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A Novel Therapeutic Approach in Breast and Hematopoietic Cancers: Inhibition of SH2-Domain Containing Inositol 5' Phosphatase (SHIP)

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A Novel Therapeutic Approach in Breast and Hematopoietic Cancers: Inhibition of SH2-Domain Containing Inositol 5' Phosphatase (SHIP)

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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Abstract

Aberrant hyperactivation of the phosphatidylinositol 3-kinase (PI3K) cell-signaling pathway, one of the most prominent cell growth, proliferation and survival pathways in the human body, has been observed in many forms of cancer. Upon deregulation, this pathway can facilitate the evasion of programmed cell death, the stimulation of autonomous growth, and the evasion of regular growth-inhibitory signals. While it was initially believed that the dephosphorylation activity of lipid phosphatases served to counteract the pathway by hydrolyzing phosphatidylinositol(3,4,5)trisphosphate (PtdIns(3,4,5)P_3) to phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P_2), emerging evidence suggests that SH2-domain containing inositol 5' phosphatase 1 and 2 (SHIP1/2) may in fact facilitate, rather than suppress, oncogenesis in certain contexts. We hypothesize, therefore, that chemical inhibition of SHIP would decrease phosphatidylinositol bisphosphate levels, thus reducing downstream activation of cell survival and proliferation effectors. Previous work in the Kerr lab identified a SHIP1 selective inhibitor (3AC) that was capable of killing malignant hematological cells but was ineffective against other, non-hematological malignancies. Therefore, this study sought to identify further, more effective inhibitors, particularly those capable of targeting both SHIP1 and SHIP2, as well as to investigate the overall effect such pan-SHIP inhibition has on blood and breast cancers. In this study, we identify several lead pan-SHIP inhibitors capable of preventing the hydrolysis of PtdIns(3,4,5)P3 to PtsIns(3,4)P2. Furthermore, we demonstrate the ability of these pan-SHIP inhibitors to reduce cell viability and to effectively kill leukemia, multiple myeloma, and breast cancer cells. In conclusion, this study suggests that the inhibition of SHIP1 and SHIP2 may hold great clinical promise as a novel therapeutic approach in the treatment of many different cancers.

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Acknowledgements

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To Dr. Belote, my Capstone project reader, faculty advisor, beloved professor, and overall life mentor, where do I even begin? From my very first day, you have played such an influential role in my experience at here Syracuse University; it certainly would not have been the same without you! To say I genuinely appreciate all you have done would be an understatement.

And last but not least, I thank my beautiful mother, herself a breast cancer survivor. It is success stories like yours that remind cancer researchers like us that the work we are doing truly makes a difference. On the days (or weeks) that my experiments did not go as planned, results did not turn out as expected, etc., *you* kept me going, *you* made me fall so in love with my work that I am having the hardest of times saying goodbye.

Introduction

The phosphotidylinositol 3-kinase (PI3K) pathway, a prominent cell growth and proliferation signal transduction system, is perhaps the most commonly activated signaling pathway in human cancers (Liu et al., 2009). The phosphatidylinositol 3-kinase pathway is activated upon the binding of an epidermal growth factor to a membrane-bound receptor tyrosine kinase. This binding induces dimerization of the receptor monomers, which are subsequently autophosphorylated. These phosphorylated monomers serve as binding and activation sites for PI3-kinases, whose primary biochemical function is to phosphorylate inositol phospholipids in the plasma membrane. Specifically, these activated PI3-kinases migrate along the cytosolic side of the cellular membrane and bind to phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P_2), adding a phosphate group to its inositol ring at the 3' position. This results in the formation of second messenger, phosphatidylinositol(3,4,5)trisphosphate (PtdIns(3,4,5)P_3, or "PIP3"), which, by acting as a docking site, further activates the serine/threonine kinase, Akt (also known as protein kinase B, or PKB) (Alberts et al., 2010).

Activated Akt has many different substrates, and hence, can result in a multitude of effects. Several of the most notable include inhibition of apoptosis, evasion of regular growth-inhibitory signals, and the autonomous stimulation of cellular proliferation. Consistent with these effects, many tumors, including both hematopoietic and tissue malignancies, present with constitutive activation of the

PI3K-Akt pathway (Fuhler et al., 2012). Therefore, targeting this signal transduction system with specific inhibitors may have broad clinical application in the treatment of multiple cancers (Bunney and Katan, 2010; Harvey and Lonial, 2007).

Common belief maintains that PI3K signaling is naturally counteracted by the dephosphorylation activity of lipid phosphatases. Phosphatase and tensin homolog, PTEN, hydrolyzes phosphatidylinositol(3,4,5)trisphosphate to phosphatidylinositol(4,5)bisphosphate by removing the phosphate group at the 3' position of the inositol ring (see figure 1). This hydrolysis reduces the activation of the proto-oncoprotein, Akt, and its downstream effectors promoting cell growth and survival. As such, PTEN functions as a potent tumor suppressor (Ruela-de-Sousa et al., 2010). The other class of lipid phosphatase implicated in the PI3K pathway, SH2-domain containing inositol 5' phosphatase (SHIP), also hydrolyzes PIP_3 to PIP_2, but does so by removing the phosphate group at the 5' position of the inositol ring (i.e. taking PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2; see figure 1) (Kerr, 2011). Based on the well-characterized tumor suppressing action of PTEN, it has generally been assumed that the intracellular enzymatic activity of SHIP functions to counteract the PI3K-Akt pathway as well. However, several intriguing observations and a growing body of literature are beginning to challenge this view.

While a loss of PTEN has been proven to confer increased susceptibility to cancer development, a loss of SHIP has not been demonstrated to have the same effect (Kerr, 2011). Based on recent study, it seems that PtdIns(3,4)P_2 actually

binds the PH domain of Akt more effectively than does PtdIns(3,4,5)P_3 (Ma et al., 2008), hence serving to *enhance* Akt activation (Sheid et al., 2002), contrary to PTEN. These findings are further supported by notably increased PtdIns(3,4)P_2 levels observed in malignant leukemia cells (Constantini, 2009; Brooks et al., 2010).

Given that the potent Akt-activating potential of PtdIns(3,4)P_2 is directly facilitated by SHIP, we hypothesize that chemical inhibition of SH2-domain containing inositol 5' phosphatase would decrease intracellular PtdIns(3,4)P_2 levels, thus reducing the activation of downstream effectors of cell survival and proliferation. As a result, this study sought to identify compounds capable of inhibiting both paralogs of SHIP: SHIP1 and SHIP2. Their 38% sequence homology (Fuhler et al., 2012) suggests that these two copies of the gene evolved from a common ancestral gene, but now carry out slightly different functions within the body. SHIP1 expression seems to be restricted to cells from the hematopoietic lineage and osteolineage (blood and bone marrow-forming cells, respectively), whereas SHIP2 seems to be ubiquitously expressed in all cells (Hazen et al., 2009, Rohrschneider et al., 2000). Previous work in the Kerr lab identified a SHIP1 selective inhibitor, 3 α-aminocholestane (3AC), that effectively killed multiple myeloma cells *in vivo*; however, this compound was relatively insoluble and was largely ineffective against non-hematological cancers. Via high throughput screening strategies, this study identified several lead pan-SHIP inhibitors that circumvented the challenges associated with 3AC

and demonstrated great promise as novel therapeutic agents in the treatment of breast and hematopoietic cancers.

Materials and Methods

Potential Inhibitors

All potential SHIP1/2 inhibitory compounds were synthesized in the organic chemistry laboratory of Dr. John D. Chisholm at Syracuse University, in Syracuse, NY. Compounds were derivatives of known SHIP inhibitors belonging to one of three groups: aminosteroids, tryptamines, or quinolones based on chemical structure. In total, more than 100 compounds were synthesized and screened at varying concentrations. Compounds were stored in solid state at -20°C until ready for use, at which time working stocks of a given concentration were made according to molecular weight and solubility, as provided by Dr. Chisholm and lab.

SHIP1

Expression and purification of human recombinant SHIP1 was performed according to a protocol previously established in the Kerr laboratory. A SHIP1 cDNA expression construct was amplified by PCR from the pMIGR1 (SHIP1) vector (Tu et al., 2001) and was then inserted into the pET24b bacterial expression vector at the *Eco*R1 and *Xho*1 restriction sites (see figure 2). This specific insertion created a SHIP1-His tag fusion at the C terminus. The His-tagged SHIP1 was then expressed in *Escherichia coli* Rosetta Gami 2 (DE3) pLys cells by induction after the addition of 0.5mM IPTG to trigger transcription. Lastly, SHIP1 was purified using Ni-chelating bead chromatography (Brooks et

al., 2010). To avoid the effects of repeated freeze/thaw cycles, SHIP1 protein stock was aliquoted and stored at -20 °C for use within 2 months and at -80 °C for use up to 6 months. It was found that stocks stored at -80°C maintained significantly greater enzymatic activity, and therefore, all but stocks currently in use were stored at -80°C.

SHIP2

While members of the Kerr lab worked to finalize a human SHIP2 protein purification protocol to be performed in the lab (like that which they have established for human SHIP1) SHIP2 Lipid Phosphatase, active (E-1000) was obtained from Echelon Biosciences, Inc. (Salt Lake City, UT). This N-terminal, His-tagged recombinant human SHIP2 (a truncated form containing SH2 domain and inositol 5-phosphatase catalytic domain) was also purified from *E. coli* using nickel column chromatography. This protein stock had approximately twice the enzymatic activity of the SHIP1 protein stock purified in-house and therefore, was used at half the concentration throughout all assays. To avoid the effects of repeated freeze/thaw cycles, SHIP2 protein stock was aliquoted and stored at -20°C for use within two months and at -80°C for use up to six months.

PIP3

D-*myo*-Phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) was obtained from Echelon Biosciences, Inc. (Salt Lake City, UT), and served as SHIP1/2's substrate in all assays. As per manufacturer's instructions, 1mg PIP_3 (P-3908) was

reconstituted in 1020µl ddH_2O. Reconstituted PIP_3 was aliquoted and stored at -20°C for up to three months. PIP_3 was used at concentration of 1mM in all assays.

Malachite Green Assay to determine SHIP1/2 enzymatic activity

To determine the enzymatic activity of SHIP *in vitro*, Malachite Green Solution (K-1501) was obtained from Echelon Biosciences, Inc. and was stored at 4°C, away from light, for use within six months. Through the course of this project we found the shelf life of Echelon's Malachite Green Solution to be shorter than manufacturer's suggestion, and used solutions within three to four months. Malachite Green Phosphatase Detection Assay was conducted as previously described (Brooks et al., 2010) in accordance with the manufacturer's protocol. In this assay, SHIP1/2 was mixed with substrate PtdIns(3,4,5)P_3 in the presence of potential inhibitor in 96-well plates. Inhibitors were dissolved in respective solvents and tested at final concentrations of 1mM, 500µM, 250µM, and 125µM, respectively (see figure 3). SHIP1 was prepared using ratio of 15µl SHIP1:285µl buffer, and SHIP2 was prepared using ratio of 7.5µl SHIP2:292.5µl buffer. Buffer solution consisted of 50mM HEPES pH 7.4, 100mM NaCl, 1mM $MgCl_2$, and 0.25mM EDTA. 10µl of potential inhibitor was then mixed with 90µl of SHIP/buffer solution. 3µl PIP_3 (1mM) was added to each well, followed by 22µl of inhibitor/SHIP/Buffer solution. Each reaction was tested in triplicate. The 96-well plate was then incubated at 37°C for 20 minutes. After incubation, 100µl of Malachite Green Solution was introduced to each reaction, which were then allowed to react in the dark for 20 minutes. Final results were quantified via Gen5

plate reader at absorbance of 620nm. The darker the color/higher the reading, the more inorganic phosphate has been liberated (see figure 3), hence indicating a higher activity of SHIP.

Cell lines studied, obtained from American Type Culture Collection (ATCC) (Manassas, VA)

K562: 53-year old female, chronic myelogenous leukemia (CML), NB4: 23-year old female, acute premyelocytic leukemia (APL), HSB2: 11.5-year old white male, acute lymphoblastic leukemia (ALL), 697: 12-year old male, acute lymphocytic leukemia (ALL), OPM-2: 56-year old female, myeloma relapse, MCF-7: 69-year old Caucasian female, breast cancer, MDA MB 231: 51-year old Caucasian female, breast cancer.

K562, NB4, HSB2, and 697 leukemia cells, as well as OPM-2 multiple myeloma cells, were routinely maintained in Iscove's Modified Dulbecco's Medium (IMDM) (ATCC) supplemented with 10% fetal bovine serum (Mediatech, Manassas, VA, USA), whereas MDA-MB-231 and MCF-7 breast cancer cells were cultured in Eagle's minimal essential medium (EMEM) with 10% fetal calf serum and L-glutamine.

Dojindo Cell Counting Kit-8 (CCK8)-Cell Viability/Cytotoxicity Assay

Cell viability was determined with Cell Counting Kit-8 from Dojindo as per manufacturers' instructions (Dojindo Molecular Technologies, Rockville, MD). Cells were treated in triplicate for 46 hours at 37°C with increasing concentrations

of inhibitory compounds or vehicle (5,000 cells/well, 10µl inhibitor/well). Dojindo CCK-8 solution was added (10µl/well) and cells were allowed to incubate at 37°C for another 2 hours. During this time, Dojindo's highly water soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] forms a water-soluble formazan dye upon reduction in the presence of an electron mediator. The solution changes from colorless to orange upon the production of formazan dye, and therefore, stronger color corresponds to greater cell viability. This colorimetric assay is also quantified via the Gen5 plate reader program at an absorbance of 450nm. The optical density (OD) of compound-treated cells was divided by the OD of their vehicle control, and the viability was expressed as a percentage of untreated cells. Results are expressed as mean ± standard error of the mean (SEM) of three individual experiments (Fuhler et al., 2012).

In vivo toxicity and tumor challenge studies

Prior to challenging mice with malignancies and subsequently treating them with inhibitors, we first ran toxicity screens of our lead compounds in wildtype C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME). This step is crucial in determining the ultimate effect of SHIP1/2 inhibition; for example, should we challenge mice with tumor cells, treat them, and the mice die, how do we know if it was the cancer that killed them or toxicity of the compound? These initial toxicity screens provide insight into how well each inhibitor is tolerated by a living organism. Dosages were dependent upon the particular compound, and

were administered at several different concentrations per inhibitor. These dosages ranged from 7.63mg/kg (compound K103) to 28.9mg/kg (compound K102).

Upon completion of toxicity screening, NOD/SCID/γcIL2R (NSG) mice (The Jackson Laboratory, Bar Harbor, ME) were challenged with 1×10^7 OPM2 cells via intraperitoneal (IP) injection. T cells and B cells of the adaptive immune system are impaired in these models and therefore, any decrease in tumorigenesis can de attributed directly to the compounds administered. "Treatment" began 6 hours post IP injection with either compound or vehicle. In our initial study, 3AC was suspended in a 0.3% Klucel/H_2O solution at 11.46 mM and administered via 200µl IP injection. Vehicle-treated mice received 200µL injection of 0.3% Klucel/H_2O solution. The final concentration of 3AC in the treated mice was 60 µM. The mice were then treated with 3AC or vehicle daily for the next 6 days and then twice per week in the remaining 15 wks of the survival study. Effects were analyzed via direct observation and measurement, via IVIS imaging (a visualization method utilizing luciferase bioluminescence), via Hemavet 950S automated blood cell analyzer, and via flow cytometry (a laser-based, biophysical technology useful for cell counting, sorting, and biomarker detection). Lead pan-SHIP inhibitors identified throughout the course of this study are currently being tested at 5-15mg/kg in 5% DMSO:Saline, 5% DMSO:H_2O, or in 100% H_2O, depending upon their solubility.

All *in vivo* studies were performed with approval from the Committee on the Humane use of Animals (CHUA) at SUNY Upstate Medical University.

Results

High-throughput screening identifies novel pan-SHIP inhibitors

To date, the three-dimensional molecular structure of SHIP is not known, and therefore, designing a particular inhibitor to "fit" and block the SHIP molecule's activity is not feasible. Therefore, a high throughput screening method was adopted such that a multitude of derivatives of known SHIP1/2 inhibitors could rapidly be analyzed for their ability to prevent the hydrolysis of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 *in vitro*. The colorimetric Malachite Green Assay allows for easy visualization of phosphate release, indicative of phosphatase (in our case, SHIP) activity. Upon dephosphorylation by SHIP at the 5' position, a free inorganic phosphate group is released into the solution, which reacts to form a colored complex with molybdate/malachite green. This color is then quantified by reading the absorbance at 620 nm. In other words, the stronger the color, the more potent the activity of SHIP. In this particular assay, we hoped to see little to no color change of the Malachite Green Solution. A colorless result (or one with a very low OD) indicates that SHIP has been inhibited, keeping PIP in the PtdIns(3,4,5)P_3 form, as desired. Throughout the course of this study, approximately 1/3 of the 100+ compounds screened were determined to inhibit the dephosphorylation activity of SHIP1 and/or SHIP2 at 1mM. These top "hits" were then tested against SHIP1 and SHIP2 at lower concentrations, specifically at 500μM, 250μM, and 125μM. After testing each compound at each concentration in triplicate, in three separate experiments, several compounds emerged as "lead inhibitors," capable of blocking the activity of *both* SHIP1 and SHIP2 better than

the parent compounds from which all new inhibitors were derived (see figure 4). The lead pan-SHIP inhibitors that we moved forward with in our study included four aminosteroids: K118, K141, K142, K179, and one tryptamine: K149, based on percent inhibition and solubility.

Pan-SHIP inhibition reduces the viability of blood and breast cancer cells ex-vivo

Upon identifying the most potent pan-SHIP inhibitors *in vitro* using the Malachite Green Assay, we then wanted to test the ability of these compounds to kill live cancer cells of both the hematopoietic and non-hematopoietic domains. Previous work in the Kerr lab demonstrated the ability of SHIP1-selective inhibitor 3AC to reduce the viability of malignant hematopoietic cells (OPM2 cells-multiple myeloma) *in vitro* and *in vivo* (Brooks et al., 2010). However, because SHIP1 expression is restricted to the hematopoietic compartment, the potential clinical applicability of 3AC is likewise restricted, feasible only in the treatment of blood cancers. By also targeting SHIP2, which is ubiquitously expressed in cells throughout the body, it was our hope that lead pan-SHIP inhibitors would demonstrate the ability to target and kill a broader range of malignancies.

As shown in figure 5, the parent pan-SHIP inhibitors from which our new compounds have been derived (3AC, 1PIE, 2PIQ, 6PTQ) demonstrate notable killing potential against OPM2 multiple myeloma cells, as well as against MDA-MB-231 and MCF-7 breast cancer cells. Consistent with its selectivity, 3AC was

found to lack any killing activity in the latter two, as neither breast cancer cell line expresses SHIP1.

Of greater significance, the lead pan-SHIP inhibiting derivatives themselves (K118, K141, K142, K149, K179) demonstrate even more potent cell-killing potential than their parent compounds (see figures 6-7). In nearly all experiments against leukemia and breast cancer cells, aminosteroid derivative K118 exhibits more notable cell killing than not only parent compound 3AC, but all other aminosteroid derivatives as well (see figure 6). Even at concentrations as low as 5μM, less than 35% of 697 cells survived; when looking at the NB4 cell line, K118 killed approximately 85% cells at a slightly increased concentration of 12.5μM. Furthermore, it is important to note that this compound is water soluble, making its potential even more exciting, especially from a pharmacological/ clinical perspective. The lead tryptamine derivative, K149, demonstrated more potent cell killing ability than its parent (K103) and all other derivatives, against every cell line, at nearly every concentration (see figure 7). Overall, 3AC (aminosteroid) derivatives were more effective than 2PIQ (tryptamine) derivatives (compare figures 6 and 7), and as may be expected, hematopoietic cancer cells expressing both SHIP1 and SHIP2 showed a higher sensitivity to pan-SHIP inhibitors than they did to 3AC which is selective only for SHIP1.

Inhibition of SHIP combats tumorigenesis *in vivo*

While the lead pan-SHIP inhibitors identified through this study are just starting to be introduced to *in vivo* models, the effects of their parent compounds (3AC, 2PIQ) have been well-characterized for comparison in this study. Dosages

were adjusted based on each particular compound, but for the initial toxicity screens, 3AC (also identified as K100) was administered and well-tolerated at 25-26.5mg/kg, 2PIQ (also identified as K103) was administered and well-tolerated at 7.63mg/kg and 15.25mg/kg, but proved toxic at 22.88mg/kg (resulted in death). Upon determining the maximum therapeutic dose in these otherwise healthy C57Bl/6 mice, SCID mice, lacking the T cells and B cells of their adaptive immune system, were challenged with multiple myeloma cells via intraperitoneal injection then treated with the predetermined dosage of inhibitory compound (or vehicle) six hours later (25mg/kg for 3AC, 12.5mg/kg for 2PIQ, and 0.3% Klucel/H_2O or 50% DMSO/PBS for vehicle). Mice were then injected daily for one week (known as the induction phase) followed by biweekly injections of 3AC, 2PIQ, or vehicle for the remaining 15 weeks of the study (known as the maintenance phase). In the mice treated with 2PIQ, we saw an increase in neutrophils and myeloid derived suppressor cells, MDSCs, (consistent with our findings from the initial toxicity study) (see figure 8), which helped to combat tumorigenesis. However, 2PIQ was not well-tolerated by the hosts and many died within the first several weeks of the study. Treatment with 3AC did not confer such toxic effects and notably reduced the viability of multiple myeloma cells *in vivo*. 100% of vehicle-treated mice (those not receiving any inhibitory compound) developed significant tumor growth, while only 40% of 3AC-treated mice showed any tumor growth whatsoever, many of which were minor (see figure 9). Most importantly, we found that treatment with 3AC results in significantly enhanced survival of mice post-multiple myeloma challenge (see figure 10). At the

culmination of the tumor challenge study, more than twice the number of 3AC-treated mice survived than vehicle-treated mice. Again, it is important to remember that 3AC is SHIP1 selective, and therefore, shall only confer results of this nature to cancers of the blood and bone marrow. Hoping to expand the clinical applicability of SHIP inhibition, we are currently beginning the same *in vivo* trials with the lead pan-SHIP inhibitors identified thus far in the study, K118, K141, K142, K149, K179. K118, K141, K142, and K179 are all derivatives of 3AC and hopefully will be tolerated as well, while K149 is a derivative of 2PIQ, raising some concern regarding toxicity. These lead pan-SHIP inhibitors are currently being tested at 5-15mg/kg in 5% DMSO:Saline, 5% DMSO:H_2O, or in 100% H_2O, depending upon their solubility.

Discussion

The prevalent belief that PI3K signaling is counteracted by all lipid phosphatases (Hamilton et al., 2011) is slowly being challenged (Kerr, 2011; Fuhler et al., 2011). It has recently been shown that the hydrolysis of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 actually results in more efficient binding of Akt (Franke et al., 1997), hence enhancing cell proliferation and survival effects. Given the implication of the PI3K-Akt pathway in many different malignancies, targeting its intricate components, such as SH2-domain containing inositol 5' phosphatase, presents both an opportunity and a challenge for novel cancer therapies. Because SHIP directly facilitates formation of the PtdIns(3,4)P_2 product, researchers are now seeking ways to inhibit its intracellular enzymatic activity. By decreasing PtdIns(3,4)P_2 levels, it has been hypothesized that growth, division, and survival signals will be decreased as well (Artemenko, 2009), ultimately resulting in increased cell death. Targeting this phosphatase in cancer treatment can be complicated by the existence its two paralogs; SHIP1 seems to be expressed predominately in the hematopoietic compartment, while SHIP2 appears to be ubiquitously expressed. While previous work in the Kerr lab identified the ability of a SHIP1 selective inhibitor to reduce viability of malignant hematopoietic cells, little study has been done on the effects of SHIP2 inhibition, or more intriguingly, pan-SHIP inhibition. While we are not the first to identify pan-SHIP inhibitors, to the best of our knowledge, we are the first to

propose pan-SHIP inhibition as a novel therapeutic approach in the treatment of both hematopoietic and breast cancers.

In this study, we sought to expand upon work done previously in the lab demonstrating the ability of 3 α-aminocholestane to reduce the viability of multiple myeloma cells *in vitro* and *in vivo.* Via high throughput screening methods, it was our goal to identify compounds capable of inhibiting both SHIP1 and SHIP2 simultaneously, hence affecting a broader range of malignancies. This screen yielded five lead pan-SHIP inhibitors, which we refer to as K118, K141, K142, K149, and K179. Throughout the course of this study, we have demonstrated the ability of these pan-SHIP inhibitors to prevent the hydrolysis of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 *in vitro*, as well as to effectively reduce the viability of leukemia, multiple myeloma, and breast cancers *ex vivo*. *In vivo* studies are currently under way as described, but according to the preliminary data we are obtaining, several of the lead pan-SHIP inhibitors appear to be both well-tolerated by the host and effective at killing the multiple myeloma malignancies with which the mice have been challenged.

In conclusion, findings of this study suggest that the inhibition of SHIP1 and SHIP2 provides a feasible technique for targeting and killing cancer cells of various origins. While development of inhibitory compounds is still in preliminary phases, the potency of lead pan-SHIP inhibitors to kill breast and blood cancer cells has been clearly demonstrated. Our studies are currently being pursued and extended and will hopefully lead to clinical trials using enhanced derivatives of the most promising pan-SHIP inhibitors identified to date. Overall,

our findings suggest that the inhibition of SHIP1 and SHIP2, collectively, may provide a promising novel therapeutic approach in the treatment of both hematopoietic and non-hematopoietic cancers. We are beyond excited to see where these studies may lead us in the near future.

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FIGURE 1.[1]

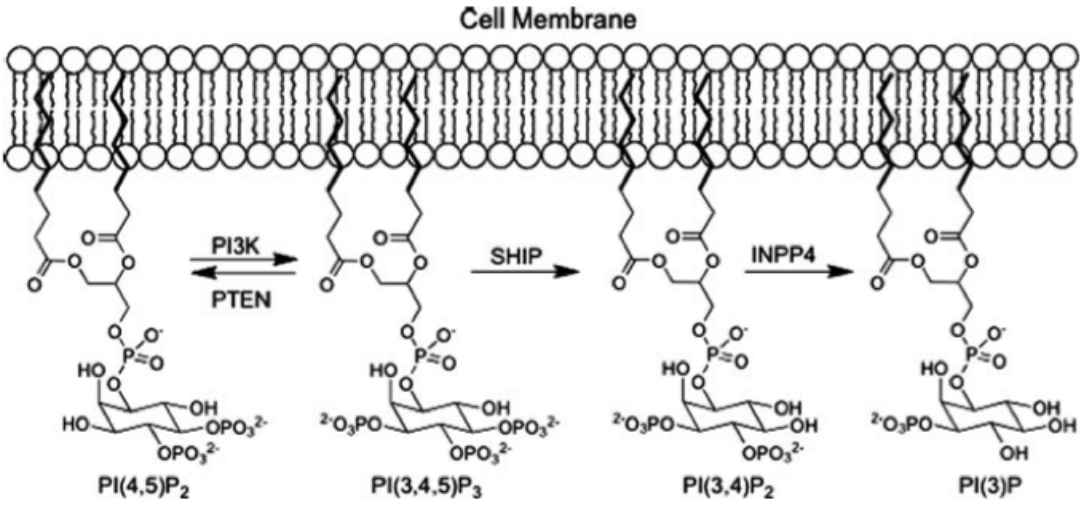

Figure 1. Enzymatic generation and hydrolysis of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ at the plasma membrane by PI3K, PTEN, SHIP, and INPP4.

Figure 1. Enzymatic generation and hydrolysis of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ at the plasma membrane by lipid phosphatases. As one can see, PI3-kinases first phosphorylate $PI(4,5)P_2$ at the 3' position of the inositol ring. This results in the formation of PI(3,4,5)P3. Two classes of phosphatases can then act on the PIP3 molecule in opposing ways. PTEN, a known tumor suppressor, dephosphorylates PIP3 at the 3' position, returning PI(3,4,5)P3 to PI(4,5)P2. SHIP, on the other hand, dephosphorylates PIP3 at the 5' position, resulting in the PI(3,4)P2 product. (INPP4 is negligible in the context of this study).

[1] Taken from Kerr WG. (2011). Inhibitor and activator: dual functions for SHIP in immunity and cancer. *Annals of the New York Academy of Sciences*. 1217: 1–17. doi: 10.1111/j.1749-6632.2010.05869.x

FIGURE 2.[2]

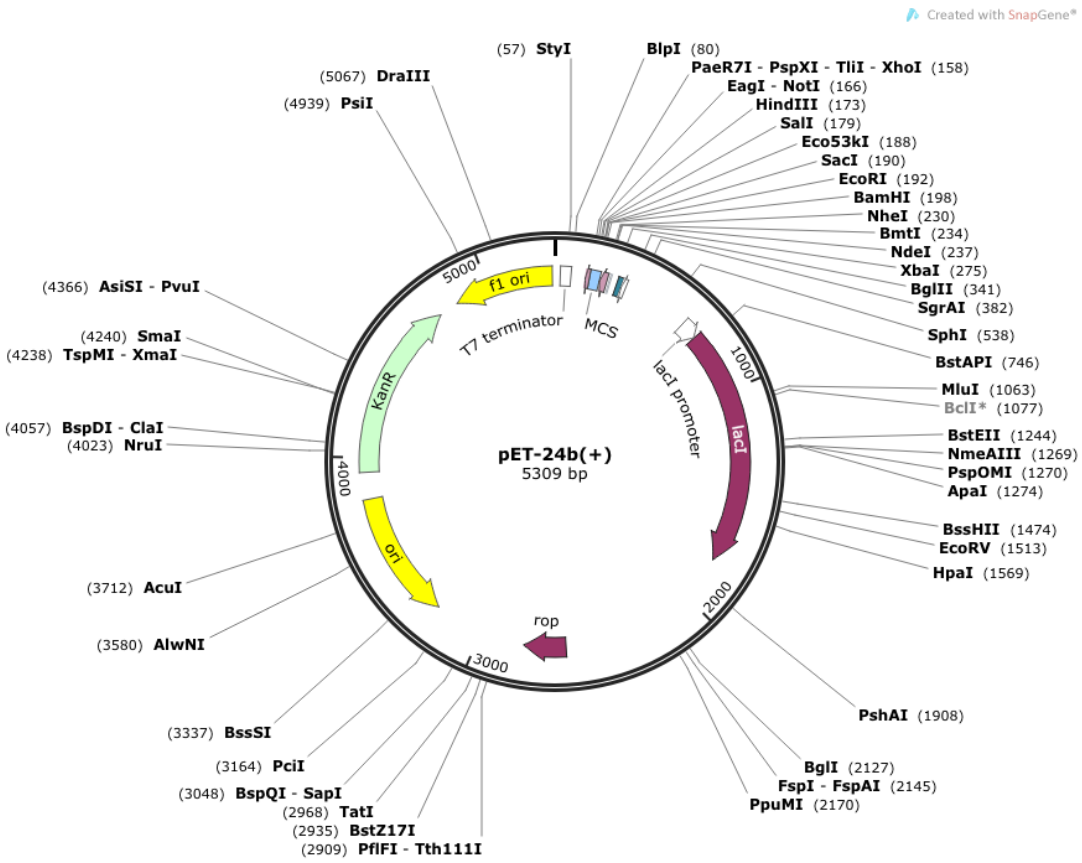

Figure 2. Restriction map of pET-24b(+) bacterial expression vector. In expression and purification process of human recombinant SHIP1, the SHIP1 cDNA expression construct was amplified by PCR from the pMIGR1 vector and was subsequently inserted into the pET24b bacterial expression vector at the *Eco*R1 and *Xho*1 restriction sites (see top right).

[2] http://www.snapgene.com/resources/plasmid_files/pet_and_duet_vectors_(novagen)/pET-24b(+)/

FIGURE 3.

A.[3]

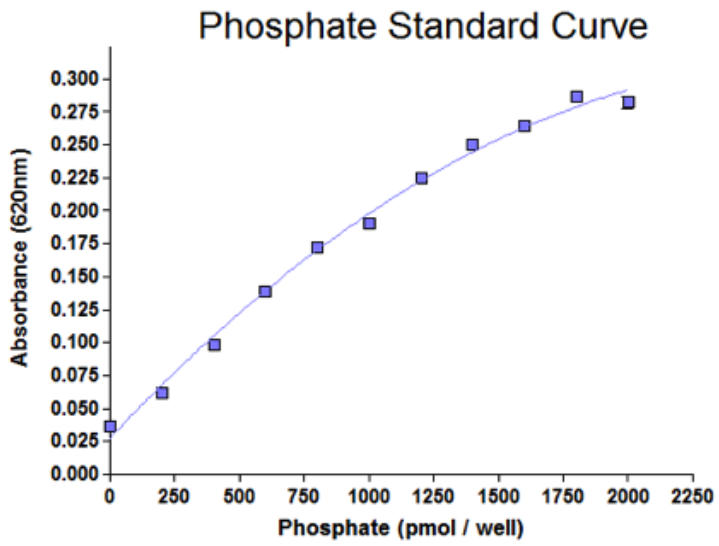

Figure 3A. Malachite Green standard curve measuring free phosphate in solution. The standard curve was generated using polynomial second order non-linear regression analysis. In simplest terms, the greater the level of free inorganic phosphate (in our study, liberated by SHIP) the higher the absorbance.

B. Working stock calculation

$$\text{___mg inhibitor} \times \frac{1\text{g}}{1000\text{mg}} \times \frac{1\text{mol}}{\text{mol. weight(g)}} \times \frac{1\text{L}}{0.010\text{M}} \times \frac{10^6\,\mu\text{l}}{1\text{L}} = \text{___ µl solvent}$$

Figure 3B. Calculation used to determine amount of solvent needed to make 10mM working stock of each inhibitory compound. This working stock was used in the Malachite Green Assay at a final concentration of 1mM. For dilution screens resulting in final concentrations of 500µM, 250µM, and 125µM, this stock was subsequently added to equal volume of pure solvent (i.e. 200µl of 10µM stock + 200µl DMSO = 5µM working stock, final concentration 500µM).

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[3] Taken from Echelon Biosciences, Inc. website at http://www.echelon-inc.com/content/EBI/product/files/TDS_K-1501_rev1.pdf

FIGURE 4.

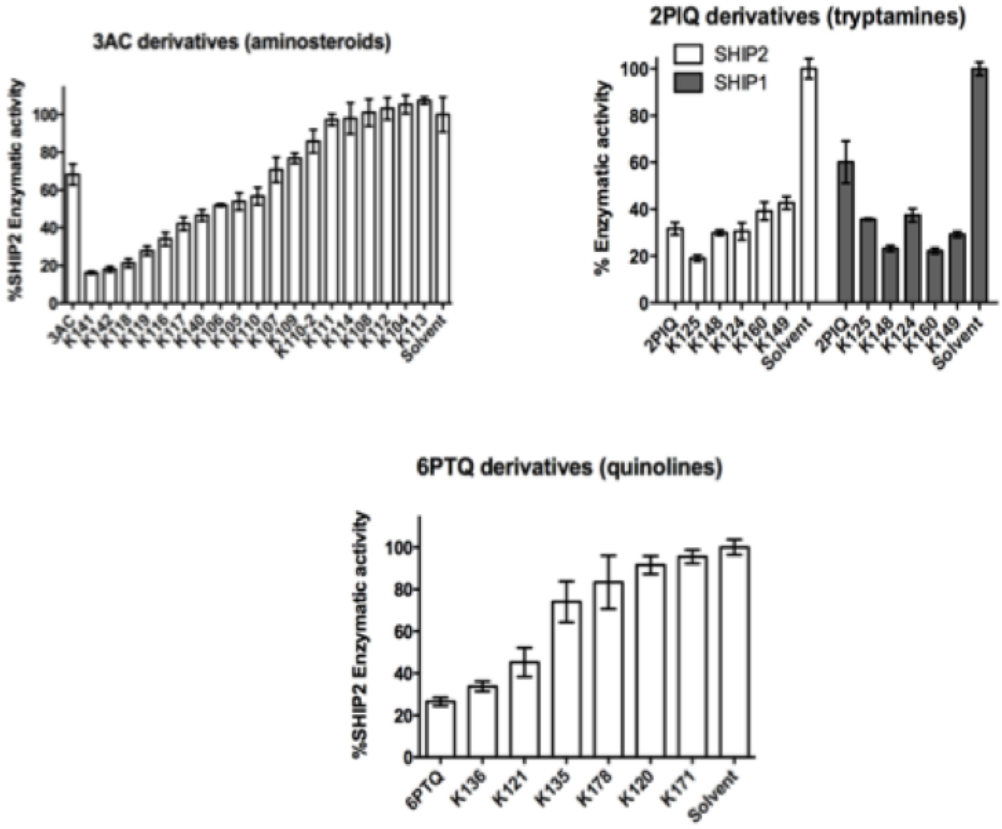


Figure 4. Relative enzymatic activity of recombinant SHIP1/2 protein with derivatives of parent compounds, as measured by Malachite Green (phosphate release) assays. Derivatives of aminosteroids (top left), tryptamines (top right) and quinolines (bottom center) have been developed and tested in vitro by Malachite Green assay and expressed as relative to solvent only control. In each graph, the parent compound is shown at leftmost position along x axis (i.e. 3AC, 2PIQ, 6PTQ). Only aminosteroid (3AC) and tryptamine (2PIQ) derivatives showed notable pan-SHIP inhibition, and hence, were moved forward with in study. Of particular interest at the culmination of all Malachite screens were compounds K118, K141, K142, K149, and K179.

FIGURE 5.

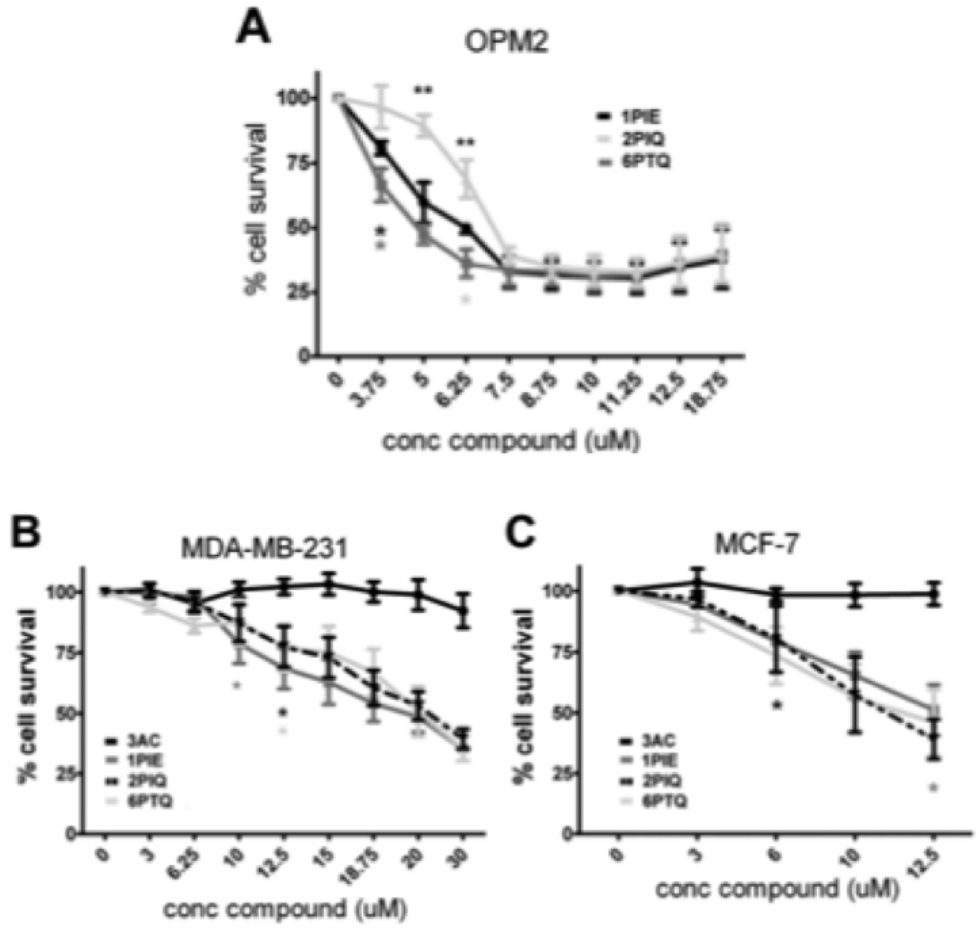
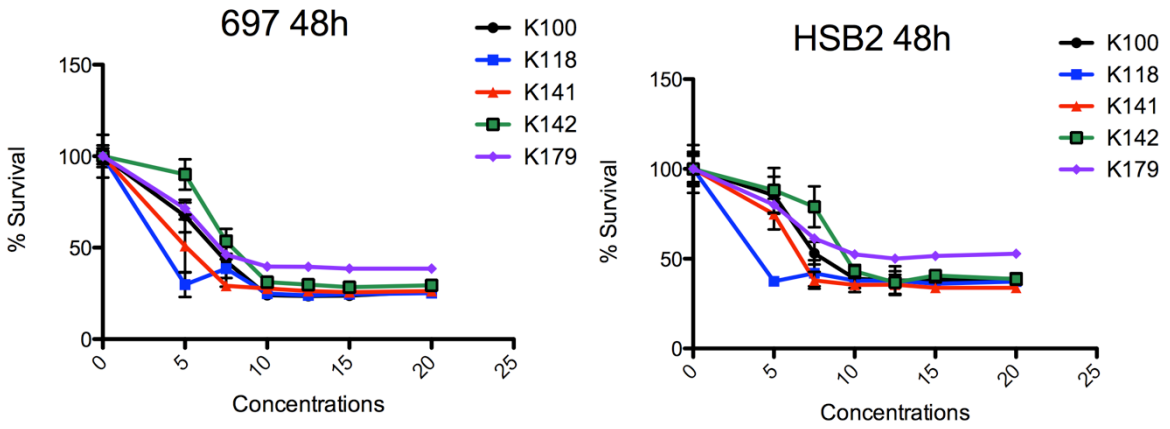
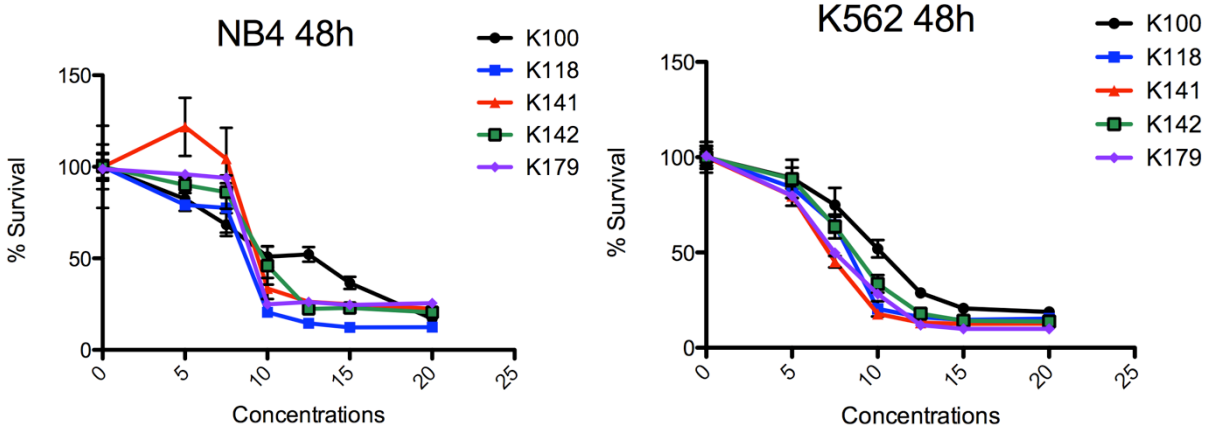


Fig. 5. Pan-SHIP inhibition reduces cell viability/induces cell death of multiple myeloma and breast cancer cells *ex vivo*. **(A-C)** Treatment of **(A)** OPM2 multiple myeloma cells, **(B)** MDA-MB-231 breast cancer or **(C)** MCF-7 breast cancer cells with 3AC, 1PIE, 2PIQ or 6PTQ decreased cell survival in a dose-dependent manner. Consistent with its selectivity for SHIP1, 3AC lacked any killing activity in the latter two breast cancer cell lines **(B, C)** which, as cancers outside of the hematopoietic compartment, do not express SHIP1.

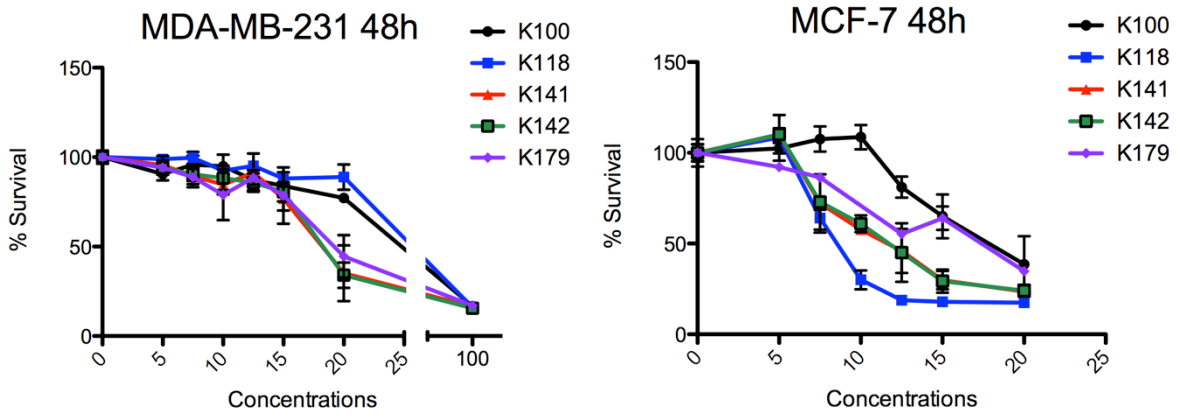
FIGURE 6. Lead aminosteroid panSHIP1/2 inhibitors vs. parent compound against leukemia and breast cancer cells



Lead aminosteroid derivatives vs. parent against pediatric leukemia cells

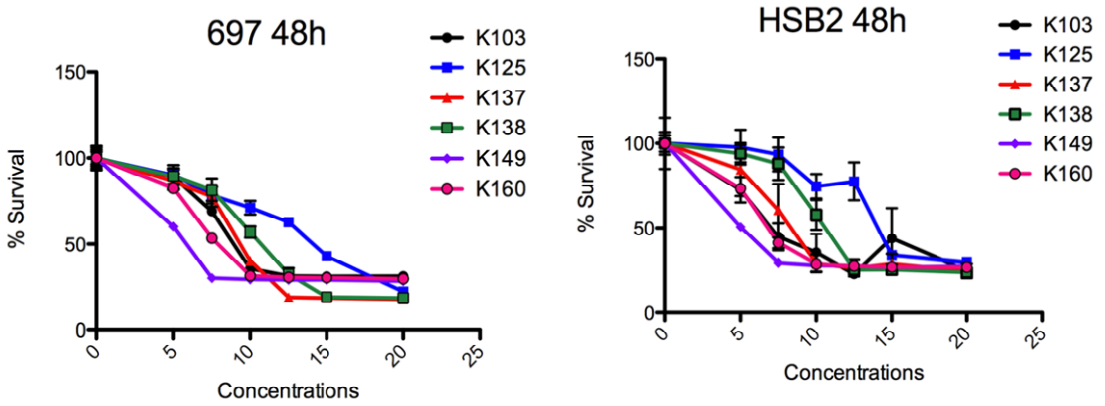


Lead aminosteroid derivatives vs. parent against adult leukemia cells



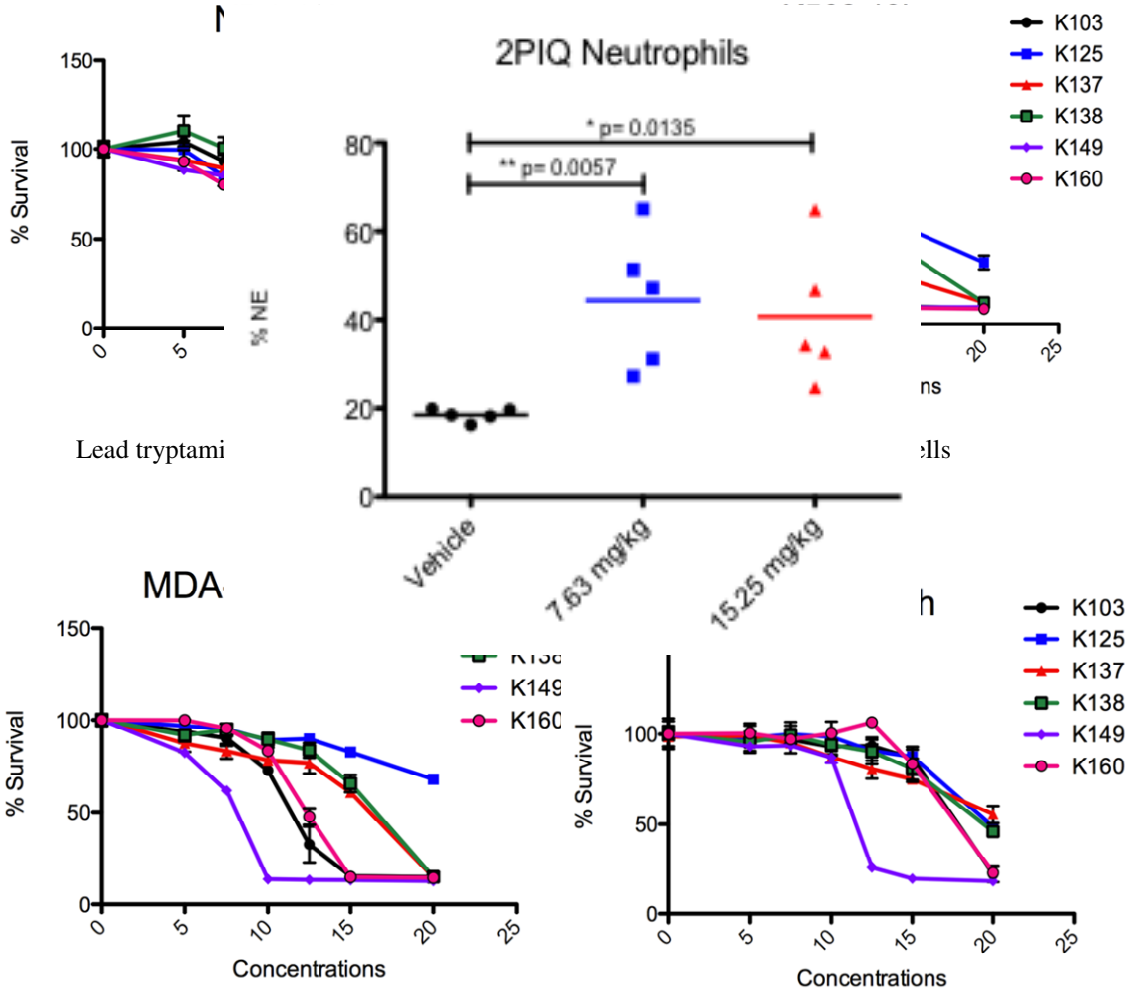
Lead aminosteroid derivatives vs. parent against breast cancer cells

FIGURE 7.



Lead tryptamine, K149 vs. parent tryptamine K103 against pediatric leukemia cells

FIGURE 8.



Lead tryptamine, K149, vs. parent tryptamine, K103, against breast cancer cells

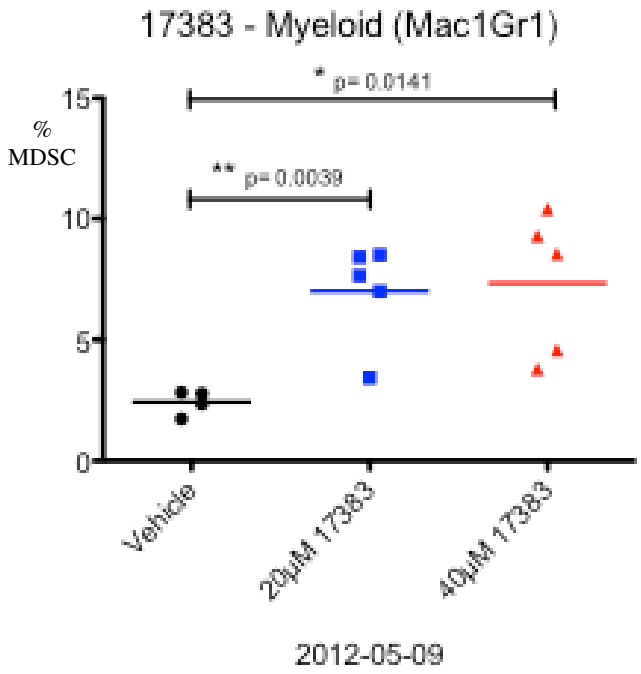


Figure 8. Hemavet analysis of mice receiving 2PIQ treatment. According to blood samples taken from mice treated with 2PIQ at two different concentrations, we saw an increase in both neutrophils and myeloid derived suppressor cells (MDSCs) when compared to mice receiving vehicle treatment.

FIGURE 9.

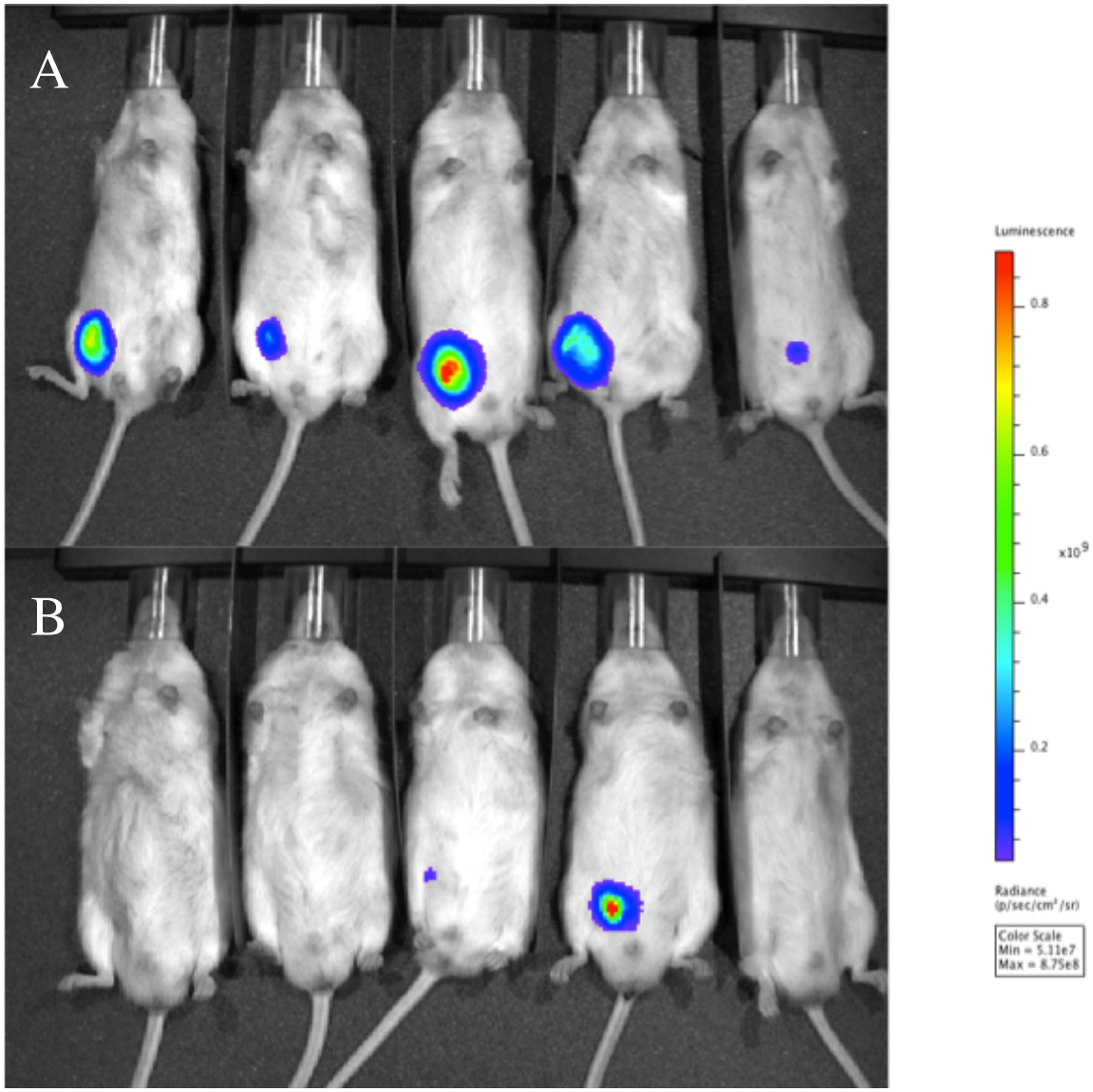


Figure 9. Vehicle vs. 3AC-treated NSG Mice, Week 3, Xenogen IVIS-200 Optical *In Vivo* Imaging System. Based on luciferase bioluminescence, it is apparent that 3AC significantly reduces the viability of OPM2 multiple myeloma cells *in vivo*. **(A)** 100% of mice challenged with OPM2 cells and treated with vehicle only (0.3% Klucel) exhibit significant tumor growth. **(B)** Only 40% of mice challenged with OPM2 multiple myeloma cells then treated with 3AC have developed any visible tumor growth. Of the two mice in this image demonstrating tumorigenesis, one displays minimal tumor growth.

FIGURE 10.

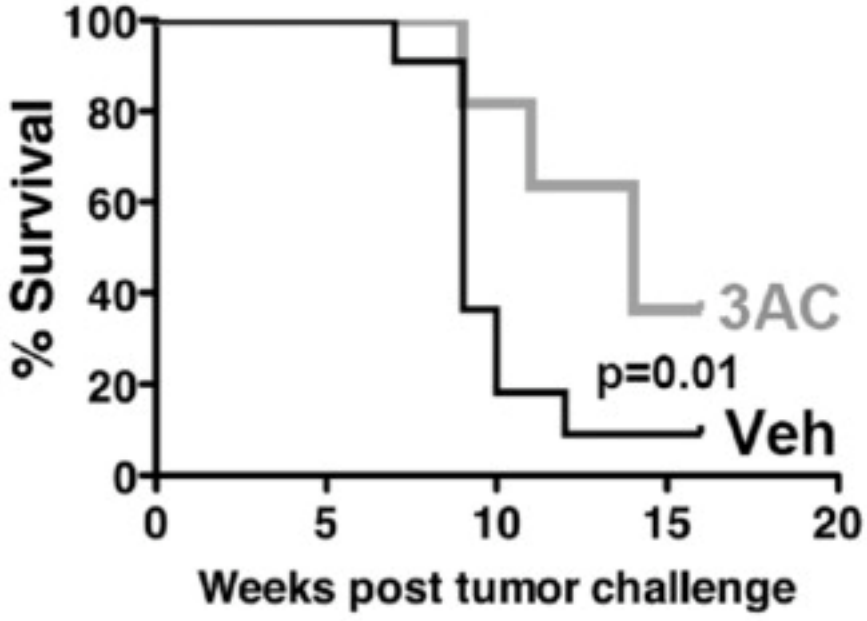


Figure 10. Treatment with 3AC results in significantly enhanced survival of mice post-multiple myeloma challenge. 3AC postponed the initial death event for the group receiving treatment to 10 weeks, while the vehicle group began to experience fatality at 7 weeks. More importantly, at the culmination of the 16-week tumor challenge study, more than twice the number of 3AC-treated mice survived than did vehicle-treated mice.

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Summary of Capstone Project

The phosphotidylinositol 3-kinase, or PI3K, pathway is one of the most prominent cell proliferation and survival pathways in the human body. In a healthy model, activation and deactivation of this pathway is carefully regulated so as to allow for normal growth and the replacement of cells as needed. Upon deregulation (typically due to mutations) however, this signal transduction system can lead to the evasion of programmed cell death, the stimulation of a cell's own growth, and the ability of a cell to replicate indefinitely. Given that one of the most fundamental definitions of cancer is an uncontrolled growth and proliferation of abnormal cells, it is not surprising that many tumor cells are observed to have hyperactivated PI3K signaling.

Through meticulous study, it has been found that the phosphorylation of a regular cell membrane component, known as phosphatidylinositol(4,5) bisphosphate to phosphatidylinositol(3,4,5)trisphosphate ("PIP3") activates this pathway, hence leading to increased cell growth and survival. For many years, it has been assumed that if phosphorylation turns the pathway "on", then dephosphorylation ought to turn the pathway "off". Dephosphorylation of PIP3 back to PIP2 is facilitated via two classes of lipid phosphatases: PTEN (phosphatase and tensin homolog) and SHIP (SH2-domain containing inositol 5' phosphatase). PTEN has been very well-characterized and functions as a known tumor suppressor. By removing a phosphate group from the 3' position of the inositol ring of PIP3, PTEN returns PIP3 to PIP2 in the "4,5" form (i.e.

phosphatidylinositol (3,4,5) trisphosphate→phosphatidylinositol (4,5) bisphosphate). In doing so, PTEN prevents downstream components of the pathway (primarily the protein kinase, "Akt") from binding to PIP3 and subsequently, signaling is discontinued. More recently discovered, the other class of phosphatase, SHIP, removes a phosphate group from the 5' position of the inositol ring, hence returning PIP3 to PIP2 in the "3,4" form (i.e. phosphatidylinositol (3,4,5) trisphosphate→phosphatidylinositol (3,4) bisphosphate). Unlike the "4,5" product, this "3,4" product actually seems to bind the downstream component Akt even more efficiently that does PIP3, and hence, enhances signaling leading to cell growth, proliferation, and survival.

Because SHIP directly facilitates hyperactivation of the PI3K pathway, cancer biologists in the Kerr laboratory at SUNY Upstate Medical University are now looking for ways to inhibit its functioning. At this time, the molecular structure of SHIP has not been determined; therefore designing specific compounds to bind to and block the protein's action is not a feasible option. Further challenging to the development of a specific and effective inhibitor is the fact that SHIP has two paralogs, or two copies of the gene that vary slightly. SHIP1 expression seems to be restricted to cells of the hematopoietic compartment (meaning those that form your blood and bone marrow cells), whereas SHIP2 seems to be ubiquitously expressed in cells throughout the body. Based on the properties of the SHIP molecule that *are* known, previous work in the Kerr lab identified a SHIP1-selective inhibitor known as 3 α-aminocholestane (3AC), that proved capable of killing hematopoietic malignancies in mice. This inhibitor was far from perfect,

however. As mentioned, 3AC is SHIP1-selective, meaning that it does not target the other form of the protein, SHIP2, which in the compound's presence is still able to hydrolyze PIP3→PIP2. Given that SHIP1 expression is limited only to the hematopoietic domain, 3AC provides a feasible approach only in the treatment of blood cancers, and remains ineffective in the treatment of other non-hematopoietic cancers (i.e. tissue cancers). Likewise, this compound is relatively insoluble; in order to work with 3AC in any *in vitro* or *in vivo* trials, it first must be dissolved in 100% ethanol at 100 °C, clearly not advantageous from a pharmacological perspective. Furthermore, during *in vivo* studies, 3AC was observed to confer slight toxicity to its hosts.

Therefore, the goal of this study over the past two years has been to identify a new, more effective inhibitor that circumvents the challenges associated with 3AC. In close collaboration with Dr. John Chisholm's organic chemistry lab at Syracuse University, who have synthesized more than 100 potential inhibitors, I have pursued a high throughput screening strategy to identify compounds with a higher solubility and lesser toxicity, capable of inhibiting the intracellular enzymatic activity of *both* SHIP1 and SHIP2. Through an *in vitro* technique known as the Malachite Green Assay, I was able to identify compounds that prevented SHIP1/2 from removing the phosphate group at the 5' position of the PIP3 inositol ring. Upon completion of the Malachite screen on all compounds, those demonstrating the most significant inhibition were then carried into *ex vivo* trials. During this phase, compounds were introduced directly on to cell lines at varying concentrations to test their ability to reduce cell viability. Cell lines

studied included adult and pediatric leukemia, multiple myeloma, and breast cancer, allowing us to observe the effects of pan-SHIP inhibition on both hematopoietic and non-hematopoietic cancers. All of our lead pan-SHIP inhibitors demonstrated notable cell-killing activity against blood and breast malignancies. Lastly, the lead inhibitors identified through *in vitro* and *ex vivo* studies were then entered into *in vivo* trials. Compounds were initially administered at predetermined dosages to otherwise healthy mice in order to observe inherent toxic effects, if any. Upon completion of the toxicity screens, SCID (severe combined immunodeficient) mice were challenged with multiple myeloma cells, then subsequently treated with our panSHIP inhibitors. These mice are deficient in the T cells and B cells of the adaptive immune system, and therefore, if tumor growth is prevented or reduced, we know it is a direct result of the treatment they are receiving. This phase of the study is currently underway, but according to the preliminary data we are obtaining, several of the lead pan-SHIP inhibitors appear to be both well-tolerated by the host and effective at killing the malignancies with which the mice have been challenged.

Findings of this study suggest that the inhibition of SHIP1 and SHIP2 provides a feasible technique for targeting and killing cancer cells of various origins. While development of inhibitory compounds is still in preliminary phases, the potency of lead pan-SHIP inhibitors to kill breast and blood cancer cells has been clearly demonstrated. Our studies are currently being pursued and extended and will hopefully lead to clinical trials using enhanced derivatives of the lead pan-SHIP inhibitors identified to date. Overall, our findings suggest that

the inhibition of SHIP1 and SHIP2 may provide a promising novel therapeutic approach in the treatment of both hematopoietic and non-hematopoietic cancers, revolutionizing chemotherapy as it is currently known.