1* Alterations in Leukemia Cell Lines

To address the possibility of null mutations that may explain the anomaly of the presence *SHIP1* transcript but no protein expression, Jurkat and K562 were sequenced for the entire coding region of *SHIP1* cDNA. In K562, no significant mutations are found, suggesting that other mechanisms of SHIP1 inactivation may be occurring. On the other hand, sequencing results of Jurkat reveal a number of polymorphisms, some of which have been previously documented in literature, while others are novel silent and missense mutations. In Jurkat, three exclusive alterations are found: at codon 345, there is a hetereozygous change that leads to a null mutation; at codon 1133, a hetereozygous change from CCG (Pro) to C(C/T)G (Pro/Leu) that affects the proline rich domain is observed; and at codon 413, the spice site for exon 11/12, a 47 bp deletion exists that result in a frameshift and subsequent premature termination.

Cloning of SHIP1 PCR product and sequential sequencing reveals the fashion in which the Jurkat mutations segregate. The codon 345 wild-type allele coincides with the codon 1133 CTG (Leu) allele, and the codon 345 nonsense mutation allele coexists with the codon 1133 wild-type allele. The 47 bp deletion found at the junction of exon 11 and 12 coincided with the codon 345 wild-type allele, generating a nonsense mutation apart from the codon 345 null mutation found on the other allele. Thus, both alleles of *SHIP1* in Jurkat, one way or another, harbor a severe mutation. These alterations of SHIP1 in Jurkat are of particular significance as they reveal the mechanisms previously unknown that explain the absence of SHIP1 protein.

Another mutation found in Jurkat is a CAT (His) to (C/T)AT (His/Tyr) substitution at codon 1168. The codon 1168 wild-type allele co-segregates with codon

345 null mutation, and the codon 1168 TAT (Tyr) mutation is found with codon 345 wild-type. Unlike the other alterations, this is not unique to Jurkat as it is also found in several cell lines and normal tissue [Gilby et al. 2007]. Table 1 summarizes the sequence alterations found in Jurkat and other cell lines.

3.4 Sequence Analysis of Normal Cells

As a reference for the mutations seen in Jurkat, normal thymocytes and MNCs were studied. Correlating with presence of *SHIP1* transcript and expression of wild-type SHIP1 protein, most normals do not harbor significant mutations (Fig. 3; Fig. 4; Table 1). However, out of 11 samples, a deletion of exons 25 and 26 is observed in one clone, and an exon 12 deletion along with an intron 14 insertion is found in another.

Table 1: *SHIP1* sequence alterations in normals and cell lines

Table 1 | ***SHIP1* sequence alterations in normals and cell lines**

Codon	Change	Conserved	Reported	Cell Line (Genotype)
162	ACA (T)-->ACG (T)	Yes	No	Molt4 (A/G)
272	TCA (S) --> TCG (S)	Yes	No	SB (A/G), K562 (A), Ramos (G), Molt4 (A/G), Molt16 (G), CEM (A/G), Jurkat (G), Thy (G), MNC (A/G)
278	GTC (V) --> ATC (I)	Yes	No	CEM (G/A)
345	CAT (Q) --> TAG (Stop)	No	No	Jurkat (C/T)
346	CTC (L) -->CTG (L)	Yes	Yes	SB (C/G), K562 (C), Ramos (C), Molt4 (C/G), Molt16 (C/G), CEM (C), Jurkat (G), Thy (G), MNC (C/G)
362	ACA (T) --> ACG (T)	Yes	No	SB (A/G), Jurkat (G), Thy (A/G)
413	47bp Deletion	No	No	Jurkat
1036	CAG (Q) --> AGG (R)	Yes	No	MNC (A/G)
1086	ACG (T) --> ACA (T)	Yes	Yes	Ramos (G/A), Jurkat (G/A)
1133	CCG (P) --> CTG (L)	No	No	Jurkat (C/T)
1168	CAT (H) -->TAT (Y)	No	Yes	Ramos (C/T), Jurkat (C/T)

3.5 *SHIP1* Transcript Levels in Primary B- and T-ALL

Because of the lack of SHIP1 protein in *SHIP1* expressing cell lines and the presence of functional mutations, we assessed SHIP1 in 62 T-ALL and 20 B-precursor ALL samples (Fig. 5; data now shown). Using PCR primers designed to amplify the entire coding region of *SHIP1*, less than half of the T-ALL primaries (48%; 30 of 62) express high levels of transcript, while the majority have either low levels (32%; 20 of 62) or are undetectable (19%; 12 of 62). In contrast, most B-precursors have high levels of *SHIP1* transcript (70%; 14 of 20), though some samples express low levels (10%; 2 of 20) or are undetectable (20%; 4 of 20).

Because it is possible that the low to undetectable levels of transcript in some samples may be due to an alternative 5' transcription site not detected by the sense primer, *SHIP1* transcript was also assessed using a forward primer designed to anneal to the enzymatic domain of *SHIP1*. Results show that both samples that express and those that do not express full-length SHIP1 are able to amplify the partial-length *SHIP1* transcript, often along with other smaller sized fragments as well (Fig. 5). Those that amplify partial-length *SHIP1*, but not full-length, may have forms of *SHIP1* that harbor an alternatively spliced 5' end. In both the full-length and partial-length amplifications of some samples, multiple bands in addition to the expected amplicon are detected, suggesting the presence of other forms of *SHIP1*.

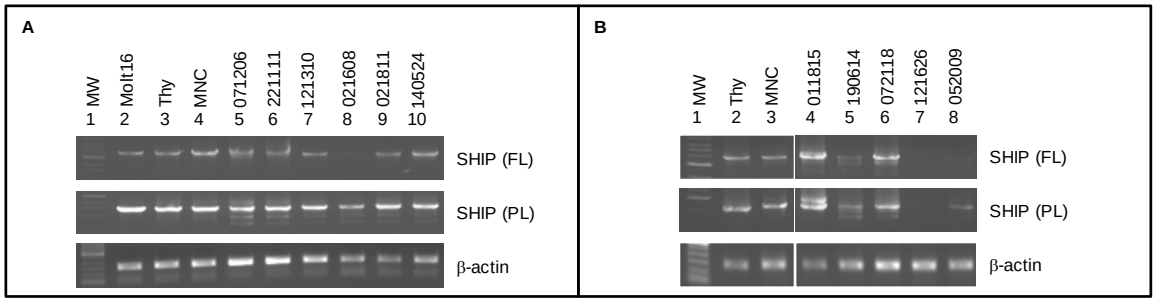

Figure 5: Expression of *SHIP1* transcript in primary ALL. Full-length and partial-length *SHIP1* transcript was assessed in (A) T-ALL and (B) B-ALL primary samples.

3.6 Expression of SHIP1 Protein in Primary ALL

To evaluate if levels of transcript expression in ALL are reflected on the protein level, several samples representing different levels of *SHIP1* transcript expression were tested via Western blot. In 15 of 20 T-ALL primaries, SHIP1 protein is not found using a C-terminal SHIP1 antibody (Fig. 6A; data not shown). The remaining samples have SHIP1 protein levels much lower than those seen in normal cells and some cell lines. To address the possibility that some of the primaries may be expressing SHIP1 protein not detected by the C-terminal antibody, an N-terminal antibody was used as well. Results confirm that T-ALL primaries do not harbor full length SHIP1 protein, but instead, have lower molecular weight forms of SHIP1. Notably, some samples remain absent for SHIP1 protein.

In B-ALL samples, utilizing the C-terminal antibody reveals that full length SHIP1 protein is not found in most samples (12 of 18), while some (2 of 18) harbor high levels that are similar to those found in B-cell leukemia lines (Fig. 6B; data not shown). The remaining samples (4 of 15) have low levels with the exception of one sample, which instead expresses a SHIP1 protein of lower molecular weight. However, probing 8 of the 18 samples with the N-terminal antibody shows that all 8 harbored some form of SHIP1 with the expected 145 kDa species being a minority species. Given the overall poor expression of wild-type SHIP1 protein in primary ALL and varying levels of *SHIP1* transcript, there appears to be no clear correlation between protein and transcript levels.

Similar to the multiple bands seen on the transcript level of *SHIP1* in some primaries, smaller species of SHIP1 protein in both B- and T-ALL samples are also

observed. The smaller sized amplicons of *SHIP1* may be connected to the multiple bands on the protein level, suggesting that these alternative transcripts form stable proteins.

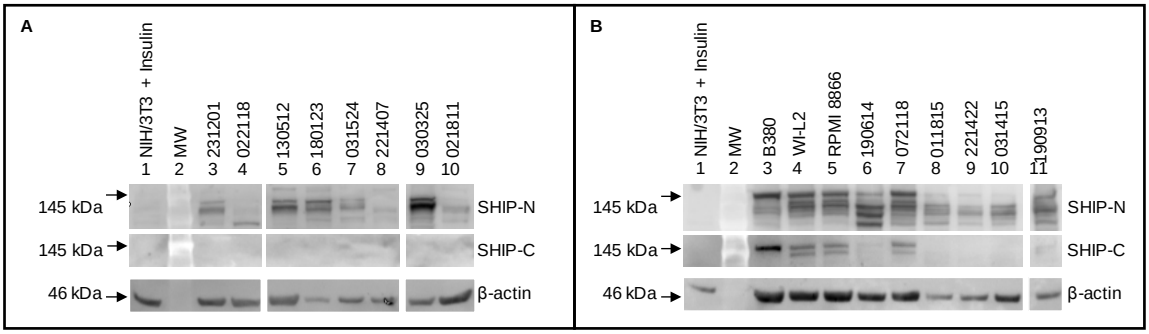

Figure 6: Expression of SHIP1 protein in ALL. SHIP1 protein, utilizing both an N- and C-terminal antibody, was assessed in (A) T-ALL and (B) B-ALL primaries. In panel B, lanes 3-5 represent cell lines and lanes 6-11 are primary samples.

3.7 Mutational Analysis of *SHIP1* in Primary Samples

In light of the lack of wild-type SHIP1 protein in primary ALLs expressing the gene, mutations on the transcript level were considered. To assess this, sequencing was conducted on B- and T-ALL primaries expressing low and high levels of *SHIP1*. Results reveal that all 20 of the ALL samples are negative for the codon 1133 missense mutation found in the Jurkat cell line. However, upon cloning several of the PCR products, various deletions and/or insertions are observed at exon junctions. A 47 bp deletion in exon 12, the same mutation seen in Jurkat, and a 624 bp insertion of intron 14 between exons 14 and 15 are found. Additionally, a partial insertion of intron 10 between exons 10 and 11, an in-frame deletion of exon 8, a deletion of exon 25, a deletion of exon 26, and a deletion of both exons 25 and 26 are observed. Despite these many possible alterations found, some clones maintain full-length *SHIP1*. Figure 7 summarizes and illustrates the variants.

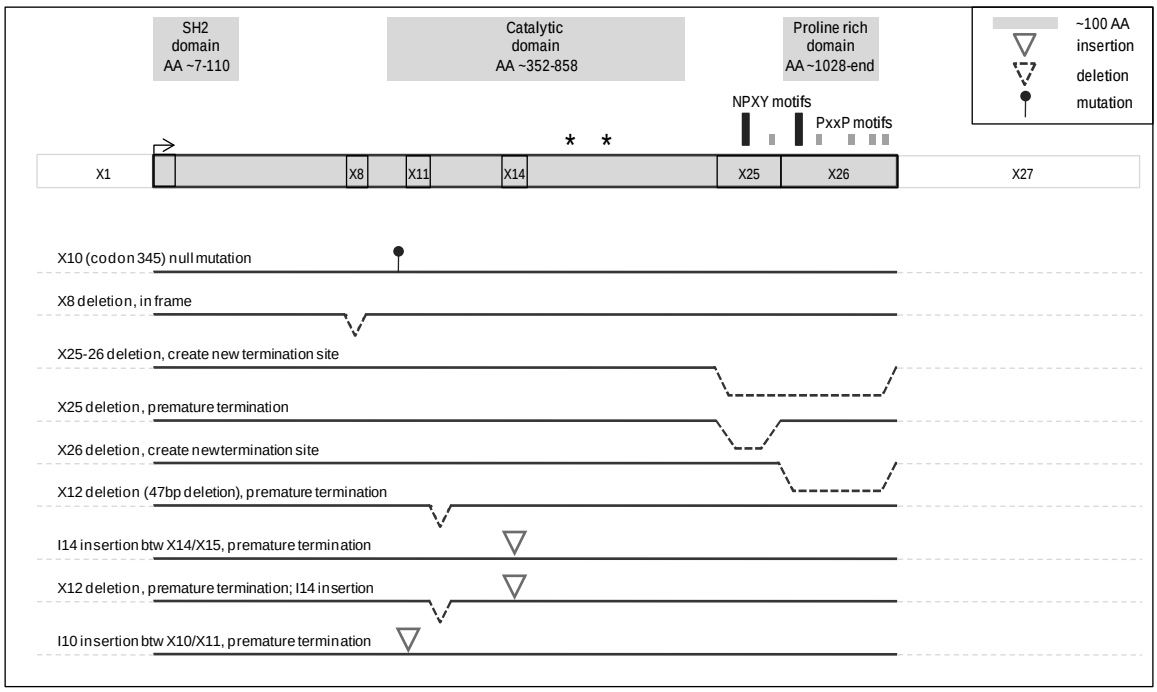

Figure 7: Map of various alterations found in ALL. Illustrations of *SHIP1* exons, introns, and splice sites are based on the 145,886 bp *SHIP1* gene comprised of 27 exons and 1188 residues created by blasting the 5274 bp *SHIP1* cDNA (GI:64085176) against the chromosome 2 genomic sequence (GI:157724517). Both B- and T-ALL express alternative spliced forms of *SHIP1*, most of which harbor premature terminations leading to the translation of truncated protein.
*, highly conserved region of homology with all 5'-phosphatases;
AA, amino acids

3.8 Alternative Splicing of *SHIP1* in ALL

SHIP1 protein is detected as full-length in both B- and T-ALL cell lines, but in primaries, various truncated forms are more abundant as full-length make up a small, if any, portion of the SHIP1 detected. On a molecular level, the ALL samples harbor several variants of *SHIP1* that possess deletions, insertions, and other mutations. These alterations occur directly at splice sites, suggesting alternative spicing as the source of these variations. Most of these mutations are observed to be nonsense or result in a frameshift that lead to premature termination and could give rise to the observed truncated forms of SHIP1 protein. The variants are detectable on the protein level, implying that they form stable proteins. These smaller sized SHIP1 proteins lack a wild-type C-terminal and exist in multiple forms seen on both the transcript and protein level.

Although a few normal cells exhibit mutations similar to those found in primary samples, the frequency of these mutations is much higher in primaries. This is reflected on the protein level: primaries exhibit a greater level of potentially nonfunctional SHIP1 than full-length protein, which is predominately expressed in normals. Although full-length *SHIP1* is detectable at low levels in primaries, the amount cannot be quantified because efficiency of the cloning system used to study these *SHIP1* variants is dependent, in part, on the size of the fragment or insert. Overall, the predominance of truncated SHIP1 protein in most samples suggests that SHIP1 is largely inactivated in ALL.

Chapter 4: PTEN and Akt in Acute Leukemia

4.1 Background: Connecting SHIP1 with PTEN and Akt

Because both SHIP1 and PTEN act to negatively regulate Akt, inactivation of either of these phosphatases would affect Akt activation. Having already observed inactivating mutations in SHIP1 in ALL, the protein expression profile for PTEN along with Akt was determined via Western blot to study how these proteins correspond with one another.

4.2 Akt Activity and PTEN Inactivation in ALL

Both B- and T-ALL cell lines exhibit similar profiles for Akt expression: all express Akt with half of each of the samples harboring detectable levels of the phosphorylated (activated) form (Fig. 8; Fig. 9, lanes 3-5; Table 2). However, there is a notable difference between primary samples. Of the 9 T-ALL samples that express Akt, 6 are phosphorylated, whereas only 1 of 6 B-ALL samples harbor detectable levels of Akt activation (Fig. 9; Table 3; data not shown).

PTEN is expressed in all (4 of 4) B-ALL cell lines, but is only found in several (2 of 6) T-ALL lines (Fig. 8; Fig. 9, lanes 3-5; Table 2). In general, the T-ALL cell lines lacking PTEN expression are accompanied by activated Akt (Fig. 8; data not shown). In primaries, PTEN is present in most B- (14 of 18) and T-ALL (17 of 21) samples (Fig. 8; Table 3; data not shown). However, in primaries, some PTEN-expressing cell lines maintain Akt activation. A recent report suggests PTEN phosphorylation as a source of inactivation, which could contribute to Akt activation [Silva et al., 2008]. Utilizing a

phosphorylated PTEN (p-PTEN) antibody reveals that all cell lines and primaries expressing PTEN harbor the phosphorylated form.

Normal thymocytes also express phosphorylated PTEN, and thus, it remains to be determined the extent of PTEN phosphorylation required for Akt activation. In contrast to cell lines, normal thymocytes do not have detectable levels of Akt activation perhaps because the PTEN phosphorylation levels detected represent normal cellular levels, which are not enough to trigger aberrant Akt phosphorylation (Fig. 8, lane 4).

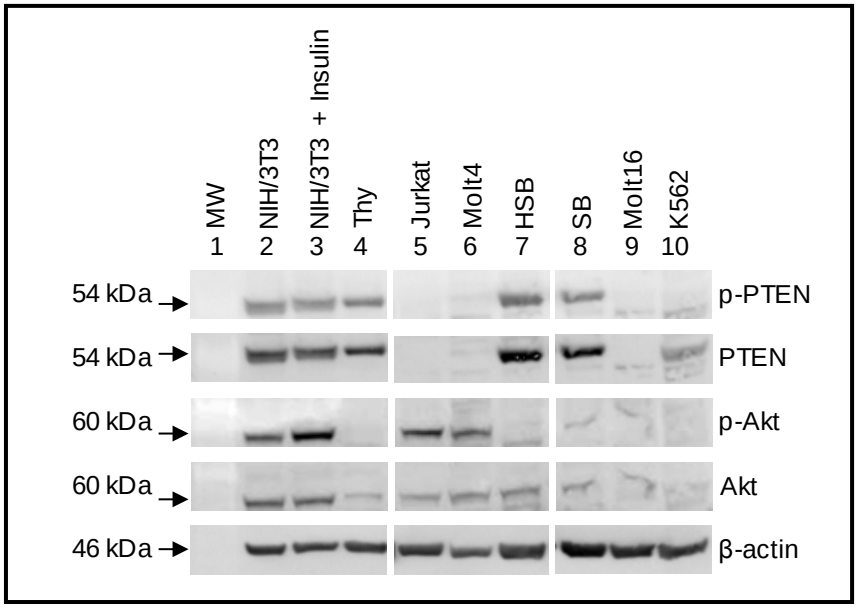

Figure 8: PTEN and Akt levels in cell lines. PTEN inactivation (p-PTEN) and Akt activation (p-Akt) were evaluated. NIH/3T3 cells were treated with insulin and used as a positive control for p-Akt.

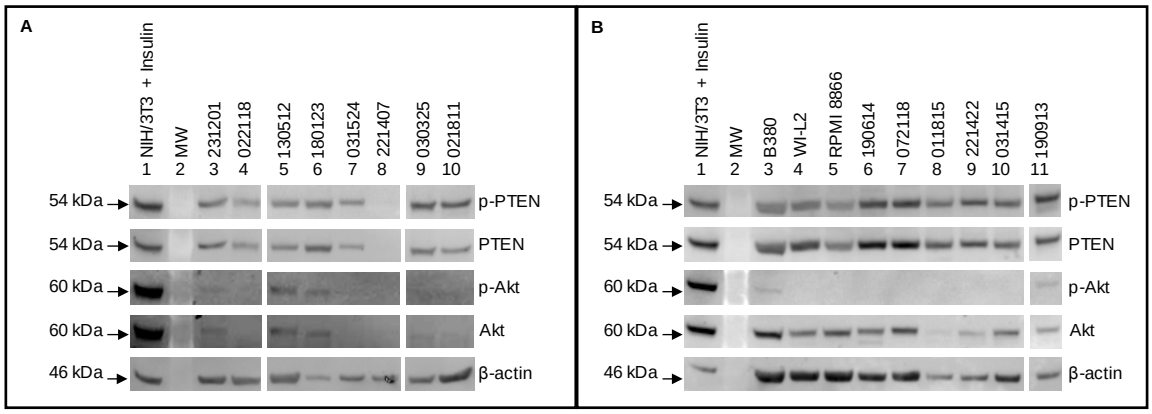

Figure 9: PTEN and Akt levels in primary ALL. Phosphorylated PTEN and Akt were qualititated in (A) T-ALL and (B) B-ALL samples.

4.3 PTEN Inactivation and SHIP1 in Akt Signaling

With the observation of PTEN inactivation and absence of wild-type SHIP1 protein in ALL, the Akt pathway becomes more significant. A recent report shows PTEN posttranslational inactivation leading to constitutive activation of Akt in ALL [Silva et al., 2008], but fails to assess the status of SHIP1. Because SHIP1 acts as a phosphatase similar to PTEN, the inactivation of SHIP1 could function autonomously or concurrently with PTEN inactivation to deregulate Akt signaling. In studies of adult T-cell leukemia/lymphoma (ATLL), both PTEN and SHIP1 are found to be downregulated and correlate with the general expression of phosphorylated Akt [Fukuda et al., 2005].

The striking difference in Akt activation between B- and T-ALL primaries highlighted in the previous section is speculated to be due to the fact that more B-cell than T-cell samples harbor some form of SHIP1 (Table 4). Molecularly, although B-ALL cells lack proper PTEN function due to inactivation, the cells have SHIP1 as a backup mechanism, allowing them to circumvent the consequences of PTEN deregulation and prevent Akt activation. In contrast, void of PTEN function and having limited, if any, SHIP1 activity because of generally lower levels of SHIP1 protein, T-ALL cells would not be able to reduce Akt activity. In terms of prognosis, the greater levels of SHIP1in B-cells may also be linked to the better outcome of B-ALL compared to T-ALL patients.

Tables 2 and 3 highlight the expression profiles of leukemia cell lines and primary samples, respectively. In comparing differences between the two tables, it is notable that primary ALL express truncated forms of SHIP1 as well as inactivated PTEN, whereas cell lines only harbor inactivated PTEN. The presence of PTEN inactivation without Akt activation in thymocytes and some cell lines may be due to an intact SHIP1, which is not

commonly found in primaries. Also, these discrepancies in protein expression between cell lines and primaries caution that cell lines may not accurately mirror ALL pathology in primaries.

Table 2: Expression profile of normals and cancer cell lines
-, -/+, +; undetectable, weakly detectable, and moderate to high gene or protein expression, respectively;
* a second band of lower molecular weight was detected in these samples;
N/D, not determined

Cell line	Cell type	*PI3K* mutations		*SHIP1*	SHIP1 protein		PTEN	p-PTEN	Akt	p-Akt
		PI3K exon 9	*PI3K* exon 20	mRNA	N	C	protein	protein	protein	protein
CEM	T-ALL	WT	WT	-/+*	N/D	-/+*	-	-	+	-
HSB	T-ALL	WT	WT	+	+*	+	+	+	+	-
Jurkat	T-ALL	WT	WT	+	-	-	-	-	+	+
Molt4	T-ALL	WT	WT	+	+	+	-	-	+	+
Molt16	T-ALL	WT	WT	+	+*	+*	-	-	+	+
Supt3	T-ALL	N/D	N/D	+	+	+	+	+	+	-
B380	B-precursor ALL	N/D	N/D	N/D	+	+	+	+	+	+
SB	B-precursor ALL	WT	WT	+	+	+	+	+	+	+
RPMI 8866	B-precursor ALL	N/D	N/D	+	+*	+	+	+	+	-
WI-L2	B-precursor ALL	N/D	N/D	+	+*	+	+	+	+	-
Ramos	Burkitt's Lymphoma	WT	WT	+	N/D	+	+	N/D	+	N/D
K562	CML	WT	WT	+	-	-	+	-	+	-
HL60	AML	WT	WT	-/+	-	+*	+	+	+	-
MNC	Normal PBLs	WT	WT	+	+	+	+	N/D	+	N/D
Thy	Normal Thymocytes	WT	WT	+	+*	+	+	+	+	-

Table 3: Expression profile of some primary ALL samples

Primary	Cell type	*SHIP1* mRNA	SHIP1 protein N	SHIP1 protein C	PTEN protein	p-PTEN protein	Akt protein	p-Akt protein
021811	T-ALL	+	-	-	+	+	-	-
022004	T-ALL	+	-	-	-	-	+	+
022118	T-ALL	+	-	-	+	+	-	-
030325	T-ALL	+	+*	-/+	+	+	-/+	-
031524	T-ALL	-/+	+*	-	+	+	-	-
031907	T-ALL	+	-	-	-/+	-/+	-	-
121310	T-ALL	+	-	-/+	+	N/D	-/+	-
121516	T-ALL	N/D	+*	+	+	+	+	-/+
130512	T-ALL	-/+	+*	-	+	+	-/+	-/+
180123	T-ALL	+	+*	-	+	+	-/+	-/+
191104	T-ALL	N/D	+*	+	+	+	+	-/+
221407	T-ALL	+	-	-	-	-	-/+	-
231201	T-ALL	-/+	+*	-	+	+	+	-/+
011815	B-precursor ALL	+	+*	-	+	+	-/+	-
031415	B-precursor ALL	-	+*	-	+	+	+	-
072118	B-precursor ALL	+	+*	+*	+	+	+	-
190614	B-precursor ALL	+	+*	-/+*	+	+	+	-
190913	B-precursor ALL	N/D	+*	-/+	+	+	+	+
221422	B-precursor ALL	+	+*	-	+	+	-/+	-

Table 4: Percentage of ALL primaries with expression. Values reflect samples that express detectable levels of the indicated protein on a Western blot. Percentages for proteins in their native form are relative to the total number of samples of the specified cell type. Percentages for phosphorylated protein are relative to the number of samples expressing the native form.

Cell type	SHIP1 protein		PTEN	p-PTEN	p-Akt
	N	C	protein	protein	protein
T-ALL	50%	26%	86%	100%	67%
B-ALL	100%	33%	82%	100%	17%

Chapter 5: Analysis of SHIP1 Isoforms

5.1 Background: Functions of SHIP1 Variants

Because of the vast forms of alternatively spliced SHIP1 observed in ALL and their frequent premature terminations, it is important to evaluate the functional integrity of these truncated species. Although predictions can be made about SHIP1 activity by studying how different motifs and domains are affected on the transcript level, it is difficult to apply the same predictions on the protein level as factors such as protein folding complicate these conclusions. For example, phosphatase activity should only be affected if the catalytic domain is mutated. However, a study of the phosphatase activity of various SHIP1 deletion and truncation mutation models demonstrate that subtle changes independent of the catalytic domain affect SHIP1's ability to convert PtdIns(3,4,5)P_3, suggesting that the phosphatase activity required proper function of regions outside of the enzymatic region [Aman et al., 2000]. Thus, it is possible that most, if not all, of the truncated isoforms observed in ALL have compromised abilities to hydrolyze lipids even if they are not mutated in the central region.

In addition, SHIP1 harbors other domains that interact with a myriad of proteins, which may contribute to functions less well-defined. Although catalytic activity is a major role of SHIP1, the ability of SHIP1 to act through its other domains can contribute to functions just as important, and thus, should not be overlooked.

5.2 Protein Stability of Alternatively Spliced SHIP1 Species

Before studying the function of SHIP1 isoforms, protein stability was tested. Expression constructs were created for wild-type (WT) SHIP1, SHIP1 harboring a 47 bp deletion located in exon 12, and SHIP1 lacking exons 25 and 26. The constructs were transfected into HEK293T cells, which are non-hematopoietic cells. Both alternatively spliced forms of *SHIP1* form stable proteins: *SHIP1* harboring the 47 bp deletion generates a truncated SHIP1 species about 45 kDa due to premature termination, and *SHIP1* lacking exons 25 and 26 translates to a protein of approximately 110 kDa, a smaller species of SHIP1 often seen primary ALL (Fig. 10).

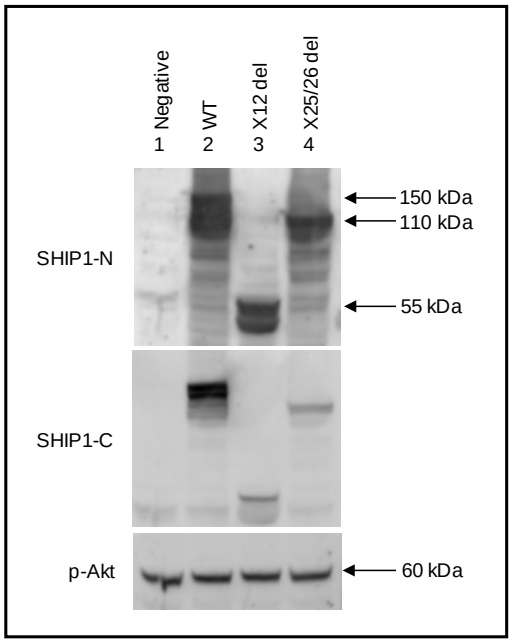

Figure 10: Protein stability of SHIP1 isoforms. SHIP1 constructs were transfected into HEK293T cells. Probing with an N-terminal-specific SHIP1 antibody show that all formed stable proteins. As expected, using a C-terminal antibody only detects SHIP1 in the WT construct. Faint bands seen in the other two constructs is likely due to incomplete stripping of the blot. Phosphorylated Akt is also assessed to determine effects of the constructs on Akt activity.

5.3 Growth Effects of SHIP1 Constructs

Cell counts were also conducted of the SHIP1-transfected cells to see if the constructs affect cell proliferation. Interestingly, those transfected with some form of SHIP1 exhibit a 40% average growth decrease, with WT SHIP1 having the greatest effect at nearly 50% (Fig. 11). Although this may illustrate the importance of SHIP1 in cellular signaling, it should be noted that HEK293T cells do not normally express SHIP1 and thus, should not have the required machinery to allow for normal SHIP1 function. SHIP1 phosphatase activity relies upon its recruitment to the membrane by receptor signaling of B- and T-cells (see section 1.5.2). Without this, the enzymatic abilities of SHIP1 are compromised, and it cannot act to decrease Akt activity through the regulation of PtdIns(3,4,5)P_3 levels. In support of this, there is no visible change in p-Akt levels of SHIP1-transfected cells compared to the control (Fig. 10).

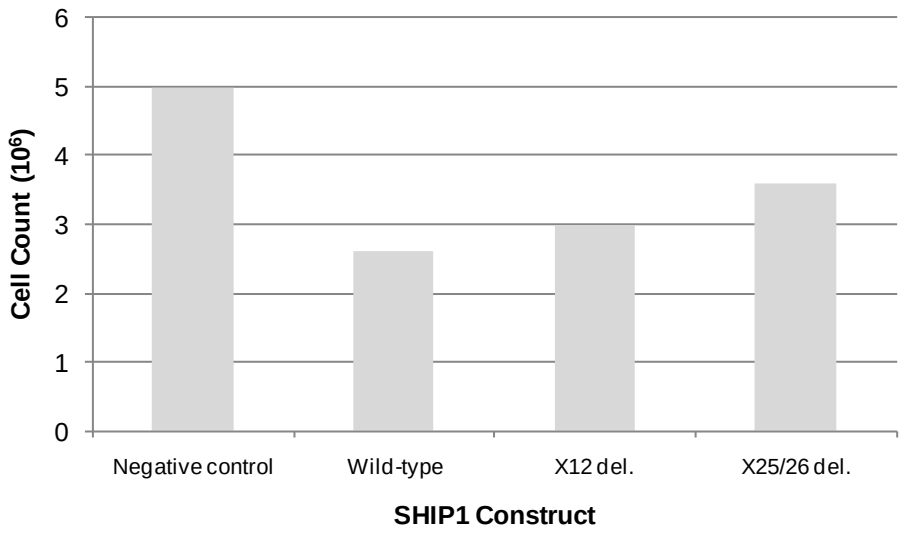

Figure 11: Cell counts of SHIP1 construct transfections. Cell viability of HEK293T cells transfected with SHIP1 constructs was determined using trypan blue exclusion test. All transfections yield an approximate viability of 90%.

5.4 Future Assessments

Although SHIP1 does not have the necessary machinery for proper phosphatase function in HEK293T cells, its other structural domains should still be able to facilitate interactions. Although the constructs vary in size, all constructs harbor at least the SH2 domain. Because all constructs exhibit some inhibition on cell growth, the SH2 domain of SHIP1 may be interacting with factors responsible for this effect. It would thus be informative to create a construct lacking the SH2 domain to see if its effects on cell growth are maintained. Also, because HEK293T cells do not naturally express SHIP1, the true function of these constructs cannot be understood unless a hematopoietic model such as Jurkat is used.

5.4.1 SHIP1 Isoforms and Oncogenic Activity

Many of the mutations found in the *SHIP1* variants include insertions and deletions located in the catalytic domain that may affect phosphatase activity. Thus, this would hinder proper SHIP1 phosphatase function and possibly lead to a subsequent increase in Akt activation. In order to assess the status of phosphastase activity in SHIP1, phosphatidylinositol phosphate levels can be measured. Specifically, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 amounts can be compared to determine if SHIP1 is properly converting the PI3K product to a biphosphate. As mentioned previously, regions outside of the catalytic domain may affect SHIP1 abilities to hydrolyze PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 [Aman et al., 2000]. Thus, SHIP1 variants harboring alterations independent of the enzymatic region also have the potential to influence lipid levels. Furthermore, Akt activity should

be measured to determine the degree in which the conversion of phospholipids is affecting Akt activation.

The C-terminal region, which includes two NPXY motifs and a proline rich domain, is primarily comprised of exons 25 and 26 (Fig. 7). Several *SHIP1* variants harbor significant deletions in this region, either losing one or both exons. Also, some variants contain mutations upstream of the C-terminal that result in premature terminations, preventing the translation of SHIP1 with a proper C-terminal. As described in section 1.6, SHIP1 utilizes its SH2 domain and NPXY motifs to downregulate Ras activity (Fig. 2). Thus, SHIP1 species missing either of these regions could have elevated Ras activity. It would be informative to test how the different alternatively spliced forms of SHIP1 compared to wild-type affect activation of Ras.

Results of the different Akt and Ras activity levels in the isoforms can be linked to various cell growth, viability, and apoptosis assays to see how cell proliferation is affected by alterations in the signaling pathways.

Chapter 6: Combination Therapeutics in Treatment of Cancer

6.1 Background: Current Modalities in the Treatment of ALL

Treatment for ALL typically consists of three phases, one of which is the remission-induction phase. The goal of this phase is to eliminate leukemic cells and reconstitute normal hematopoietic function. To accomplish this, a regimen of three drugs is employed: a glucocorticoid (prednisone or dexamethasone), vincristine, and an anthracycline (doxorubicin or daunorubicin) [Pui et al., 2008]. A variety of other drugs are also incorporated in treatment depending on the prognosis of the patient. However, even with a large repertoire of drugs, more and more children confer resistance to treatments and despite the high success rates, fall to relapse.

6.1.1 Combining Pathway Inhibitors with Chemotherapeutic Agents

Various reports demonstrate that drug resistance is enhanced by oncogenic signaling, suggesting that cross-talk between oncogenic signaling pathways and chemoresistance mechanisms control cellular responses to drugs. The PI3K inhibitor LY294002, which interacts with the ATP-binding site of PI3K, is commonly used to study the PI3K/Akt pathway and is shown to have antiproliferative and proapoptotic effects both *in vitro* and *in vivo* [Vlahos et al., 1994; Cheng et al., 2005]. Several reports show LY294002 sensitizing glioblastoma and colon carcinoma to doxorubicin [Opel et al., 2008; Abdul-Ghani et al., 2006]. Thus, in regards to the therapy of ALL, use of signaling inhibitors in conjunction with current drugs can sensitize cells and subsequently enhance the efficacy of chemotherapeutic agents.

The anthracycline agent doxorubicin is used to treat a wide range of malignancies. Although its exact mechanism of action is unknown, it is proposed that doxorubicin induces growth arrest and cell death by inhibiting DNA-topoisomerase II [Gewirtz, 1999]. Studies show that pediatric ALL patients who are given doxorubicin along with other agents have better event-free survival [Silverman et al., 2000]. However, reports suggest doxorubicin therapy has long-term effects that affect myocardial growth in a dose-dependent manner, a side effect believed to be caused by the generation of free radicals at high doses of the drug [Nysom et al., 1998; Gewirtz, 1999]. Hence, a way to minimize these effects would be to find alternative methods of administration that allow for a smaller dose of doxorubicin without compromising its efficacy.

Given the various studies that show improved drug sensitivities of chemotherapeutic agents after PI3K inhibition and the effectiveness of doxorubicin in ALL, the combination of LY294002 with the anthracycline may interact synergistically. This would allow for a lower dosage of doxorubicin while maintaining its efficacy and increasing its therapeutic index.

6.1.2 Exploiting the Expression of SHIP1 for Therapeutic Purposes

With deregulation of both PTEN and SHIP1 in ALL, the Akt pathway becomes an important target for therapeutics. Most ALL demonstrate the lack of wild-type SHIP1, and instead express truncated isoforms. Many of these variants harbor alterations that lead to premature terminations, which may impair phosphatase activity and other functions of SHIP1, leading to the aggravation of important cellular signals involved in pathways such as Akt and Ras. Success in therapeutic targeting of these oncogenes is

limited, perhaps due to the lack of consideration in how these pathways are deregulated. The understanding that both PTEN and SHIP1 are inactivated in ALL may provide new insight into how pathway inhibitors should be administered to make ALL more susceptible to drugs.

6.2 Effects of LY294002 and Doxorubicin in T-ALL Cell Lines

To test how SHIP1 expression affects the sensitivity of cells to drugs, T-ALL cell lines Molt16 and Jurkat were used as models. Molt16 cells express functional SHIP1, but lack PTEN, while Jurkat cells are absent for both proteins. In correlation with their expression profiles, Jurkat cells express higher levels of detectable p-Akt compared to Molt16 (Fig. 4). While it was not known the resistance of these cell lines to drugs, the difference in SHIP1 expression between the two cell lines would allow for the exploration of how the presence of SHIP1 could affect the efficacy of drugs. Peripheral blood MNCs are included in this study to assess the cytoxicity of the drugs in normal cells.

Utilizing an XTT assay to measure cell proliferation and viability, cells were subjected to various combinations of LY294002 and doxorubicin. Normal cells are generally insensitive to doxorubicin with no apparent effect on cell growth and only a limited effect is observed from LY294002 treatment (Fig. 12). Combining the two drugs did not show any greater inhibition in growth. The general lack of effect in normal cells suggests that the dosages of the drugs used are not very cytotoxic. However, it should be noted that the normal cells are subjected to slightly lower dosages of doxorubicin than those given to cell lines.

In Molt16 cells, both drugs alone are effective, each decreasing growth by about 50% at the highest dosages (Fig. 13A). Despite the effectiveness of the drugs alone, combining increased concentrations of the drugs do not achieve an effect much greater than the drugs alone. This “leveling off” effect suggests that the two agents could be interacting antagonistically. In Jurkat cells, both drugs alone exhibit similar effects seen in Molt16 cells (Fig. 13B). However, combining the drugs elicited a greater effect, achieving approximately 80% decrease in cell growth. Increasing concentrations of the agents correlated with more cell death.

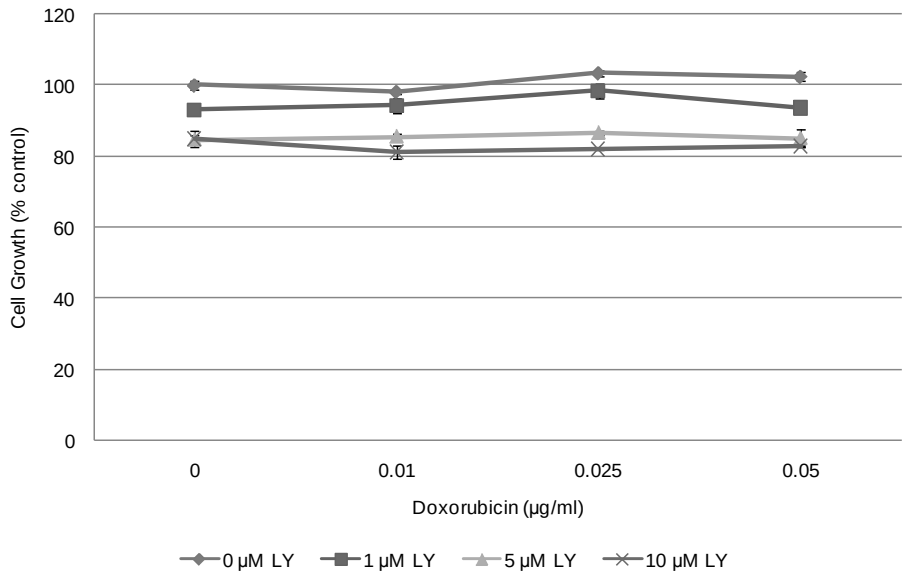

Figure 12: The effect of LY294002 and doxorubicin in peripheral blood MNCs. Cells were cultured with the indicated suboptimal concentrations of LY294002 and doxorubicin. Cell proliferation and viability were assessed after 72 h using an XTT assay. Values reflect cell growth relative to percent of control.

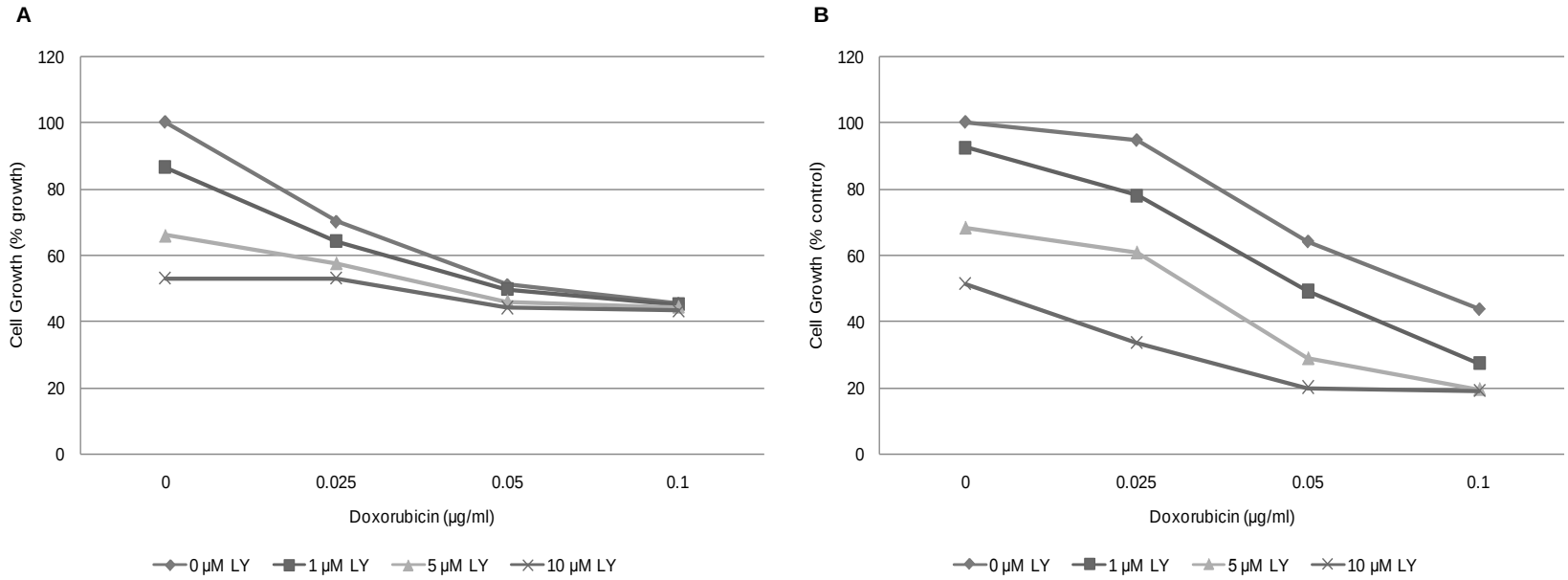

Figure 13: The effect of LY294002 and doxorubicin in T-ALL. (A) Molt16 and (B) Jurkat cells were cultured with the indicated suboptimal concentrations of LY294002 and doxorubicin and assayed in similar conditions as normal cells.

6.2.1 Drug Interaction of LY294002 and Doxorubicin

To characterize the drug interaction between the two agents, two different models were utilized: the effect addition model and the Bliss independence model. In general, these models determine synergism and antagonism by comparing the actual effects of drug combinations to predicted effects based on the drugs alone (see section 2.7). In agreement with the observed effects in Molt16 cells from Figure 13A, both the effect addition model and effect multiplication model predict an antagonistic effect of the drugs at all concentrations and combinations (Fig. 14A; Fig. 15A). In Jurkat cells, the effect multiplication model predicts synergism at all concentrations and combinations of the agents (Fig. 15B). Similarly, the effect addition model suggests that the two agents act in a synergistic manner, except at high doses of both drugs (Fig. 14B).

A

Fraction of Cells Dead

LY294002 (μM) \ Doxorubicin (μg/ml)	0	0.025	0.05	0.1
0	0.000	0.300	0.489	0.545
1	0.135	0.357^{-}	0.505^{-}	0.548^{-}
5	0.340	0.424^{-}	0.539^{-}	0.557^{-}
10	0.471	0.472^{-}	0.558^{-}	0.568^{-}

Effect Addition Model

LY294002 (μM) \ Doxorubicin (μg/ml)	0	0.025	0.05	0.1
0	-	-	-	-
1	-	0.434	0.624	0.680
5	-	0.640	0.830	0.886
10	-	0.771	0.961	1.017

B

Fraction of Cells Dead

LY294002 (μM) \ Doxorubicin (μg/ml)	0	0.025	0.05	0.1
0	0.000	0.055	0.359	0.562
1	0.075	0.222^{+}	0.509^{+}	0.727^{+}
5	0.319	0.393^{+}	0.712^{+}	0.805^{-}
10	0.487	0.664^{+}	0.799^{-}	0.809^{-}

Effect Addition Model

LY294002 (μM) \ Doxorubicin (μg/ml)	0	0.025	0.05	0.1
0	-	-	-	-
1	-	0.130	0.434	0.638
5	-	0.373	0.677	0.881
10	-	0.542	0.846	1.049

Figure 14: Drug interactions predicted through the effect addition model. The interaction between LY294002 and doxorubicin was determined in both (A) Molt16 and (B) Jurkat cells. Actual values greater than the predicted value suggest synergism ($^{+}$), and values lesser suggest antagonism ($^{-}$).

A

Fraction of Cells Alive

LY294002 (μM) \ Doxorubicin (μg/ml)	0	0.025	0.05	0.1
0	1.000	0.700	0.511	0.455
1	0.865	0.643^{-}	0.495^{-}	0.452^{-}
5	0.660	0.576^{-}	0.461^{-}	0.443^{-}
10	0.529	0.528^{-}	0.442^{-}	0.432^{-}

Effect Multiplication Model

LY294002 (μM) \ Doxorubicin (μg/ml)	0	0.025	0.05	0.1
0	-	-	-	-
1	-	0.606	0.442	0.393
5	-	0.462	0.337	0.300
10	-	0.370	0.270	0.240

B

Fraction of Cells Alive

LY294002 (μM) \ Doxorubicin (μg/ml)	0	0.025	0.05	0.1
0	1.000	0.945	0.641	0.438
1	0.925	0.778^{+}	0.491^{+}	0.273^{+}
5	0.681	0.607^{+}	0.288^{+}	0.195^{+}
10	0.513	0.336^{+}	0.201^{+}	0.191^{+}

Effect Multiplication Model

LY294002 (μM) \ Doxorubicin (μg/ml)	0	0.025	0.05	0.1
0	-	-	-	-
1	-	0.874	0.593	0.405
5	-	0.644	0.437	0.298
10	-	0.485	0.329	0.225

Figure 15: Drug interactions predicted through the effect multiplication model. The interaction between LY294002 and doxorubicin was determined in both (A) Molt16 and (B) Jurkat cells. Actual values lesser than the predicted value suggest synergism ($^{+}$), and values greater suggest antagonism ($^{-}$).

6.2.2 Significance of SHIP1 in Drug Response

The noticeable differences in drug sensitivity between Molt16 and Jurkat suggest that the presence of SHIP1 may play a role in determining the response of cells to agents. As mentioned earlier, studies show that cellular responses to drugs are related to oncogenic signaling, suggesting a link between oncogenic pathways and chemosensitivity mechanisms. As such, the loss of SHIP1 in Jurkat cells could lead to stimulation of the Akt pathway, and in turn, elicit change in drug sensitivity allowing for a greater response than SHIP1-expressing Molt16 cells.

However, because Molt16 and Jurkat are two different cell lines, variables such as the expression of proteins other than SHIP1 must be considered. This complicates the relation between SHIP1 and drug sensitivity as other factors may influence cellular responses as well. Thus, to determine if SHIP1 truly affects chemosensitivity, Jurkat cells untransfected and transfected with SHIP1 could be compared. It would also be informative to see if the various SHIP1 isoforms confer different responses to drug treatment. Perhaps cells harboring specific truncated forms of SHIP1 are more susceptible to drug treatments, while others are more resistant.

Another caveat in these experiments is that although cell lines are convenient models to study pathology, the discrepancies observed in PTEN and SHIP1 expression between cell lines and primaries warrant the need to assess these sensitivity studies in primary ALL. Additionally, the predominance of SHIP1 isoforms in primaries would give a more realistic representation of how SHIP1 affects drug response of cells.

6.3 Alternative Pathway Inhibitors

Aside from SHIP1's effects in the Akt pathway, SHIP1 may also function through the Ras pathway. Thus, deregulation of SHIP1 would lead to abnormal activated levels of Ras, justifying the use of a Ras inhibitor for potential treatment purposes. As briefly mentioned before, increased Ras activation is found in T-ALL, but not B-ALL [von Lintig et al., 2000]. The increased frequency of activation in T-ALL as compared to B-ALL may reflect the greater expression of some form of SHIP1 in B- than in T-ALL. Thus, although SHIP1 exists primarily as truncated forms in ALL, the greater amount in B-cells may suffice for proper inhibition of Ras activity, supporting its lower frequency of activation compared to T-cells. Additionally, as suggested by the SHIP1 transfections of HEK293T cells (section 5.1; Fig. 9), the decrease in cell growth may be caused by the effects of SHIP1 through the Ras pathway. The lack of machinery in HEK293T cells required for proper SHIP1 phosphatase activity suggests that this effect on cell growth acts through an Akt-independent pathway, such as Ras. Similar to its effects on the Akt pathway, deregulation of SHIP1 can lead to Ras activation, substantiating the study of Ras inhibitors in the treatment of ALL.

Chapter 7: Concluding Remarks

7.1 Importance of SHIP1 in the Akt Pathway

The Akt pathway, which is associated with cell survival, is often deregulated in ALL. In most cancers, Akt deregulation is caused by activating and inactivating mutations of PI3K and PTEN, respectively. However, in ALL, these mutations are infrequent, and thus, the cause for abnormal Akt activity remains a question. SHIP1, which acts similarly to PTEN and is not commonly studied in ALL, is a possible answer.

In leukemia cell lines, SHIP1 seems largely unaffected with the exception of Jurkat and K562, which fail to express protein despite the presence of transcript. Mutational analysis of Jurkat reveals significant alterations that explain the lack of protein. In primary ALL, mutations are found at a much larger scale. Most samples do not have wild-type SHIP1, but instead, express truncated forms that arise from alternative splicing. A comparison between ALL shows a greater level of these truncated species in B- than T-ALL. Because most these variants harbor deletions of critical regions of SHIP1, they are largely considered nonfunctional.

With a new understanding of SHIP1 status in ALL, activated Akt was studied. In primaries, a notable difference in Akt activity levels is seen between B- and T-ALL: two-thirds of T-ALL samples are detectable for p-Akt, whereas it is less than one-fifth in B-ALL (Table 4). An analysis of PTEN expression shows absence of 15% of primary ALL, but those that express PTEN harbor the phosphorylated (inactivated) form. Thus, with PTEN inactivated, the difference of activated Akt levels reflect and may be accounted for by the contrasting levels of SHIP1 expression between B- and T-ALL. With inactivation

of PTEN and infrequent PI3K mutations, the extensive mutations observed in SHIP1 make SHIP1 inactivation a prime candidate for Akt deregulation and could contribute to the pathogenesis of ALL.

7.2 Possible SHIP1 Implications in Therapeutics

Despite the success in the treatment of ALL, patients continue to relapse. This, along with the secondary diseases caused by toxicity of current modalities, calls for the necessity of new diagnostic and therapeutic guidelines. Studies link specific genetic ALL abnormalities to prognosis, illustrating the importance of risk assessment for optimization of therapeutic regimens [Mancini et al., 2005]. For example, in B-ALL, certain fusion genes caused by chromosomal translocation categorize the disease as either high or low risk. Genetic aberrations such as these provide crucial prognostic information, demonstrating the connection between genetics and outcome. Thus, improvement upon current treatment protocols requires a better understanding of the molecular biology of ALL.

In ALL, several differences are seen between primary B- and T-ALL: B-ALL generally have higher expression levels of SHIP1 than T-ALL; more T-ALL tend to have detectable activated Akt levels than B-ALL; and studies show T-ALL have elevated levels of Ras, whereas B-ALL do not [von Lintig et al., 2000]. Connecting these together suggest that the greater overall expression of SHIP1 in B-cells may be responsible for the lower number of samples detectable for p-Akt and also for the lack of abnormal Ras activity. In terms of prognosis, this connection fits well with the fact that T-ALL patients

historically have a poorer outcome and are more difficult to treat than those with B-ALL. Thus, the expression of SHIP1 may potentially contribute to risk assessment of ALL.

Preliminary drug sensitivity studies in Jurkat and Molt16 cells suggest that the absence of SHIP1 causes cells to have a greater response to drugs. As demonstrated by the application of LY294002 and doxorubicin, possible synergy of drugs can be achieved in these cells lacking SHIP1. Because most patients probably lack SHIP1, as suggested by the evaluation of primary samples, these experiments demonstrating greater sensitivity in SHIP1-absent cells may provide insight into how future therapeutics are administered. Potentially, the achievement of synergy would allow for lower drug dosages, avoiding the secondary effects caused by drug toxicity.

Studying the SHIP1 isoforms and how they play into drug response will also be informative as most patients harbor these truncated forms of SHIP1. Establishing a difference in sensitivity between the different forms would allow a greater risk assessment that can be utilized for therapeutic protocols. Potentially, a protein expression fingerprint can be developed to predict sensitivity to drugs and determine the most favorable avenue of drug treatment for patients. For example, those with prognostically unfavorable expression profiles may be placed on aggressive drug regimens, while those deemed low risk may be subjected to less harsher conditions to avoid any unnecessary side effects.

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