INHIBITION OF THE PHOSPHOINOSITIDE 3-KINASE PATHWAY IN
MULTIPLE MYELOMA MEDIATED THROUGH ACTIVATION OF SHIP

by

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ABSTRACT

Multiple myeloma (MM) is a B-lymphocyte neoplasia that remains incurable due in part to intrinsic and acquired drug resistance, despite the numerous conventional therapies available. The growth, survival and anti-apoptosis signals resulting from adhesion and cytokine-mediated interactions between the malignant clones and the bone marrow microenvironment are transduced chiefly through the elevated phosphoinositide 3-kinase (PI3K) signaling pathway, which has been demonstrated essential for disease progression. Many biologically-based therapeutics are in development for MM, but the therapies aimed at abrogating this cascade have shown modest success and often have problems with toxicity. A novel alternate approach in controlling this signaling is activation of an endogenous negative regulator, the inositol phosphatase SHIP. A benefit of targeting SHIP is its restricted expression to hematopoietic cells, thereby limiting potential toxicity to surrounding tissues. Further, the PI3K pathway is involved in the development of drug resistance, and abrogating the cascade can re-sensitize MM cells to conventional therapeutics. Here we demonstrate activation of SHIP is sufficient to inhibit proliferation and induce apoptosis of MM cells in vitro, while having no significant effects on non-hematopoietic cancer cells or lymphocytes lacking SHIP. We also show that SHIP activators enhance the cytotoxicity of current chemotherapeutic agents and provide preliminary results of efficacy in a murine xenograft model. These results not only provide the basis for the further study of a new therapeutic agent to improve MM patient outcome but also propose a new model for studying signal transduction through activating the endogenous negative regulators of the PI3K pathway.
TABLE OF CONTENTS

ABSTRACT ................................................................................................................ ii

TABLE OF CONTENTS .......................................................................................... iii

LIST OF FIGURES ................................................................................................. v

LIST OF ABBREVIATIONS ..................................................................................... vi

INTRODUCTION ...................................................................................................... 1

Multiple Myeloma .................................................................................................. 1
  A. Epidemiology .................................................................................................... 1
  B. Diagnosis ........................................................................................................ 1
  C. Pathophysiology .............................................................................................. 2

Biology of disease ................................................................................................ 4
  A. The bone marrow microenvironment ............................................................ 4
  B. Signaling pathways in MM ............................................................................ 6
    Ras/Raf/MAPK ................................................................................................. 6
    Jak2/STAT3 ...................................................................................................... 7
    PI3K .................................................................................................................. 8

Therapy .................................................................................................................. 9
  A. Conventional therapy and the development of resistance ......................... 9
  B. Drug resistance ............................................................................................... 10
  C. Anti-MM drugs .............................................................................................. 11
    Thalidomide and immunomodulatory derivatives ....................................... 11
    Lenalidomide .................................................................................................. 12
    Bortezomib ...................................................................................................... 12
    Arsenic trioxide .............................................................................................. 13
    Other targeted therapies ............................................................................... 14

The PI3K pathway .................................................................................................. 15
  A. Endogenous regulation .................................................................................. 15
  B. Targeting the PI3K pathway ......................................................................... 18
  C. SHIP as a target ............................................................................................ 19
  D. SHIP activators ............................................................................................. 19

Hypothesis ............................................................................................................. 20

MATERIALS AND METHODS ............................................................................ 21
  SHIP activators ................................................................................................. 21
  Animals ............................................................................................................... 21
  Cells and reagents ............................................................................................. 21
  Primary murine B-lymphocyte purification .................................................... 22
  Thymidine incorporation ................................................................................... 23
LIST OF FIGURES

Figure 1. The PI3K pathway ................................................................. 17
Figure 2. The lead candidate SHIP activators ................................................. 29
Figure 3. SHIP activation inhibits phosphorylation of Akt and downstream targets .......... 31
Figure 4. SHIP activation inhibits thymidine incorporation of MM cell lines but does not inhibit thymidine incorporation in SHIP-deficient carcinoma lines ........................................ 34
Figure 5. Viable MM cell numbers are significantly reduced by SHIP activation .......... 38
Figure 6. SHIP activation induces MM cell apoptosis ...................................... 41
Figure 7. SHIP activation selectively acts on wild-type murine B-lymphocytes and mast cells ........................................................................ 44
Figure 8. The cytotoxicity of current therapeutic agents is enhanced by SHIP activation .... 46
Figure 9. SHIP activation inhibits MM cell growth in vivo ................................. 48
LIST OF ABBREVIATIONS

As₂O₃  arsenic trioxide
Bcl-X<sub>L</sub>  B cell lymphoma X<sub>L</sub>
BCR  B cell receptor
bFGF  basic fibroblast growth factor
BM  bone marrow
BMMC  bone marrow-derived mast cell
BMSC  bone marrow stromal cell
BSA  bovine serum albumin
CAM-DR  cell adhesion-mediated drug resistance
CML  chronic myeloid leukemia
CRAB  hypercalcemia, renal insufficiency, anemia and bone lesions
DMEM  Dulbecco's modified Eagle's medium
DNA  deoxyribonucleic acid
ECM  extracellular matrix
EDTA  ethylenediamine tetraacetic acid
EGFR  epidermal growth factor receptor
FGFR3  fibroblast growth factor receptor 3
FKHR  forkhead transcription factor
GPCR  G-protein coupled receptor
GSK-3β  glycogen synthase kinase-3β
ICAM-1  intracellular adhesion molecule-1
Ig  immunoglobulin
IGF1  insulin-like growth factor 1
IGF1R  insulin-like growth factor 1 receptor
IgH  immunoglobulin heavy chain
IL-6  interleukin-6
IL-6R  interleukin-6 receptor
IMDM  Iscove's modified Dulbecco's medium
IMiDs  immunomodulatory derivatives
JAK  Janus kinase
LFA-1  lymphocyte function-associated antigen-1
MAPK  mitogen-activated protein kinase
Mcl-1  myeloid cell leukemia sequence-1
MGUS  monoclonal gammopathy of undetermined significance
MM  multiple myeloma
mTOR  mammalian target of rapamycin
NF-kB  nuclear factor-kB
NOD  non-obese diabetic
NSCLC  non-small cell lung cancer
p70S6K  p70S6 kinase
PAGE  polyacrilamide gel electrophoresis
PARP  poly(ADP-ribose) polymerase
PBS  phosphate-buffered saline
P-gp  P-glycoprotein
PI  propidium iodide
PI3K  phosphoinositide 3-kinase
PI-3,4-P₂  phosphatidylinositol-3,4-bisphosphate
PI-4,5-P₂  phosphatidylinositol-4,5-bisphosphate
PIP₃  phosphatidylinositol-3,4,5-trisphosphate
PTEN  phosphate and tensin homologue deleted on chromosome 10
RPMI  Roswell Park Memorial Institute
SCID  severe combined immunodeficient
SDF1  stromal derived factor 1
SDS  sodium dodecyl sulfate
SHIP  Src homology 2-containing inositol 5'-phosphatase
STAT  signal transducers and activators of transcription
TBS  tris-buffered saline
TGF-β  transforming growth factor-β
TNF-α  tumor necrosis factor-α
VAD  vincristine, doxorubicin and dexamethasone
VEGF  vascular endothelial growth factor
VLA-4  very-late antigen 4
INTRODUCTION

Multiple Myeloma

A. Epidemiology

Multiple myeloma (MM) is a plasma cell malignancy characterized by the accumulation of transformed B cells in multiple sites, known as plasmacytomas, in the bone marrow along with elevated levels of monoclonal immunoglobulin (Ig) proteins in the serum and urine. The tumors originate from post-germinal centre plasma cells and rely on the bone marrow microenvironment for their growth, survival, migration and acquired drug resistance.

The annual incidence of MM in the United States is 4.3 cases per 100,000 people, with 15,000 new diagnoses each year. The median age at time of diagnosis is 66 years, with only 2% younger than 40 years. It is seen twice as often in African Americans compared to Caucasians, and affects men more often than women in all racial categories. Although it is still not clear, genetics may play a role based on some familial studies. The mean patient survival time using conventional therapeutics is 3-4 years, and this can be prolonged by about one year if high-dose treatment is followed by autologous stem-cell transplantation. However, MM remains incurable due to the development of resistance to all current therapies, highlighting the need for further treatment options to improve patient outcome.

B. Diagnosis

Patients will most often present symptoms of fatigue, bone pain and frequent infections. A diagnosis of MM requires a plasma cell content of 10 percent or more in the bone marrow (or...
a positive biopsy of a plasmacytoma), along with monoclonal proteins in the serum or urine and evidence of organ damage. Monoclonal protein levels are measured by serum protein electrophoresis and by immunofixation in suspect patients. In approximately 3% of cases, patients will not have detectable levels of monoclonal protein in the serum or urine and are considered to have nonsecretory MM. End-organ damage for diagnosis includes hypercalcemia, renal insufficiency, anemia, and bone lesions, together referred to as CRAB. Anemia is seen in 70% of cases at diagnosis, while hypercalcemia and elevated serum creatinine are seen in around 13% and 19% of patients, respectively. Bone lesions are detected in approximately 80% of the patients by radiography.

C. Pathophysiology

The initial pathogenic event in the development of MM is the formation of pre-malignant tumor consisting of a small number of clonal plasma cells, known as monoclonal gammopathy of undetermined significance (MGUS). Although MGUS is present in 1% of adults over the age of 25, the development of this condition is age-dependent and is rarely seen in people under 40 years. The tumor cells secrete low levels of monoclonal Ig, but patients show no symptoms of disease or organ damage. MGUS progresses to malignant myeloma at a rate of approximately 1% per year. Amyloidosis is related to MGUS by having the same pathology but the secreted monoclonal proteins form fibrous depositions in various tissues, often leading to death due to heart, kidney or other organ failure within 18 months of diagnosis. Both these disorders occur in the bone marrow and are distinguished from multiple myeloma by a tumor-cell content below 10 percent. Smoldering myeloma has a stable level of intramedullary tumor cells above 10 percent but patients show no other signs of disease such as bone lesions, and therefore are
considered asymptomatic. The progression to symptomatic multiple myeloma occurs as the secondary features of the condition appear, such as anemia, immunodeficiency, and osteolytic bone disease. Plasma cells proliferate at a relatively low rate, with less than 1% of tumor cells actively synthesizing DNA during normal progression. Later stages of the disease exhibit greater proliferation as more mutations occur in the plasma cells and growth conditions in the bone marrow become more ideal.

MM cells are the transformed progeny of germinal and post-germinal centre plasma cells. Numerous cytogenetic changes are thought to be involved in the development of MGUS and the progression to MM. These genetic transformations have been divided into primary and secondary translocations, responsible for initiation and progression of the disease, respectively.

It is thought that the primary translocations occurring early on in tumorigenesis are mediated by errors in B-cell DNA modification processes involving Ig loci, primarily immunoglobulin heavy chain (IgH) switch recombination but also somatic hypermutation and VDJ recombination. These result in one or more genes being placed near strong Ig enhancers, DNA elements that increase the use of promoters near their location on the chromosome. Common groups of genes involved in primary translocations include cyclin D1 and D3, fibroblast growth factor receptor 3 (FGFR3), the nuclear protein MMSET, and the c-MAF and MAFB transcription factors.

Secondary translocations generally occur in post-germinal mature plasma cells no longer undergoing Ig rearrangements. The resulting translocations further dysregulate the expression or function of the cell, resulting in the molecular pathogenesis seen in the progression of both MGUS and MM. Two common examples of these translocations are c-MYC and an unidentified tumor suppressor on chromosome 13q – possibly unique to MM. These
mutations are associated with enhanced proliferation and a poorer prognosis, and not surprisingly their frequency is correlated with the stage of disease\textsuperscript{13}. Activating mutations of nRAS and kRAS are also common in MM and genetically distinguish it from MGUS\textsuperscript{23,24}. These mutations enhance signaling through the mitogen-activated protein kinase (MAPK) and PI3K pathways, enhancing growth and decreasing the interleukin-6 (IL-6) dependence\textsuperscript{25} of tumor cells. Other secondary translocations exist and are seen with greater frequency as the tumors become more proliferative at later stages of progression.

Biology of disease

A. The bone marrow microenvironment

The interactions between MM cells and the bone marrow (BM) microenvironment are responsible for much of the growth and survival signals mediated through various signaling pathways. The pathogenesis of MM is dependent on the continuous mutual communication between the malignant clones and the microenvironment, made up of bone-marrow stromal cells (BMSCs), vascular epithelial cells, osteoblasts, osteoclasts and lymphocytes along with extracellular matrix (ECM). This communication is mediated by both adhesion and growth factor receptor initiated signaling.

Homing of the myeloma cells to the bone marrow occurs by chemotaxis and selective binding of adhesion molecules on the plasma cells to the ECM and BMSCs. The very-late antigen-4 (VLA-4) on MM cells binds fibronectin, while the lymphocyte function-associated antigen-1 (LFA-1) on MM cells binds to the intracellular adhesion molecule-1 (ICAM-1) on BMSCs\textsuperscript{26}. Stromal-derived factor 1 (SDF1) and insulin-like growth factor 1 (IGF1) are
identified chemoattractants secreted by bone marrow endothelial cells and stromal cells that are involved in the homing and migration process. Also, vascular endothelial growth factor (VEGF) secreted by the BMSCs is involved the migration of tumor cells. Adhesion is also enhanced by the inflammatory cytokine tumor-necrosis factor-α (TNF-α) secreted by MM cells, which upregulates the cell-surface adhesion molecules on MM cells and BMSCs, a process mediated by nuclear factor-κB (NF-κB).

The adhesion of tumor cells enhances the reciprocal secretion of numerous paracrine growth factors necessary for pathogenesis, most importantly IL-6, IGF1, VEGF and TNF-α. IL-6 is the most well characterized cytokine related to MM. It is implicated in both autocrine and paracrine growth of tumor cells in the BM microenvironment, with increased transcription and secretion by BMSCs in the presence of MM cell-derived VEGF, TNF-α, and transforming growth factor-β (TGF-β). IL-6 is a strong survival and anti-apoptotic factor in myeloma cells, acting through the MAPK, Janus kinase (JAK) 2/signal transducers and activators of transcription (STAT) 3 and PI3K signaling cascades. IL-6 is necessary for development of B-cell neoplasms in vivo as shown in IL-6 knockout mice. Further, resistance to dexamethasone-induced apoptosis has been observed with IL-6 treatment, thereby implicating this cytokine in the conventional drug resistance seen in this disorder. IGF1 is another growth factor secreted by BMSCs that regulates proliferation and prevents apoptosis of myeloma clones, and it has been shown to be more potent than IL-6 in these roles. This is due to the stand-alone effects of IGF1 and its ability to augment the effects of IL-6 through the MAPK and PI3K pathways. Like IL-6, IGF1 inhibits dexamethasone-induced apoptosis, but IGF1 also is able to diminish the effects of other anti-MM drugs such as proteasome inhibitors. VEGF, previously mentioned in its role as a chemoattractant, is a known angiogenic factor in myeloma. It is produced by both
MM cells and the BMSCs, leading to increased angiogenesis in the BM environment. VEGF directly affects proliferation and apoptosis resistance of MM cells through the MAPK and PI3K pathways and indirectly through the upregulation of IL-6 production\textsuperscript{44,46}. TNF-\(\alpha\) does not have a significant effect on MM cell growth or drug resistance, but is the most potent stimulus of IL-6 and VEGF transcription and secretion by BMSCs, as well as upregulation of adhesion molecules on both MM and BMSCs, leading to cell adhesion-mediated drug resistance (CAM-DR)\textsuperscript{47}.

B. Signaling pathways in MM

The aforementioned adhesion of MM cells in the BM microenvironment and the subsequent stimulation of autocrine and paracrine cytokines activate a broad range of proliferative and anti-apoptotic signal transduction pathways. Although the initiation signals vary considerably between direct integrin binding-triggered cascades and between cytokines and their receptors, the downstream biological targets converge into a few major pathways that are responsible for the cellular events seen in MM.

Ras/Raf/MAPK

The proliferation of MM cells initiated by growth cytokines such as IL-6 and IGF1 is mediated through a number of signaling pathways including the Ras-dependent MAPK cascade\textsuperscript{30,41,44,48}. In addition, it has been shown that this pathway can be active during co-culture with BMSCs in the absence of IL-6\textsuperscript{49}, suggesting activation through cell-adhesion. However, abolition of MAPK cascade activity with various inhibitors is not sufficient to induce apoptosis\textsuperscript{41,49}. Ras protein transduces signals through the MAPK cascade, and activating Ras
mutations are seen at greater frequencies in advanced myeloma, which explains the enhanced proliferation seen at later stages and the poorer prognosis. Growth and survival signals also are transduced by oncogenic Ras through other pathways, most importantly PI3K, confirming the observation that MAPK inhibition alone is not a viable treatment option in MM. Further, significant cross-talk between the PI3K and the MAPK pathways exists and targeted disruption of the MAPK pathway is only effective in conjunction with PI3K signal abortion. Although MAPK signaling is constitutively activated in MM cells, its significance seems to be dwarfed by the PI3K pathway.

Jak2/STAT3

IL-6 is a potent activator of the Jak2/STAT3 pathway. As a result, this pathway is continuously active in many MM cell lines and patient samples while having minimal activity in healthy plasma cells lacking an IL-6-rich environment. This transduction pathway plays an important role in MM cell proliferation and drug resistance. STAT3 activation is necessary for transcription of the anti-apoptotic proteins B-cell lymphoma (Bcl)-XL and myeloid cell leukemia sequence (Mcl)-1, and this is emphasized by the observation that blocking the IL-6 receptor (IL-6R) induces apoptosis in some MM cell lines. However, co-culturing the tumor cells with BMSCs survive this IL-6R blockade, questioning the importance of IL-6 induced STAT3 signaling. Interaction with the BM microenvironment stimulates the production of other growth factors, activating IL-6-independent pathways that exert proliferative and survival responses in MM cells. This is consistent with the findings that MM cells can become IL-6-independent with disease progression.
The PI3K signal cascade is the most important transduction pathway in the pathogenesis of MM. This pathway is redundantly stimulated by IL-6, IGF1, VEGF, and the other aforementioned growth factors produced as a result of the complex interactions within the BM microenvironment\textsuperscript{37,41,60,61}. Activation of the PI3K enzyme produces the potent lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP\textsubscript{3}) from phosphatidylinositol-4,5-bisphosphate (PI-4,5-P\textsubscript{2}). PIP\textsubscript{3} is localized to the inner leaflet of the plasma membrane, where its initiation of downstream signaling through recruitment of proteins containing the lipid-binding pleckstrin homology (PH) domain, most importantly the serine/threonine protein kinase Akt (also known as protein kinase B)\textsuperscript{62,63}. Activated Akt subsequently phosphorylates downstream target molecules including glycogen synthase kinase (GSK)-3\(\beta\), forkhead transcriptional factor (FKHR), p70S6 kinase (p70S6K), NF-\(\kappa\)B and Bad, which mediate the proliferation, survival, cell cycle progression, migration and drug resistance seen in MM cells\textsuperscript{37,40,41,60}. Cross-stimulation of the PI3K enzyme and its downstream targets from other pathways is also observed\textsuperscript{41,51,64,65}, which further confirms the role of this cascade in disease progression.

Constitutive activation of PI3K and Akt through BM microenvironment stimulation and/or oncogenic mutations has been found in MM cell lines and in primary patient samples, while normal hematopoietic cells isolated from the same patients do not show elevated levels of activated Akt\textsuperscript{37,66,67}. Further, the level of Akt phosphorylation is correlated to the progression of the disease\textsuperscript{66,68}. Pharmacological inhibition of PI3K signaling with the Akt inhibitors LY-294002 or wortmannin inhibits proliferation and induces apoptosis in MM cells\textsuperscript{37,66}. This inhibition of signaling is also seen if a dominant-negative Akt construct is expressed\textsuperscript{66,69}. Conversely, the expression of a constitutively active Akt construct lead to increased downstream
signaling, enhanced proliferation, and protection from dexamethasone-induced apoptosis\textsuperscript{69}. Taken together, it is evident that the PI3K cascade is the most significant transduction pathway in neoplastic plasma cells. Moreover, abrogating this elevated signaling is an appealing and promising approach in MM therapy.

Therapy

A. Conventional therapy and the development of resistance

The treatment of MM is commenced upon diagnosis of symptomatic MM as there is no evidence of benefit in treating patients with asymptomatic MM\textsuperscript{70,71}. Conventional induction therapy has consisted of regimens of alkylating agents (eg. cyclophosphamide, melphalan), alkaloids (eg. vincristine), anthracyclines (eg. doxorubicin) and glucocorticoids (eg. dexamethasone, prednisone)\textsuperscript{7,8,72}. Prednisone and melphalan was the standard therapy for many years, with response rates of approximately 50\% and median survival of around 3 years\textsuperscript{73}. The addition of autologous stem cell transplantation in combination with high dose chemotherapy improved patient outcome in numerous studies\textsuperscript{9,74-76}. However, stem cell harvests were difficult after melphalan treatment due to its suppressive effects on the bone marrow. As a result, pretransplantation induction therapy more recently was a regimen of vincristine, doxorubicin and dexamethasone (VAD)\textsuperscript{72,77}. It was later found that high-dose dexamethasone was responsible for most of the response from VAD\textsuperscript{78}. 
B. Drug resistance

The vast majority of MM patients are responsive to these treatments initially, however, drug resistance is acquired in most cases\textsuperscript{79}. One mechanism of resistance is through the expression of P-glycoprotein (P-gp), a transmembrane protein encoded by the multidrug resistance (MDR) gene that functions as an efflux pump of substances thought to be harmful in the cell, such as chemotherapeutics and steroids. Expression of this protein and subsequent unresponsiveness to treatment are correlated with chemotherapy\textsuperscript{80,81}. Accordingly, the ability to re-sensitize tumor cells to conventional therapeutics is an interesting possibility for treatment.

The BM microenvironment also confers resistance to MM cells in two ways as previously mentioned. First, the binding of integrins such as VLA-4 on MM cells to fibronectin in the BM milieu imparts CAM-DR in tumor cells that is linked to G1 growth arrest\textsuperscript{82} and the upregulation of anti-apoptotic proteins\textsuperscript{47}. In addition, adhesion molecules on MM cells are overexpressed in drug-resistant lines and are upregulated upon treatment with cytotoxic agents\textsuperscript{83}. Second, the cytokines produced by the BMSCs and the plasma cells induce Jak2/STAT3 and PI3K signaling which mediate the resistance to conventional and novel therapeutics through upregulation of Bcl-X\textsubscript{L} and Mcl-1 and activation of Bad and NF-κB, respectively\textsuperscript{84,85}. Growth factors including IL-6 and IGF1 protect myeloma cell lines and patient cells from dexamethasone-induced apoptosis via stimulation of these pathways\textsuperscript{37,41}. Akt signaling downstream of PI3K has been demonstrated to be the most important mediator of drug resistance, as inhibiting the kinase in normal or drug-resistant cells is sufficient to restore sensitivity and induce apoptosis by abrogating NF-κB activation and the increase of anti-apoptotic proteins\textsuperscript{40}. Further, resistance to dexamethasone was overcome using rapamycin, an mTOR inhibitor, in MM cell lines and patient samples, and exogenous addition of growth factors...
did not overcome this responsiveness\textsuperscript{86}. This highlights the significance of the PI3K pathway in drug resistance, the primary reason why MM remains incurable.

C. Anti-MM drugs

Understanding the role the interactions in the tumor microenvironment play in the pathogenesis of the disease as well as delineating the molecular mechanisms responsible for MM disease progression has allowed for the development of various therapeutics, some of which are targeted with known mechanisms of action while others are used because they empirically have efficacy. Agents targeting myeloma cells and the BM milieu environment have shown great potential, while drugs aimed at specific molecular mechanisms of the myeloma cell also are showing success. Some of these therapeutics are discussed below.

Thalidomide and immunomodulatory derivatives

Thalidomide is a synthetic derivative of glutamic acid used as a sedative in the 1950’s, but its use was ceased due to their teratogenic effects\textsuperscript{87}. Then in the early 1990’s, thalidomide was reported to inhibit TNF-α synthesis by monocytes and other cellular sources and inhibit angiogenesis induced by VEGF and basic fibroblast growth factor (bFGF)\textsuperscript{88-90}. Because of the observed increased levels of angiogenesis in the BM of patients with MM\textsuperscript{91,92} and its correlation to a poorer prognosis, thalidomide was reevaluated as an anticancer therapeutic. The first clinical trial involving patients with refractory MM showed a benefit of 30-40% using thalidomide alone\textsuperscript{93}, and similar results were reported by others\textsuperscript{94}. Thalidomide showed further efficacy in combination with dexamethasone, with response rates increasing to approximately 50%\textsuperscript{95-97}. The addition of an alkylating agent to this regimen further increased response rates to
above 70%\textsuperscript{98,99}. This combination with conventional therapies shows greater success in part due to the inhibition of TNF-\(\alpha\), which upregulates adhesion molecules on BMSCs and MM cells that impart drug resistance through CAM-DR, as previously mentioned. Other notable effects of treatment are reducing MAPK and PI3K signaling through the inhibition of growth factor production, and increased activation of T-lymphocytes targeting MM cells\textsuperscript{100}. Thalidomide is now routinely administered in combination with other agents for relapsed and refractory myeloma, and also is indicated in the initial treatment of MM\textsuperscript{101}.

**Lenalidomide**

In light of the success of thalidomide in MM therapy, more active immunomodulatory derivatives (IMiDs) that posed fewer negative effects such as teratogenesis were sought. Lenalidomide (Revlimid\textsuperscript{\textregistered}, Celgene Corp.) is a thalidomide derivative shown in preclinical studies to be many times more potent a TNF-\(\alpha\) inhibitor, as well as more potently inducing apoptosis, downregulating adhesion molecules, inhibiting angiogenesis and promoting natural killer cell-mediated toxicity\textsuperscript{102-106}. Lenalidomide showed clinical activity in 66% of patients with refractory myeloma in one trial\textsuperscript{107}, with others ongoing\textsuperscript{108}. A response rate of 91% was demonstrated when administered with dexamethasone in newly diagnosed patients\textsuperscript{73}, again emphasizing the benefit of combining agents in the treatment of MM. Lenalidomide has been approved for use for relapsed and refractory myeloma.

**Bortezomib**

Bortezomib (Velcade\textsuperscript{\textregistered}, Millenium Pharmaceuticals, Inc.) is a boronic acid compound that binds and inhibits the 26S proteasome, preventing the degradation of ubiquitinated proteins in the cell. The cell cycle is driven by the proteolysis of cyclins, and oncogenic mutations can
cause dysregulated protein levels and lead to cancer\textsuperscript{109,110}. Proteasome inhibition causes the buildup of these pro-growth cell cycle proteins, leading to apoptosis\textsuperscript{111}. Bortezomib has been found to have a direct cytotoxic effect \textit{in vitro} and \textit{in vivo} in numerous cancer models, including prostate cancer, Burkitt’s lymphoma, and multiple myeloma\textsuperscript{111-113}. Proteasome inhibitors also target the MM cell in the BM milieu through preventing the activation of NF-κB, thereby reducing the expression of adhesion molecules and preventing CAM-DR and the upregulation of adhesion-dependent transcription and secretion of cytokines\textsuperscript{84,114}. The success of bortezomib led to the rapid translation to the clinic, where it showed a 35% response rate in patients with relapsed MM that was refractory to the last therapy\textsuperscript{115}, and was confirmed by another trial\textsuperscript{116}. Bortezomib also demonstrated an improved clinical outcome over high-dose dexamethasone treatment in refractory MM\textsuperscript{117}. Prolonged exposure to proteasome inhibitors results in the development of resistance in some cases through the overexpression of antiapoptotic proteins and the expression of MDR and related genes along with the resistance conferred by the BM microenvironment\textsuperscript{40,118-120}. However, the addition of dexamethasone to patients showing a suboptimal response to bortezomib and refractory to earlier treatment, in some cases dexamethasone, was associated with an improved response\textsuperscript{121}. Bortezomib is approved for use in refractory or relapsed MM, and other targets are being investigated as targets to sensitize tumor cells to proteasome inhibition\textsuperscript{122}.

**Arsenic trioxide**

Arsenic trioxide (As\textsubscript{2}O\textsubscript{3}) recently has been used in MM due to its ability to induce apoptosis by means other than those familiar to conventional therapies. As\textsubscript{2}O\textsubscript{3} induces apoptosis of normal and drug-resistant MM cells through downregulation of antiapoptosis proteins and activation of caspase-9. It also targets MM cells in the BM microenvironment by inhibiting
binding to BMSCs and subsequent IL-6 and VEGF secretion as well as adhesion-induced proliferation\textsuperscript{123-125}. Clinical trials using As\textsubscript{2}O\textsubscript{3} in patients with refractory MM have shown some beneficial response\textsuperscript{126,127}, and preliminary trials in combination with other therapies report similar findings\textsuperscript{128}.

Other targeted therapies

There are numerous new agents in preclinical or early clinical development for MM. These agents target one or multiple facets of the tumor cell or its environment. There are intracellular signal cascade inhibitors, including Akt and mTOR inhibitors for the PI3K pathway, and p38 MAPK and MEK/ERK inhibitors for the MAPK pathway. There are also cytokine receptor kinase inhibitors under development, including those targeting the cellular receptors for IGF1, VEGF and TGF-\textbeta. Many others exist but will not be discussed here.

The knowledge of myeloma cell-BM interactions and their role in disease progression has allowed for the establishment of the vast number of targets. This has made myeloma a new paradigm for cancer treatment as a whole, whereby intimate knowledge of the pathobiology of the malignancy allows for multiple target identification, drug validation and rapid translation from the bench to the clinic\textsuperscript{129}. Novel agents are welcome in the study of MM, as it remains incurable. However, the successes reported from combination therapies mentioned earlier suggest future therapies administered in tandem with the new and old could greatly improve patient outcome.
The PI3K pathway

A. Endogenous regulation

The importance of the elevated PI3K transduction pathway in the pathogenesis of MM has already been detailed. The second messenger PIP₃ initiates the downstream cascade after being produced by the PI3K enzyme. Cellular PIP₃ levels are kept under strict homeostatic control through regulation of its production by the PI3K enzyme and by the action of inhibitory lipid phosphatases that degrade PIP₃ and terminate signaling (Figure 1). There are three main phosphatases that degrade PIP₃: the 3'-phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) produces PI-4,5-P₂, and the 5'-phosphatases SHIP (Src homology 2-containing inositol 5'-phosphatase) and SHIP2 produce PI-3,4-P₂.

PTEN is a known tumor suppressor, and loss of its expression has been well documented in numerous solid tissue cancers. In MM, some cell lines are PTEN-null and as a result show higher Akt phosphorylation. In addition, these cells are more sensitive to killing by agents targeting the PI3K pathway. It is also observed that expressing PTEN in PTEN-deficient MM lines abolishes tumor growth in vitro and in mouse xenograft models. Taken together, these data emphasize the importance of the PTEN phosphatase in controlling the PI3K pathway.

The expression of the SHIP phosphatase is limited to cells of hematopoetic origin, while SHIP2 is ubiquitously expressed. SHIP has been demonstrated to be a key regulator of signaling in hematopoetic cells by degrading PIP₃, and the expression of a SHIP construct results in increased apoptosis. Specifically, it has been shown to be essential in negative regulation of B cell receptor (BCR) signaling mediated by FcγRIIB receptor-BCR coligation and suppression of Akt signaling in stimulated B cells and mast cells. Additionally, stimulated SHIP⁻/⁻ B
cells show enhanced proliferation and reduced apoptosis\textsuperscript{145}. Studies with SHIP\textsuperscript{−/−} mice have revealed a myeloproliferative phenotype, with hyper-responsiveness to inflammatory stimuli, severe osteoporosis and a shortened lifespan\textsuperscript{146,147}, confirming the role the phosphatase plays in modulating signaling within the hematopoietic system.

The loss or decreased expression of SHIP has roles in human disease. Lower levels of SHIP has been observed in hyperallergenic patients through the increased release of histamine by basophils\textsuperscript{148}. More relevant to MM, reduced expression or the loss of SHIP has been observed in some leukemias\textsuperscript{149-153} and the activity of SHIP can differ after drug treatment in some hematological malignancies\textsuperscript{154,155}.
Figure 1. The PI3K pathway. PI3K produces PIP₃, which activates Akt. Akt then mediates the downstream signaling of many targets involved in cancer cell proliferation and survival. The phosphatases PTEN and SHIP degrade PIP₃, thereby reducing signaling through the PI3K pathway. Figure adapted from Vivanco and Sawyers, *Nature Reviews Cancer*. 2002; 2:489-501.
B. Targeting the PI3K pathway

Due to the central role that the PI3K pathway plays in many malignancies including MM, it has been the subject of intense efforts to identify and develop inhibitors of the various kinases in the cascade. The PI3K enzyme inhibitors wortmannin and LY-294002 routinely used in the laboratory globally inhibit all isoforms of the enzyme expressed in all cell types and therefore have no potential in a clinical setting. Rather, isoform-specific inhibitors of the PI3K enzyme show greater potential benefit\textsuperscript{156-159}. The PI3K\textgreek{y} isoform, primarily expressed in immune cells, is involved in mediating G-protein coupled receptor (GPCR) signaling, and a PI3K\textgreek{y} inhibitor was recently described as being protective in mouse models of inflammatory disorders including rheumatoid arthritis and lupus\textsuperscript{160,161}. The PI3K\textalpha{} isoform is mutated in numerous solid tumors\textsuperscript{162}, and a dual PI3K\textalpha{}/mTOR inhibitor\textsuperscript{163} was shown effective in a human glioma xenograft model\textsuperscript{164} with no reported toxicities, despite the role of PI3K\textalpha{} plays in insulin signaling in all tissues\textsuperscript{165}.

Experimental inhibitors are also targeting downstream kinases. Akt inhibitors also have been identified and have shown moderate success in clinical trials with some cancers\textsuperscript{166-168}, and show potential in MM based on preclinical data\textsuperscript{169}. The mTOR inhibitors rapamycin and CCI-779 have also shown \textit{in vitro} and \textit{in vivo} killing of MM cells\textsuperscript{135,170,171}. However, the activation of Akt through enhanced IGF1R signaling is also observed\textsuperscript{172}, consequently its use must be in tandem with other agents that induce apoptosis such as bortezomib. Although there is promise in targeting these kinases, their universal expression again raises toxicity issues.
C. SHIP as a target

An alternative to abrogating PI3K signaling with kinase inhibitors is through activation of the endogenous negative regulators, the lipid phosphatases PTEN and SHIP. Small molecule allosteric activators of SHIP would be an advantageous alternative due to its ability to control PI3K signaling and its hematopoietic-restricted expression. Allosteric regulation of the SHIP family molecules has been proposed through intra- or intermolecular interactions\textsuperscript{140}. Moreover, allosteric activation of the 3'-inositol phosphatases PTEN and myotubularin by selective phosphoinositides has been shown\textsuperscript{173,174}, justifying research into potential allosteric SHIP agonists for the treatment of enhanced PI3K signaling in hematopoietic disorders.

D. SHIP activators

Extracts from the marine invertebrate library of collaborator Dr. Raymond Andersen were screened to potentially identify small molecules that influence the phosphatase activity of SHIP\textsuperscript{175}. The compound showing the most promising activity, isolated from the Papua New Guinea sponge \textit{Dactylospongia elegans}, was subjected to enzyme-guided fractionation. The structure of the active component was solved and identified as the meroterpenoid, pelerol\textsuperscript{175}. This compound was independently identified by two other groups, which noted the interesting molecular structure\textsuperscript{176,177}, however neither party investigated its activity in mammalian cells as a potential phosphatase activator.

The limited natural supply of pelerol prompted the development of a method to chemically synthesize the compound in order to produce the quantities needed for study in biological assays. An efficient nine-step protocol to produce pelerol was developed from sclareolide\textsuperscript{175}. During the process, the intermediates of synthesis of pelerol were tested as SHIP...
activators. The intermediate designated AQX-016A was found to have a significantly greater effect on SHIP activation\textsuperscript{175}. However, the structural analog of AQX-016A designated AQX-MN100, also showing greater SHIP activation potential than pelerol, was primarily used for these studies due to the possibility of the off-target effects of AQX-016A, which is discussed later.

The allosteric SHIP agonists have been shown to selectively activate the SHIP phosphatase by binding an activation domain within the enzyme while having minimal effects on SHIP2\textsuperscript{178}. They also have been shown to stimulate SHIP activity in in vitro macrophage and mast cell assays and protect against endotoxemia and acute cutaneous anaphylaxis in mouse models\textsuperscript{178}.

Hypothesis

Due to the critical role PI3K signaling plays in MM pathogenesis and the successes reported using SHIP activators in inflammation, it was hypothesized that allosteric SHIP activators could offer potential as anti-neoplastic agents. Herein, we describe the ability of the small molecule allosteric activators of SHIP phosphatase activity AQX-016A and AQX-MN100 to inhibit PI3K signaling, thereby decreasing proliferation and inducing apoptosis of MM cells in cellular assays and in xenograft tumor models. This study provides the framework for clinical trials using SHIP agonists to improve MM patient outcome.
MATERIALS AND METHODS

SHIP activators

Both SHIP agonists were obtained from Dr. Ray Andersen (University of British Columbia; Vancouver, Canada). The compound AQX-016A was synthesized as previously described\textsuperscript{175}, while the compound AQX-MN100 was synthesized from AQX-016A as detailed in a forthcoming manuscript (Nodwell, M. and Andersen, R; \textit{in preparation}). The majority of experiments were carried out with AQX-MN100 solubilized in 95\% ethanol and diluted into media containing at least 10\% fetal bovine serum (FBS) for cell-based assays. The amount of AQX-MN100 actually in solution was quantified using tritiated compound in parallel experiments. The compound AQX-016A was delivered using cyclodextran (Cyclodex Technologies; High Springs, FL) as a caging carrier vehicle in cellular assays at a concentration of 6 mM (2 mg/mL). For animal administration, AQX-MN100 was solubilized in walnut oil and administered at 50 mg/mL.

Animals

Non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice (6-8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). They were maintained in a specific pathogen-free area in the Jack Bell Research Centre animal facility. The University of British Columbia Animal Care Committee approved all animal studies conducted.

Cells and reagents

Five MM cell lines and 2 control carcinoma lines were used in this study. The MM lines RPMI 8226 and U266 and the prostate carcinoma line LNCaP were obtained from the American
Type Culture Collection (Rockville, MD). The MM lines OPM1 and OPM2 were kindly provided by Dr. Jonathan Keats (Mayo Clinic; Scottsdale, AZ). The MM.1S line was provided by Dr. Steven Rosen (Northwestern University; Chicago, IL). The breast carcinoma line LCC6-Her2 was provided by Dr Michael Cox (University of British Columbia; Vancouver, Canada). Primary murine B-lymphocytes were obtained from wild type and SHIP⁻/⁻ C57B1/6J spleens kindly provided by Dr. Laura Sly (University of British Columbia; Vancouver, Canada), who also provided the mast cells from wild type and SHIP⁻/⁻ C57B1/6J mice. The preparation of mast cells has been previously described¹⁷⁹. MM cell lines and primary murine B-lymphocytes were maintained in RPMI-1640 medium containing 10% FBS and 2 µM L-glutamine. The experiments involving MM cell lines were carried out in RPMI-1640 medium containing 5% FBS and 2 µM L-glutamine. Non-hematopoetic cell lines were maintained in DMEM supplemented with 9% FBS. Mast cells were maintained in IMDM medium containing 15% FBS and 30 ng/mL interleukin-3. All cells were propagated at 37°C and 5% CO₂. All chemicals and reagents were obtained from Sigma-Aldrich (Oakville, Canada) unless otherwise indicated.

Primary murine B-lymphocyte purification

B-lymphocytes were isolated from total spleen cells of wild type and SHIP⁻/⁻ C57BL/6J mice. The generation of the knockout strain has been previously described¹⁴⁶. Spleens were first removed from the mice, and dispersed into a single cell suspension by maceration through a 0.45 µm cell strainer. Mononuclear cells were then purified by layering the cell suspension over ficoll-paque and centrifuging for 25 minutes at 1800 revolutions per minute. The collected mononuclear cells were then subjected to further purification by suspension in a hypotonic red cell lysis buffer for 5 minutes to remove any remaining erythrocytes. B-lymphocytes were purified from the mononuclear cells by negative selection using the EasySep separation kit.
(StemCell Technologies; Vancouver, Canada) and following the provided protocol of the manufacturer.

**Thymidine incorporation**

DNA synthesis was measured by thymidine incorporation. The MM cell lines were cultured in 96 well plates seeded with \(3 \times 10^4\) cells suspended in 200 µL of medium along with various concentrations of AQX-MN100 or AQX-016A (and associated cyclodextran vehicle control), with LY294002 serving as a positive control in the indicated experiments. Experiments with AQX-016A ran for 24 hours total, while experiments with AQX-MN100 were for 48 hours; both had 1 µCi of \(^{3}H\)-thymidine (GE Healthcare, Baie D’Urfe, Canada) being added for the final 8 hours of the culture. Non-hematopoetic carcinoma lines were plated at \(1 \times 10^4\) cells/well, allowed to adhere overnight, and then cultured with fresh media containing AQX-016A, AQX-MN100 or LY294002 for 32 hours (AQX-MN100 experiments) or 24 hours (AQX-016A experiments), with 1 µCi of \(^{3}H\)-thymidine added for the final 6 hours of culture. Freezing the plates terminated the experiments and aided in cell lysis. Cells were then harvested onto glass fibre filters using an automatic cell harvester (TomTech; Orange, CT) and radioactivity was measured via liquid scintillation counting using a Wallac Microbeta counter (Perkin-Elmer; Boston, MA). Proliferation is determined indirectly by the quantification of radiolabeled thymidine incorporated into the genomes of cells in each culture. Wells were plated in triplicate.

**Analysis of cell survival**

The ability of SHIP activators to reduce tumor cell survival was assessed in MM cell lines treated with AQX-MN100 or AQX-016A. The lines OPM1, OPM2, MM.1S and RPMI 8226 were plated at a density of \(1 \times 10^5\) cells/mL in 200 µL of medium with various
concentrations of AQX-MN100, and viable cell numbers were determined on day 3 and day 5 by trypan blue exclusion. The lines RPMI 8226 and U266 were plated at a density of $1 \times 10^6$ cells/mL in 250 µL of medium with various concentrations of AQX-016A. At day 4, the medium of each culture was replaced by fresh medium containing the same concentration of AQX-016A. At day 7, the viable cell number of each culture was determined by trypan blue exclusion. Wells were plated in triplicate.

Detection of apoptosis

The induction of apoptosis by treatment with SHIP activators was detected by propidium iodide (PI) staining. OPM2 and MM.1S cell lines were plated at $5 \times 10^5$ cells/mL in 200 µL of medium along with various concentrations of AQX-MN100 for 1, 4 and 6 hours. Then, each culture was washed and resuspended in fresh, compound-free medium for the remainder of a 24-hour period. RPMI 8226 and U266 cell lines were plated at $1 \times 10^6$ cells/mL in 250 µL of medium containing various concentrations of AQX-016A and cultured for 24 hours.

At the end of the 24-hour period for both SHIP activator assays, cells were washed once with phosphate-buffered saline (PBS) and fixed with ice-cold 80% ethanol for 24 hours at 4°C. Samples were then washed with staining buffer (1x PBS, 0.1% Triton X-100, 0.1 mM EDTA) before being resuspended in staining buffer with 50 µg/mL DNase-free RNase (Roche Diagnostics; Montreal, Canada) and 50 µg/mL PI. After incubation at room temperature for one hour, the percentages of apoptotic and cycling cells were determined by flow cytometry using an Epics XL cytometer (Coulter Immunology; Hialeah, FL). Sub-G₀ gated cells are considered to be apoptotic. Results are representative of triplicate samples.
SHIP specificity

To assess the specificity of AQX-MN100 to the SHIP phosphatase, freshly-purified primary murine B-lymphocytes isolated from wild-type and SHIP<sup>−/−</sup> C57Bbl/6J mice were cultured overnight in a 10-cm Petri dish with 10 μg/mL of the F(ab')<sub>2</sub> fragment of goat anti-mouse IgM antibody (Jackson Immunoresearch; West Grove, PA) to initiate cycling.

To assess viability, the following day cells were seeded at 5x10<sup>5</sup> cells/mL in 200 μL of medium along with 10 μg/mL IgM F(ab')<sub>2</sub> fragment and a range of AQX-MN100 drug concentrations. After 24 hours of culture, SHIP specificity was determined by the percentage of viable cells relative to the untreated groups of wild type and SHIP<sup>−/−</sup> B-lymphocytes as established by trypan blue exclusion. Experiments were performed in triplicate samples.

To assess the degree of apoptosis, wild type and SHIP<sup>−/−</sup> B-lymphocytes were seeded at 1 x 10<sup>6</sup> cells/mL in 200 μL of medium along with 10 μg/mL IgM F(ab')<sub>2</sub> fragment and a range of AQX-MN100 drug concentrations. After 24 hours of culture, cells were washed once with PBS and fixed with ice-cold 80% ethanol for 24 hours at 4°C. They were then stained with PI and analyzed by flow cytometry as described above.

The specificity of AQX-016A to the SHIP phosphatase was tested using mast cells isolated from wild-type and SHIP<sup>−/−</sup> C57Bbl/6J mice. Cultures were seeded with 3 x 10<sup>4</sup> cells in 200 μL medium along with various concentrations of AQX-016A for 48 hours with 20 μL of Alamar Blue reduction dye (Biosource International; Camarillo, CA) being added for the final 6 hours of culture. Proliferation was determined by the percentage of reduction relative to the untreated groups of wild type and SHIP<sup>−/−</sup> mast cells as measured on a microtitre plate spectrophotometer. Wells were plated in triplicate.
Western blot analysis

To examine the effect of AQX-MN100 on PI3K signaling and cleavage of pro-apoptotic proteins, OPM2 and MM.1S cell lines were treated with various concentrations of AQX-MN100 at a density of 1 x 10^6 cells/mL for various times. Samples were lysed at 1x10^7 cells/mL in sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris, 80 mM SDS, 5% mercaptoethanol, 8.75% glycerol), then sonicated for 20 seconds on ice. Equal volumes of sample were boiled, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto an Immobilon polyvinylidene difluoride membrane (Millipore; Bedford, MA) for 1 hour at 15 V. Blots were blocked with 3% bovine serum albumin (BSA) in tris-buffered saline (TBS) solution containing 0.1% Tween-20 for 1 hour before overnight incubation with primary antibodies (Cell Signaling; Mississauga, Canada) against pAkt (Ser 473), Akt, pGSK3β, pFKHR, p-p70S6 kinase, poly(ADP-ribose) polymerase (PARP), and cleaved PARP. Following this, the blots were incubated with fluorescing secondary antibodies (Invitrogen; Burlington, Canada) and imaged on a LI-COR Odyssey system (LI-COR; Lincoln, NB).

Enhanced cytotoxicity of current therapeutics

OPM2 MM cells were seeded with 3 x 10^4 cells/well in 200 μL medium along with various concentrations of dexamethasone or bortezomib (Millennium Pharmaceuticals; Cambridge, MA). AQX-MN100 or control media was also added to these samples to assess whether activation of SHIP enhances the cytotoxicity of conventional or emerging therapeutics. The cells were then cultured for 48 hours, with 1 μCi of [3H]-thymidine added for the final 8 hours of the assay. The plates were then frozen to terminate the experiment, and proliferation
was quantified using liquid scintillation counting as previously described. Results are representative of triplicate samples.

Lentiviral infection of MM cells

A firefly luciferase construct was introduced into OPM2 and MM.1S cell lines using a lentiviral vector. Cells were plated in a 96-well round-bottom well at $1 \times 10^5$ cells/well in 20 µL of medium. A lentiviral stock was then added to the wells, along with 8 µg/mL protamine sulfate to aid in infection. Cells were left overnight and diluted with fresh medium the following day. Successful infection was determined by adding firefly D-luciferin (Xenogen Corporation; Alameda, CA) to in vitro cultures and qualifying luminescence with a Xenogen IVIS 200 imaging system (Xenogen Corporation; Alameda, CA).

Xenograft murine model

Mice were inoculated with two tumors of $3 \times 10^6$ OPM2 cells expressing a luciferase construct suspended in 50 µL of growth medium and 50 µL of Matrigel basement membrane matrix (Becton Dickenson; Bedford, MA). Tumors were injected subcutaneously in the upper and lower flanks of the mice and allowed to establish for 2 weeks. After 2 weeks, AQX-MN100 or control vehicle was administered in a subcutaneous oil depot at a dose of 50 mg/kg every 3 days.

Tumors were measured using bioluminescence imaging on the Xenogen IVIS 200. Mice received intra-peritoneal injections of 200 µL of D-luciferin at 3.75 mg/mL in sterile PBS. Mice were then anesthetized with isofluorane and imaged 15 minutes post-injection of luciferin. Quantification of tumor size was performed using the Living Image™ software provided with the machine.
RESULTS

Two candidate SHIP phosphatase agonists were used in this report. AQX-016A was the original synthetic intermediate of pelerol identified as an allosteric activator, and initial studies were completed with this compound. However, the presence of a catechol moiety within the structure of AQX-016A raised the issue of potential off-target effects (Figure 2a). Catechols can exert effects unrelated to the binding of the agonist in the activation domain of the enzyme, such as their oxidation to form damaging reactive oxygen species\textsuperscript{180}. To address this concern, a structural analog of AQX-016A with the catechol group removed was synthesized, AQX-MN100 (Figure 2b). This analog displayed increased SHIP enzyme activity \textit{in vitro} and showed similar activity in inflammation assays as was seen with AQX-016A\textsuperscript{178}. Therefore, further studies – comprising the majority of experiments – were completed using AQX-MN100. For each experiment, the results with the lead candidate AQX-MN100 are described first, followed by the data using AQX-016A if applicable.
Figure 2. The lead candidate SHIP activators. (A) The SHIP activator AQX-016A is a synthetic intermediate of pelerol. (B) AQX-MN100 was synthesized from AQX-016A and is the lead drug candidate.
Activation of SHIP inhibits phosphorylation of Akt and downstream effectors

We first wanted to see the ability of SHIP agonists to inhibit the phosphorylation of Akt and the kinases downstream of Akt that mediate the growth and survival signals for MM progression. All MM cell lines used in this study show elevated Akt phosphorylation at the serine 473 residue, which is required for Akt activation\(^{181}\), but the OPM2 line was used in the signaling experiments because it showed the greatest basal Akt phosphorylation due to the loss of PTEN expression\(^{137}\). OPM2 cells treated with 9.6 \(\mu\)M AQX-MN100 for 0.5 to 6 hours showed significant inhibition of Akt phosphorylation in a time-dependent fashion (Figure 3a), while no major differences in Akt protein levels were observed, which also served as the loading control. Phosphorylation of important downstream targets of Akt, including GSK3\(\beta\), FKHR and p70S6 kinase, was also largely inhibited as expected. We then looked at the effect of treatment with a range of AQX-MN100 concentrations (2.4-9.6 \(\mu\)M) for 6 hours and observed a dose-dependent inhibition of Akt phosphorylation and the same target kinases as before (Figure 3b). The results indicate that activation of SHIP using AQX-MN100 is sufficient to inhibit the Akt activity seen in MM cell lines, despite the elevated levels resulting from loss of the PTEN phosphatase, thereby preventing signaling in this critical pathway.
Figure 3. SHIP activation inhibits phosphorylation of Akt and downstream targets. (A) OPM2 cells were cultured with AQX-MN100 (9.6 μM) for the indicated time periods and (B) with the indicated concentrations of AQX-MN100 for 6 hours, then lysed in SDS-gel sample buffer and subjected to SDS-PAGE. Immunoblotting was performed with antibodies against pAkt (Ser 473), Akt, pGSK3β, pFKHR and p-p70S6K, with Akt serving as a loading control. This experiment was performed two times.
Activation of SHIP inhibits thymidine incorporation of human MM cell lines

To assess the ability of AQX-MN100 to inhibit DNA synthesis, an indirect measurement of proliferation, uptake of \(^{3}\text{H}\text{-thymidine}\) was measured in cultures of OPM2 and MM.1S cells treated with a range of doses of the experimental compound for 48 hours. All cell lines showed significant inhibition of DNA synthesis in a dose-dependent fashion (Figure 4a-b). The kinase inhibitor LY-294002 was used as a positive control. This potent suppressor of PI3K signaling showed similar inhibition at 10 \(\mu\text{M}\) as that of 9.6 \(\mu\text{M}\) AQX-MN100. A concentration of 4.8 \(\mu\text{M}\) was needed to hinder thymidine incorporation in OPM2 cells, while the MM.1S line showed much greater sensitivity with almost complete inhibition seen at the same concentration. Similar results were obtained with the OPM1 and RPMI 8226 myeloma cell lines (data not shown).

To address the specificity of AQX-MN100 as an activator of SHIP, the non-hematopoetic prostate carcinoma cell line LNCaP and the breast carcinoma line LCC6-Her2 were treated with the experimental compound for 32 hours. These two lines also have enhanced PI3K signaling that is required for their survival; therefore targeting this signal cascade would effectively inhibit DNA synthesis. In addition, LNCaP cells have lost PTEN expression, suggesting an enhanced ability\(^{136,182}\) to inhibit DNA synthesis by agents targeting the pathway. Indeed this is seen using LY-294002 at the 10 \(\mu\text{M}\) dose proven sufficient in MM lines, but the SHIP activator AQX-MN100 shows no significant effect on DNA synthesis at doses that fully inhibit the hematopoetic MM lines (Figure 4c-d). These data demonstrate the ability of AQX-MN100 to specifically inhibit thymidine incorporation of PI3K-dependent MM cells expressing the SHIP phosphatase while having no major effect on non-hematopoetic cancer cells also dependent on this transduction pathway.
The SHIP activator AQX-016A was also shown to inhibit DNA synthesis of the MM lines RPMI 8226 and U266 (Figure 4e-f) in a 24-hour assay. These findings suggest that AQX-016A may be less potent a SHIP activator, as even as high a concentration as 18 μM only inhibited thymidine incorporation to 40% of the control. However, these results are from 24 hours of treatment, and a longer time of exposure would result in greater killing. It was these data produced early in this project that lead to the decision to extend the length of the assay. Further complicating the direct comparison of the efficacy of AQX-016A and AQX-MN100 is that AQX-016A was solubilized in cyclodextran, which we later realized had its own effects on MM cells. We observed cyclodextran treatment of MM cells resulted in variable effects, including stimulation of thymidine incorporation in some instances as can be seen in the U266 cell line. This effect caused by the carrier vehicle also may explain the decreased sensitivity of the MM lines to AQX-016A. This finding led us to terminate the use of cyclodextran as a delivery vehicle. As was the case with the other SHIP activator, AQX-016A showed no significant effect on DNA synthesis in the breast carcinoma line LCC6-Her2 over a 24 hour time period as compared to the cyclodextran vehicle (Figure 4g).
Figure 4. SHIP activation inhibits thymidine incorporation of MM cell lines but does not inhibit thymidine incorporation in SHIP-deficient carcinoma lines. (A-B) The MM lines OPM2 and MM.1S were cultured for 48 hours and (C-D) the non-hematopoetic prostate carcinoma line LNCaP and breast carcinoma line LCC6-Her2 were cultured for 32 hours with
the indicated concentrations of AQX-MN100 or LY-294002 (10 μM). (E-F) The MM lines RPMI 8226 and U266 and (G) the non-hematopoetic breast carcinoma line LCC6-Her2 were cultured for 24 hours with the indicated concentrations of AQX-016A or an equivalent volume of vehicle control. DNA synthesis was measured by adding [3H] thymidine for the final 8 hours (MM lines) or 6 hours (non-hematopoetic lines) of culture and measuring incorporation on a scintillation counter. Data represent mean counts per minute (cpm) ± SD of triplicate cultures. These experiments were performed at least three times.
Activation of SHIP decreases MM cell viability

To demonstrate the effect of AQX-MN100 on the survival of MM cells over an extended time frame, the lines OPM1, OPM2, MM.1S and RPMI 8226 were cultured with the experimental compound at concentrations up to 7.2 μM and viability was determined by trypan blue exclusion at day 3 and day 5. At day 3, all cell lines showed a notable reduction in viability in a dose-dependent fashion, with IC_{50} values of 4 μM or below (Figure 5a). No major differences to day 3 were seen when viability was measured at day 5 (Figure 5b), although the cultures did not increase in cell number and replicate samples showed much less variation between them. It is possible that the compound is degraded into an inactive metabolite or precipitates out of solution over time, and this is why little difference is seen between day 3 and day 5. As previously observed, the MM.1S line was the most sensitive to AQX-MN100 treatment, while the OPM1 line showed the greatest ability to resist death at both days assessed. These data demonstrate that extending exposure time to AQX-MN100 past 48 hours as done in the proliferation experiments – at least to 3 days – lowers the apparent IC_{50} value in all lines tested.

AQX-016A was also shown to decrease survival of the MM lines RPMI 8226 and U266. Cell viability decreased in a dose-dependent fashion over a 7-day assay (Figure 5c). Again, the results suggest that AQX-016A is not as effective as AQX-MN100 as a SHIP agonist, with IC_{50} values of 5 μM or above. Degradation of the compound is a possible explanation as before. However, a more likely explanation is the density of the cell cultures in this experiment. A greater number of cells in the same volume of medium would show reduced effects of the drug at the same concentrations due to the greater production of autocrine and paracrine growth factors that would enhance pro-survival signals. This experiment was completed early on in the project,
and further survival assays were done at lower cell concentrations, and for shorter time periods. Taken together with the thymidine incorporation data, it is clear that SHIP phosphatase activation with AQX-MN100 or AQX-016A is sufficient to prevent the growth and survival of MM cell cultures.
Figure 5. Viable MM cell numbers are significantly reduced by SHIP activation. The MM lines OPM1, OPM2, RPMI 8226 and MM.1S were cultured with the indicated concentrations of AQX-MN100 for (A) 3 days and (B) 5 days. IC₅₀ values for cell lines after 3 days of AQX-MN100 treatment: OPM1 3 μM; OPM2 3.4 μM; RPMI 8226 3.2 μM; MM.1S 2.8 μM. (C) The MM lines RPMI 8226 and U266 were cultured with the indicated concentrations of AQX-016A for 7 days. IC₅₀ values for cell lines treated with AQX-016A: RPMI 8226 12.5 μM, U266 4.5 μM. Viable cell numbers were determined by trypan blue exclusion. Data represent mean cell numbers ± SD of triplicate cultures. These experiments were performed at least two times.
Activation of SHIP induces MM cell apoptosis

To further confirm that AQX-MN100 was inducing cytotoxicity in MM cell lines, drug-treated cultures of OPM2 and MM.1S were stained with PI and cell cycle analysis was conducted by flow cytometry. The MM cells were treated for 1, 4 or 6 hour(s), with each culture being resuspended in fresh medium after treatment for the remainder of a 24-hour period. This was done to determine the drug concentration and the time of exposure required before MM cells committed to an apoptotic phenotype. Both OPM2 and MM.1S cells showed a dose-dependent increase in sub-G₀ PI staining, indicative of apoptosis, and the percentage of cells undergoing apoptosis at all concentrations is proportional to the length of exposure to AQX-MN100 (Figure 6a). The MM.1S cells again showed greater sensitivity to treatment with the experimental compound, while OPM2 cells were somewhat resistant to AQX-MN100 until the drug concentration rose above 5 μM.

The above findings with AQX-MN100 were confirmed by western analysis of PARP cleavage. PARP, essential for DNA repair, is an important caspase target and its cleavage is a well-established marker of cells undergoing apoptosis. High-dose (9.6 μM) treatment rapidly induced PARP cleavage in a time-dependent manner in both OPM2 and MM.1S cells (Figure 6b). These results confirm that the cytotoxicity in MM cells observed after treatment with the PI3K signaling inhibitor occurs via induction of apoptosis and is mediated by the caspase cascade.

The SHIP agonist AQX-016A induced apoptosis of RPMI 8226 and U266 MM cells in a dose-dependent manner over a 24-hour period of culture (Figure 6c). As with other experiments with this SHIP activator, the cyclodextran carrier and the higher cell density resulted in less
profound effects than seen in the thymidine incorporation assay, and these errors in experimental
design and setup were not repeated in subsequent AQX-MN100 assays.
Figure 6. SHIP activation induces MM cell apoptosis. (A) OPM2 and MM.1S cells were incubated with increasing doses of AQX-MN100 for the indicated times, then cultured in fresh media for the remainder of a 24 hour period. Cells were then fixed and stained with propidium iodide to assess cell cycle profile. Data represent the mean percentage of cells in the sub-G₁ (apoptotic) phase ± SD of triplicate cultures. (B) OPM2 and MM.1S cells were incubated with AQX-MN100 (9.6 μM) for the indicated time periods, then lysed in sample buffer and subjected to SDS-PAGE. Immunoblotting was performed with antibodies against PARP and its cleaved fragment. These experiments were performed at least 3 times. (C) RPMI 8226 and U266 cells were cultured with the indicated concentrations of AQX-016A for 24 hours, then stained with propidium iodide and analyzed by flow cytometry to assess degree of apoptosis. This experiment was performed two times.
The experimental SHIP activators selectively act on wild-type primary murine cells versus SHIP<sup>−/−</sup> cells

The ability to specifically activate the SHIP phosphatase in hematopoetic cells while having minimal effects on surrounding cells is crucial for the further development of AQX-MN100 or other SHIP agonists as a regulator of elevated PI3K signaling. Therefore, we set out to determine the effect of the experimental compound in B-lymphocytes isolated from the spleens of wild-type and SHIP<sup>−/−</sup> C57Bl/6J mice as a way to assess the specificity of the compounds for SHIP. Stimulation of the BCR has been shown to increase PIP<sub>3</sub> levels and Akt phosphorylation in B cells<sup>143</sup>, so we cultured wild-type and SHIP<sup>−/−</sup> primary cells with the F(ab')<sub>2</sub> fragment of IgM to induce PI3K activity without producing inhibitory signaling in the wild-type cells caused by the coligation of the BCR and Fcγ receptor IIB, which is mediated by SHIP<sup>141</sup>. Cells were incubated overnight with the stimulatory IgM F(ab')<sub>2</sub> fragment, then cultures were supplemented with a range of concentrations of AQX-MN100. Cell viability measured after 24 hours of culture by trypan blue exclusion yielded a significant difference in survival between the wild-type and SHIP<sup>−/−</sup> at AQX-MN100 concentrations of approximately 2 μM and above (Figure 7a). An important observation is the lack of cell death seen below this concentration; this can be attributed to the differences between the cell types used. The MM cell lines have elevated PI3K signaling and are reliant on such activity for their survival. However, the murine B-lymphocytes have been stimulated through their BCR, thereby elevating PI3K signaling, but these normal, non-transformed cells are not as dependent on the PI3K pathway as the MM tumor cell lines (as observed by their decreased sensitivity to PI3K pathway inhibitors). Further, BCR stimulation induces MAPK family signaling<sup>184</sup>, which has been reported to result to Akt-independent gene expression<sup>185</sup> and give rise to other survival signals not under the control of the SHIP

42
phosphatase. This rationale also supports the observed high concentrations of AQX-MN100 needed to induce apoptosis in wild-type B-lymphocytes (Figure 7b). The SHIP$^{-/}$ cells were relatively resistant to the effects of AQX-MN100. A greater percentage of wild-type cells would be in that sub-G$_0$ apoptotic phase if primary cells had the dependence on the pathway seen in the cancer cells, but the contribution of multiple transduction pathways attenuates their sensitivity to PI3K pathway inhibitors.

Wild-type and SHIP$^{-/}$ mast cells behaved similarly to treatment with SHIP activators. IgE-stimulated mast cells from these mice were cultured with AQX-016A for 48 hours and cell survival was measured by the relative reduction of the non-toxic dye Alamar Blue, which is reduced at a higher rate in the mitochondria of growing cells. Although not the most relevant cell model for MM research, the PI3K pathway also contributes to survival and SHIP is a negative regulator in mast cells$^{186,187}$. Compared to the cyclodextran control, activation of SHIP had a more profound effect on wild-type cells versus the SHIP$^{-/}$ cells (Figure 7c-d). The cyclodextran vehicle itself appears to inhibit mast proliferation to some extent, further justifying the termination of its use as a carrier in future experiments.
Figure 7. SHIP activation selectively acts on wild-type murine B-lymphocytes and mast cells. Splenic B-lymphocytes were purified from wild-type and SHIP<sup>−/−</sup> C57Bl/6J mice and incubated with 10 μg/mL of the F(ab')<sub>2</sub> fragment of goat anti-mouse IgM antibody overnight. Cells were then cultured with the indicated concentrations of AQX-MN100 along with 10 μg/mL of goat anti-mouse IgM F(ab')<sub>2</sub> fragment for 24 hours. (A) Cell viability was assessed by trypan blue exclusion. Data represent mean percentage of viable cells relative to the number in the untreated control ± SD. This experiment was performed two times. (B) Apoptosis was detected by PI staining and analysis by flow cytometry. Data represent the mean percentage of cells in the sub-G<sub>1</sub> (apoptotic) phase ± SD. * p < 0.05; ** p < 0.005. This experiment was performed one time. (C) Wild-type and (D) SHIP<sup>+/−</sup> mast cells from C57Bl/6J mice were cultured with the indicated concentrations of AQX-016A or control vehicle for 48 hours, with Alamar Blue reduction dye added for the final 6 hours of culture. Proliferation was determined by reduction percentage relative to the untreated control as measured by spectrophotometry. All experiments were performed in triplicate cultures. This experiment was performed one time.
Activation of SHIP enhances the cytotoxic effects of current therapeutics

Knowing the critical role the PI3K pathway plays in MM cell growth and survival, we wanted to investigate if the combination of SHIP activators with conventional and emerging therapeutics would augment the cytotoxicity of the established agents. OPM2 cells treated with the commonly used therapeutic dexamethasone were cultured in the absence or presence of AQX-MN100 for 48 hours, and cytotoxicity was measured indirectly by the inhibition of proliferation as measured by $[^3H]$-thymidine incorporation into the DNA. Activation of SHIP dramatically enhanced the killing generated by this glucocorticoid (Figure 8a) at a concentration of AQX-MN100 that does not have a substantial effect on its own (4 μM), suggesting a synergistic relationship between the two agents.

The proteasome inhibitor bortezomib has been shown to have anti-myeloma activity and is thought to target both the MM cells and the BM milieu $^{84,114,188}$. It has proven itself valuable in the treatment of refractory MM, yet many patients still do not respond to this agent $^{115}$. We investigated if activation of SHIP augmented cytotoxicity induced by proteasome inhibition in OPM2 cells in an experiment identical to the dexamethasone assay above. We found that the addition of AQX-MN100 (4 μM) to cultures with bortezomib had significantly more inhibition of proliferation than cells treated with bortezomib alone (Figure 8b). However, in contrast to dexamethasone, the effects of AQX-MN100 are more additive than synergistic, implying that SHIP activation is increasing cytotoxicity through different mechanisms. These data support the use of allosteric SHIP activators to enhance the effects of the current therapeutics used in MM therapy.
Figure 8. The cytotoxicity of current therapeutic agents is enhanced by SHIP activation. OPM2 cells were cultured with the indicated concentrations of (A) dexamethasone or (B) bortezomib in the absence or presence of 4 μM AQX-MN100 for 48 hours, and pulsed with [³H] thymidine for the final 8 hours of culture. Cells were harvested and [³H] thymidine incorporation measured by scintillation counting. Data represent mean counts per minute (cpm) ± SD of triplicate cultures. This experiment was performed two times.
Activation of SHIP inhibits MM cell growth *in vivo*

Having clearly demonstrated the anti-proliferative and pro-apoptotic effects of SHIP agonists *in vitro*, we wanted to study the ability of AQX-MN100 to mediate anti-human myeloma cell growth in a murine xenograft tumor model. In our studies, immunocompromised NOD/SCID mice are subcutaneously inoculated with luciferase-expressing OPM2 cells in their shoulder. The cells are suspended in growth medium and Matrigel basement membrane matrix, which provides structure for the tumor to establish and vascularize. Tumors are allowed to grow until consistent measurements are attainable using bioluminescence imaging. In practice, this ranges from 10 to 20 days, depending on initial cell number injected.

In this preliminary study, tumors were allowed to establish for approximately 3 weeks. At this point, the mice (n=5) were given 50 mg/kg AQX-MN100 solubilized in walnut oil, or oil alone, administered in a subcutaneous depot in the lower back every 3 days for a 12-day period. Tumor measurements were taken at days 6 and 11, after which the experiment had to be terminated due to the large tumor volumes in the control group. It appears from this study that AQX-MN100 delays the growth of human MM cells in a murine model (Figure 9). The dose of AQX-MN100 given in this study was shown to have no toxic effects on normal hematopoiesis in a previous study as determined by methylcellulose colony formation (data not shown).
Figure 9. **SHIP activation inhibits MM cell growth in vivo.** OPM2 cells expressing firefly luciferase were injected along with Matrigel basement membrane into the upper flank of NOD/SCID mice and allowed to establish for 2 weeks. AQX-MN100 or control vehicle (n=5) was administered subcutaneously in an oil depot in the lower flank at a dose of 50 mg/kg of body weight every 3 days. Tumor volume was quantified using bioluminescence imaging. This experiment was performed one time.
DISCUSSION

The vast amount of research effort devoted to understanding the signaling pathways regulating cell growth, cell cycle progression and apoptosis has led to extraordinary improvements in our knowledge of the cellular and molecular mechanisms behind cancer. This research has defined many of the key regulatory proteins and cascades that become aberrantly controlled and subsequently drive tumorigenesis and progression.

The interactions between MM cells and the BM microenvironment have been intensively studied. MM cell interactions with the BM microenvironment trigger MAPK, JAK2/STAT3, and PI3K pathway activation and are responsible for proliferation, survival, drug resistance and migration\textsuperscript{79,189,190}. The PI3K signaling pathway has been shown to be the most important cascade in regulating MM cell survival and protection from apoptosis\textsuperscript{37,41,59,60,66,67}, and this is evident by the amount of research devoted to designing therapeutics that target this pathway, both for MM and for other diseases that depend on the cascade\textsuperscript{157,158,191}. As previously mentioned, moderate successes have been shown in preclinical studies involving PI3K\textit{\alpha}, Akt and mTOR kinase inhibitors in MM and other malignancies\textsuperscript{164,169,170}. Despite the potential of these agents, novel approaches to regulating PI3K pathway signaling are warranted.

The potential of SHIP activators

Translational research in controlling PI3K activity, particularly in regards to cancer, has focused on the development of kinase inhibitors. One alternative to inhibiting protein kinases in this pathway is to activate the lipid phosphatase SHIP, an endogenous negative regulator of the pathway. SHIP activators were originally proposed in our lab as therapeutic agents for immune
disorders. However, the restricted expression of the phosphatase to blood cells combined with its role in regulating the PI3K pathway soon brought up discussion of their application to hematological malignancies. Multiple myeloma was used as a model because of the necessity of elevated PI3K signaling and the need for more therapeutics in this presently incurable neoplasia. This is the first report to detail the use of allosteric SHIP activators to inhibit proliferation and induce apoptosis in vitro and to present preliminary data of its efficacy in vivo. Future work will focus on developing the MM xenograft murine model as well as in vitro studies with other blood cancers.

Upon growth factor or immunoreceptor stimulation, SHIP is tyrosine phosphorylated and translocates to the plasma membrane\textsuperscript{192}. However, the 5'-phosphatase activity of the enzyme does not change upon this extracellular stimulation\textsuperscript{138}; instead, SHIP exerts its inhibitory biological effects upon translocation to the membrane\textsuperscript{193,194}, the location of its substrate. With regards to B cells, the degradation of the second messenger PIP\textsubscript{3} leads to reduced Akt phosphorylation\textsuperscript{142,143}, which is consistent with the observation of enhanced proliferation and survival in SHIP\textsuperscript{-/-} B cells\textsuperscript{144}. SHIP expression was observed to increase 10-fold in response to BCR activation, emphasizing its importance in controlling PIP\textsubscript{3} levels\textsuperscript{195}. Further, Akt phosphorylation levels are only slightly elevated in resting SHIP\textsuperscript{-/-} bone marrow-derived mast cells (BMMCs) versus normal BMMCs\textsuperscript{196}. This suggests that SHIP is not a constitutively active regulator of the PI3K pathway such as PTEN, rather it is a negative regulator of elevated signaling. This would be consistent with the observation that PTEN\textsuperscript{+/+} mice frequently develop multiple tumors while SHIP\textsuperscript{-/-} mice show a lymphoproliferative phenotype but no overt tumor development.
Because SHIP appears to be responsible for limiting elevated PI3K signaling in activated cells rather than maintaining the basal homeostatic state, SHIP agonists such as AQX-MN100 would preferentially target cells with greater activity in that cascade such as MM cells while having minimal effects on quiescent hematopoetic cells with little PI3K activity and low SHIP expression. SHIP agonists such as AQX-MN100 have the further advantage of being allosteric activators. It has been proposed that agents targeting allosteric binding sites possess greater selectivity than those against active sites and that more research should be devoted to identifying modulators of these sites\textsuperscript{197}. AQX-MN100 demonstrated minimal off-target effects on a screen of related kinases and phosphatases\textsuperscript{178}, and the data in this study clearly validate that finding by showing no significant effect in SHIP\textsuperscript{-} cells and in non-hematopoetic tumor cells.

**The multiple myeloma cell model**

The myeloma cell lines available are genotypically heterogeneous. Showing efficacy in various lines and understanding the significance of the differences between each cell line is important for rational drug design. Therapeutics targeting specific molecules in cancer have shown great success with minimal toxicity in patients who harbor a particular lesion, while other patients lacking it respond poorly. For example, the discovery of imatinib, a kinase inhibitor of the BCR-abl translocation found in 95% of chronic myeloid leukemia (CML) patients, shows great success in initial treatment response\textsuperscript{198,199}. Consequently, it was important to include MM cells with varying degrees of PI3K pathway activation in the study.

The lines used in this study are routinely used in MM research and are representative of different stages of disease. For example, MM.1S cells are particularly sensitive to dexamethasone treatment, while OPM2 are relatively resistant. Dexamethasone resistance
commonly develops with disease progression, and therefore MM.1S reflect an earlier stage phenotype than the other lines used\textsuperscript{200}. Also, MM.1S cells express the p53 tumor suppressor while RPMI 8226 are p53 null\textsuperscript{104,201}. The RPMI 8226 line does express the PTEN tumor suppressor though, however the OPM2 line harbors a PTEN deletion\textsuperscript{135,137}. In addition, the lines RPMI 8226 and U266 are IL-6-non-responsive\textsuperscript{202}, while the other lines are responsive to IL-6.

The primary target of the lipid messenger PIP\textsubscript{3} is Akt, and this kinase initiates the majority of downstream signaling mediated by the PI3K pathway. Therefore, the degree of PI3K activity, and the effectiveness of SHIP activators to degrade the PI3K enzyme product PIP\textsubscript{3} can be related to the level of Akt phosphorylation. The cell lines OPM1, OPM2, and MM.1S all have greater basal Akt phosphorylation than RPMI 8226 and U266\textsuperscript{169}. This is consistent with their sensitivity to cytokine stimulation by IL-6, which increases Akt phosphorylation\textsuperscript{37}. Further, OPM1 and OPM2 are PTEN-mutated and PTEN-null, respectively, and the loss of this phosphatase certainly increases PI3K pathway activity and Akt phosphorylation\textsuperscript{130,134}. Thus, we selected OPM2 and MM.1S cells for the majority of experiments using AQX-MN100 as they reflected the best model.

OPM2 cells were chosen as the best model for signaling and murine xenograft studies due to the absence of PTEN. It has been reported that loss of the PTEN phosphatase sometimes seen in advanced stages of MM is associated with increased PI3K activity\textsuperscript{136,203}, and that PTEN-null MM cells are more sensitive to inhibitors of the pathway\textsuperscript{135}. Our \textit{in vitro} studies did not note any strong correlation between sensitivity to AQX-MN100 and PTEN status, which may reflect the existence of additional points of regulation in the PI3K cascade. Thus OPM2 cells were chosen for our xenograft model because they lacked the competing PIP\textsubscript{3} phosphatase and they represent a later stage phenotype that is more resistant to glucocorticoid treatment. MM.1S
cells were observed to be the most sensitive to killing mediated by SHIP activation, most likely due to their early-stage, drug-sensitive phenotype. We felt that using a more-resistant cell line is more relevant model as diagnosis and subsequent treatment will not always occur at an early stage, and demonstrating in vivo efficacy using resistant cells is more important than using the most sensitive cell line.

Our early experiments with AQX-016A were performed with RPMI 8226 and U266 cells. The lower sensitivity observed using AQX-016A may be the result of a poor choice of a model, these cells are IL-6-non-responsive and have very low basal Akt phosphorylation, suggesting lower PI3K activity. We did not have the more optimal cell lines MM.1S and OPM2 at the time of the AQX-016A studies. This along with the differences in vehicle carrier used and assay length as discussed in the results section contributes to the apparent greater sensitivity seen using the lead candidate SHIP activator AQX-MN100. Conclusive data regarding their relative efficacy will require direct comparison in the same assay conditions.

Overcoming resistance and increasing sensitivity to current therapies

SHIP agonists can also be used to potentiate the effects of therapeutics approved for use in the treatment of MM. Dexamethasone is a widely used immunosuppressant/cytotoxic drug that induces apoptosis in MM cells mediated by caspase-9 cleavage\textsuperscript{204}. Stimulation of the PI3K pathway by cytokines such as IL-6 and IGF1 in the BM microenvironment results in resistance to dexamethasone and other conventional chemotherapeutics by inhibiting this caspase cascade activation\textsuperscript{37,41}. Therefore, the elevation of PI3K activity with disease progression is consistent with the observation that MM cells become more resistant to dexamethasone. However, inhibiting the PI3K enzyme with LY-294002 blocks the downstream signaling providing this
protective effect, thereby resensitizing the cells to glucocorticoid treatment. Here we
demonstrated that SHIP activation is sufficient to overcome dexamethasone resistance in the
later-stage phenotype OPM2 cells, suggesting the combination of such agents for clinical
therapy.

The proteasome inhibitor bortezomib demonstrates significant anti-MM effects both in
vitro and in phase 2 and 3 trials of patients with refractory MM\(^{84,114,115,117,118}\). As previously
discussed, it overcomes acquired chemotherapeutic resistance by inhibiting NF-κB activation,
thereby downregulating adhesion molecules that contribute to CAM-DR and decreasing TNF-α
production that leads to cytokine release from BMSCs that upregulate PI3K. Yet, these studies
also found some patients did not respond to treatment and others acquired resistance to the drug.
Resistance sometimes develops after prolonged exposure through upregulation of antiapoptotic
proteins such as Bcl-2 or expression of MDR genes\(^{81,118,120}\). Also, tumor cell binding in the BM
milieu and the subsequent release of growth cytokines that activate the pro-survival PI3K
pathway is in some cases not sufficiently inhibited by bortezomib alone\(^{40,119,122}\). Further, it was
observed by one group that MM treatment of cells with bortezomib triggered Akt
phosphorylation\(^{169}\). This observation was not studied further, but one could speculate that the
increased Akt phosphorylation is due to some form of feedback stimulation in response to
treatment with the proteasome inhibitor. Taken together, inhibition of the PI3K pathway in
combination with proteasome inhibition should overcome the resistance seen to bortezomib. We
demonstrated here that inhibition of the PI3K cascade mediated by SHIP activation does enhance
the effect of bortezomib. This is a promising finding, as overcoming clinical resistance to
proteasome inhibition would prove very effective in patients with relapsed or refractory
myeloma.
In vivo xenograft model

There are a few noteworthy items to address regarding this preliminary animal model. The length of treatment and the number of tumor measurements are not sufficient to draw any major conclusions, but as mentioned, this provides the basis for further animal models, which are ongoing. The administration route has also been a challenging issue in our studies. AQX-MN100 is water insoluble and previous studies have shown it to have poor pharmacokinetics in vivo. Therefore, oral gavage, intraperitoneal and intravenous dosing are impractical due to the labor required and the burden of numerous injections but such experiments may be carried out at a later time. At the time of this study, the subcutaneous oil depot showed the most promising results. Current ongoing studies are using a water-soluble analog of AQX-MN100 with better results because it is given in the drinking water and therefore higher serum levels are maintained over time. These ongoing studies involve statistically relevant numbers of mice followed over greater lengths of time. The toxic effects of AQX-MN100 will be again examined at the termination of these studies.

Mutations and oncogene addiction

The process of tumorigenesis involves the sequential acquisition of mutations in multiple genes. These transformations can be divided into driver mutations, those responsible for the initiation and progression of the disease, and passenger mutations, those not significantly contributing to pathogenesis. Numerous driver mutations exist in MM, such as the previously discussed c-Myc and Ras, any many others have yet to be identified. These transformations result in the development of an increased dependence upon the PI3K pathway, which is confirmed by Akt phosphorylation. This phenomenon has been defined as
oncogenic addiction$^{197}$, whereby inhibiting a single pathway induces growth arrest and programmed cell death. The selective kinase inhibitors imatinib, which targets BCR-abl, and gefitinib or erlotinib, which target the epidermal growth factor receptor (EGFR), have been shown to efficiently kill tumor cells that express those oncogenes in vitro$^{198,208,209}$. Additionally, CML and non-small cell lung cancer (NSCLC) patient subsets with these mutated oncogenes have shown dramatic response to these inhibitors$^{210-213}$. Consequently, inhibitors of the PI3K pathway, such as SHIP activators, show therapeutic selectivity towards MM clones versus normal tissues$^{157}$. As previously eluded to, this can be seen when looking at the reduced effects AQX-MN100 imparts on the survival of primary B-lymphocytes as compared to the MM cell lines.

The multistage accumulation of genetic alterations in carcinogenesis results in many changes in the intracellular signaling circuitry of a normal cell. The sequential activation of oncogenes and inactivation of tumor suppressor genes may occur, but it appears to be a simplified model as the regulatory circuitry adapts through various feedback mechanisms to maintain homeostatic balance in these transformed cells$^{207,214}$. This is illustrated by the very heterogeneous networks of signal cascades and cross-communication that develop during tumorigenesis. Certain proteins can gain new novel functions and interaction partners, giving rise to behavior in these cells that cannot be predicted in normal cells. The process of oncogene addiction is an example of this, where targeting one protein or pathway is sufficient to induce apoptosis in a tumor cell population. Another example is the inhibition of growth or induction of apoptosis seen in tumor cells with multiple mutations after the introduction of one tumor suppressor gene, such as p53 or PTEN$^{137,215}$. If cancer cells maintained normal transduction pathways throughout their step-wise transformation, you would not expect to see such a dramatic
effect by the addition of one negative regulator. This phenomenon presents a great opportunity for the design of targeted therapeutics. However, it is important to note that the circuitry differs in each case, therefore treatments must be individualized to reflect the cancer-specific lesions, as has been highlighted in BCR-abl transformed CML patients.

Oncogenic shock

One of the paradoxical observations seen as a result of tumor cells with numerous mutations is the upregulation of negative regulators of signaling such as tumor suppressor genes in order to cope with the growth-promoting signals and maintain homeostasis. This observation has led to a new explanation on the mechanism of oncogene addiction: multiple prosurvival signals exist from one or more driver mutations, and these are counteracted by elevated proapoptotic signals that increased due to the unusual signal circuitry in a transformed cell. Treatment of cancer cells with a targeted agent against an oncogenic pathway results in a temporal imbalance in signaling where proapoptotic signals persist in the absence of prosurvival signals, dubbed oncogenic shock. This is due to survival signals being short-lived while apoptotic signals are longer lasting. This model of signal imbalance is demonstrated using Src, BCR-abl and EGFR oncogene inactivation in several in vitro models.

It is therefore proposed that a window of opportunity exists where proapoptotic signals are unchecked by the inactivated oncogenic signals, and an apoptotic outcome can result if this window persists long enough. Further, it has been demonstrated that this outcome can result from only a few hours of proapoptotic signaling. Our findings with SHIP activation support this theory. Rapid dephosphorylation of Akt and its downstream effectors as well as the significant induction of apoptosis occurs in MM.1S and OPM2 lines after a few hours of
treatment. This proposed mechanism of oncogenic shock also suggests that cycling treatment regimens may be more beneficial than continuous administration due to the greater number of opportunity windows of favored proapoptotic signaling, however this is just speculation at this point and must be investigated further.

Future directions

The SHIP activator AQX-MN100 has been demonstrated to be effective in the treatment of MM in cultures of cell lines. Cell lines are ideal for creating and optimizing in vitro and in vivo models of disease, however their maintenance in culture for extended times is sure to induce numerous mutations not standard to the disease. Therefore, it is essential to test primary patient samples in the developed models as these cells do not harbor these acquired mutations and should be more representative of the actual transformed cells that the drug must kill in order to become a viable treatment. In addition to the ongoing xenograft models previously mentioned, AQX-MN100 must show efficacy using primary cells in the developed models described in this report. Pharmacokinetic and toxicity studies are also to be conducted to understand more about the bioactivity and dosing of the compound. All these data will be required before AQX-MN100 can be used to treat human patients.

Conclusion

In summary, we show that allosteric activation of the SHIP phosphatase is a new paradigm in controlling PI3K signaling in MM and can inhibit proliferation and induce apoptosis in vitro and in preliminary murine xenograft models. The hematopoietic restriction of SHIP

58
combined with the demonstrated specificity for its target limits its potential toxicity to surrounding tissues, an essential requirement for biologically-based therapeutic design. Moreover, intrinsic and acquired drug resistance can be overcome by the combination of lead candidate AQX-MN100 with other therapeutics, thereby resolving the main problem why MM remains incurable. Single-agent regimens for the treatment of cancers are unlikely to be successful due to drug resistant mutations\textsuperscript{219}; therefore the ability of AQX-MN100 to enhance the cytotoxic effects, possibly in a synergistic manner, is an extremely valuable finding. This study provides the framework for further \textit{in vivo} xenograft models, which can then be translated into patient trials in the clinic.
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