NOVEL STRATEGIES FOR ANTAGONIZING THE PHOSPHATIDYLINOSITOL-3-KINASE PATHWAY IN DISEASE

by

TIEN YIN YAU

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Abstract

The phosphatidylinositol 3-kinase (PI3K) signaling pathway is a critical regulator of cell physiology. This project aims to investigate several novel approaches to target the PI3K pathway. First, in order to determine the importance of PI3K regulators on normal cells, I investigated the effect of PTEN haploinsufficiency on glucose regulation in mice. Even a 50% reduction in PTEN expression was sufficient to increase phosphorylation of the downstream targets AKT and GSK3β.

Next, I wanted to see if PI3K pathway could treat idiopathic thrombocytopenic purpura (ITP). Since the established ITP therapy (IVIg) is thought to signal through SH2-containing inositol 5' phosphatase (SHIP), I tested the ability of a SHIP activator AQX-MN100 to reverse a murine model of ITP. In the classic model of ITP, AQX-MN100 was unable to rescue mice from antibody-mediated platelet destruction. However, prophylactic AQX-MN100 prevented the infection-mediated form of ITP.

I then studied the potential uses of AQX-016A/AQX-MN100 in the hematopoietic malignancies multiple myeloma (MM) and mantle cell lymphoma (MCL). AQX-016A/AQX-MN100 successfully induced apoptosis of the cancer cell lines *in vitro* in both a time and dose dependant manner.

I then investigated the potential of a small molecule ILK inhibitor to inhibit early prostatic dysplasia/hyperplasia in a murine model. Under the initial experimental parameters chosen, the ILK inhibitor was not able to inhibit dysplasia/hyperplasia. However, further studies are required to determine whether ILK inhibition may be an effective therapeutic strategy for treatment of prostate cancer.

Finally, I attempted to potentiate the effects of PI3K pathway inhibitors with borrelidin, an inhibitor of tRNA synthetase, which successfully exhibited synergy with the PI3K inhibitor

LY294002, but only exhibited additive effects with the ILK inhibitor. The results of this project show the validity of targeting members of the PI3K pathway either in alone or in combination with a synergistic pathway.

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Introduction

In order to survive, cells have to be able to be to detect and act on external factors. A cell's fitness is determined by its ability to translate internal and external conditions into the right action. Consequently, the exterior of a cell is covered in receptors that enable the cell to sense and quantify external conditions. All cells are constantly progressing through this cascade; primary sensing of external conditions leads to activation of signal transduction cascades. Signal transduction not only integrates the input from all the different external signals, but also the current internal state of the cell. Depending on the relative strengths of the contributing signals, certain downstream pathways are activated. This then results in some sort of cellular action. This can be translation of target genes, phosphorylation of target proteins, compartmentalization/decompartmentalization of specific products, or opening/closing of ion channels. Depending on the conditions this can cause the cell to undergo apoptosis, divide, grow, move, or initialize signaling pathways in other cells.

A cell is exposed to a myriad of signals; there are several mechanisms by which a cell integrates all the information into a single action. A primary level of specificity is provided by receptors; the cell can only sense and react to a signal if it has the corresponding receptor. However, the signaling pathway is a critical mediator of cell behavior because it synthesizes signals from all the individual receptors and also amplifies them into an actionable response. Consequently, there is a lot of interest in studying a single signaling pathway at multiple points.

Phosphatidylinositol-3-kinase pathway

The Phosphatidylinositol-3-kinase (PI3K) pathway is one of the most important signaling pathways in the cell. It is the primary survival pathway and is important for deciding whether

conditions are sufficient to commit to a resource-intensive action such as growth and proliferation (Entingh, Law et al. 2001). It is common to all cell types and can have a variety of downstream effects on cell physiology such as survival, motility, proliferation, glucose regulation, angiogenesis, cytoskeletal reorganization, chemotaxis, adhesion, and cytokine production (Fruman, Meyers et al. 1998). A complicated set of signaling events determine which functions get activated under which conditions and in which cell types. Ever since the significance of the PI3K pathway was discovered, there has been much interest in elucidating the details of how the PI3K pathway is regulated and how it in turn regulates its downstream effects. This is not a trivial endeavor. Even though the PI3K pathway is common to all cell types, the exact details of the pathway depend on the cell type. Differential expression of receptors or signaling mediators means that the same signal can produce different effects in different cells.

The PI3K pathway operates through manipulation of the phosphoinositol molecule (PI). PI is a phospholipid characterized by an inositol head group attached to two hydrophobic fatty acid chains. The inositol ring has five free hydroxyl groups that can be the site of phosphorylation (Fruman, Meyers et al. 1998). It can exist as the monophosphates PtdIns-3-P, PtdIns-4-P, and Ptd-Ins-5-P; the bisphosphates PtdIns-3,4-P_2 and PtdIns-4,5-$P_{2,}$, and the trisphosphate PtdIns-3,4,5-P_3 (PIP3). Phosphorylation is accomplished by a family of phosphatidylinositol kinases loosely classified as PI3Ks, PI4Ks, and PIP kinases depending on their substrate.

PI3Ks are the most intensively studied member of the family due to their central role in signal transduction. However, the other phosphatidylinositol kinases also play important roles. PIP kinases exist as two families (PIP5K and PIP4K) that are designated based on their distinct phosphorylation sites in the inositol ring (Fruman, Meyers et al. 1998). Both are capable of generating PtdIns-4,5,-P_2; a metabolic precursor to PIP3 and a second messenger in its own right (Heck, Mellman et al. 2007). *In vivo*, PIP kinases regulate such functions as insulin signaling,

stress responses, and actin assembly (Yin and Janmey 2003; Lamia, Peroni et al. 2004; Jones, Bultsma et al. 2006). PI4Ks are ubiquitously expressed and appear to be concentrated in cellular membranes (Pike 1992). They only use PI as a substrate and the resulting PtdIns-4-P can be further phosphorylated by both PI3K and PIP5K, feeding into both the PIP3 and the PtdIns-4,5-P2 pathways (Pike 1992).

PI3Ks are divided into three general classes based on structural features and substrate specificity: Class I PI3Ks are heterodimeric proteins characterized by a catalytic (110-120 kDa) and a regulatory subunit (elaborated below). These are in turn further separated into class I_A and I_B based on sequence homology and function (Fruman, Meyers et al. 1998). They function as both lipid and protein kinases, but one of their most important activities is the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PtdIns-3,4-P_2) to the second messenger PI (3,4,5)P3 (PIP3). This makes this class extremely important in disease. The larger (170-210 kDa) Class II PI3Ks are monomeric, and they preferentially phosphorylate PtdIns and PtdIns-4-P. Class III PI3Ks are heterodimeric with an adaptor (p150) and catalytic (Vps34) domain and they only phosphorylate PtdIns.

The three Class I_A PI3Ks p110α, p110β, p110δ (named as a reference to the size of the catalytic subunit) share significant homology (42-58%) in the catalytic subunit. Their regulatory subunits are generally referred to as "p85 proteins" though their molecular weights are more variable. There are two 85 kDa isoforms (p85α and p85β) and a 55 kDa isoform (p55γ). p85α also has two other splice variants (p50α, p55α), where p55α shares structural similarity to p55γ. These regulatory subunits act as adapters; they contain SH2 and SH3 domains that mediate binding to and regulation of different intracellular proteins. Different p110/p85 combinations result in differential downstream expression and impart control of specific signaling processes.

There is only a single class I_B PI3K. It consists of the catalytic subunit p110γ and the regulatory subunits p101, p84 and $p87^{PIKAP}$. p110γ only shares 36% identity to p110α and p101

and the regulatory subunits do not appear to share homology with the other p85 proteins. In fact, they only share 30% identity with each other, mostly in the N- and C- terminal ends that are important for their interaction with p110γ and G protein coupled receptors (Suire, Coadwell et al. 2005; Voigt, Dorner et al. 2006). All three of them are expressed in the same tissues, implying that there may be a subunit-dependant regulation of PI3Kγ. The class I_B subunits do not appear to interact directly with the class I_A PI3Ks.

The different PI3K classes have different roles and expression patterns. All cell types express PI3Kα and PI3Kβ, whereas PI3Kδ and PI3Kγ are mainly expressed in leukocytes. PI3Kγ is also present at low concentration in smooth muscle cells, endothelia and cardiomyocytes (Patterson, Boehning et al. 2004; Kerfant, Rose et al. 2006).

Growth factor binding activates receptor tyrosine kinases (RTKs), which in turn activate PI3K by phosphorylating its p85 regulatory subunit. This phosphorylation can be either a result of direct interaction with the RTK or through activation of adaptor kinases such as the insulin receptor substrates IRS1 and IRS2 (Vivanco and Sawyers 2002). PI3K p110-p85 complexes are located cytoplasmically when inactive. Upon activation, they translocate to the membrane and are able to phosphorylate their targets, the most critical being phosphatidylinositol-4,5-bisphosphate to form the second messenger PI (3,4,5)P3 (PIP3). PIP3 binds to a subset of pleckstrin homology domains in down stream protein targets and recruits them to the plasma membrane.

The action of PI3K is antagonized by the dephosphorylation of PIP3 by the phosphatases PTEN and SHIP, which dephosphorylate the 3', and 5' phosphates respectively. PTEN (phosphate and tensin homolog deleted in chromosome ten) hydrolizes PtdIns(3,4,5)P3 to PtdIns(4,5)P2 (Stambolic, Suzuki et al. 1998). As suggested by its alternate name MMAC (mutated in multiple advanced cancers), PTEN is often mutated or inactivated during cancer progression. In the absence of this breaking system, activation of the PI3K pathway is

unopposed, leading to a constitutive activation of downstream signals. SHIP similarly depletes the PIP3 pool by converting PtdIns(3,4,5) to PtdIns(3,4)P2 (Sly, Rauh et al. 2003).

While there are a wide variety of PI3K down stream events, most of them are mediated through AKT and mTOR. The protein kinase AKT mediates many of the survival processes of the PI3K pathway including proliferation, apoptosis and growth (Vivanco and Sawyers 2002). Diseases involving the PI3K pathway usually result from the constitutive activation of AKT.

AKT/PKB is a homolog of PKC and was discovered as an oncogene within the mouse leukemia virus AKT8 (Staal 1987; Bellacosa, Testa et al. 1991). Upon recruitment to the plasma membrane, it is phosphorylated at threonine 308 by PDK1 and at Serine 473 by either the ILK/Rictor or the mTOR/Rictor complex, activating it. It recognizes and phosphorylates intracellular proteins containing the peptide sequence R-X-R-X-X-S/T-B (Alessi, Caudwell et al. 1996). AKT/PKB is a member of the AGC (cAMP-dependent, cGMP-dependent and protein kinase C) protein kinase family, a collection of protein kinases that display a high degree of sequence similarity within their respective kinase domains. AGC kinase proteins are characterized by three conserved phosphorylation sites that critically regulate their function. The first one is located in an activation loop in the centre of the kinase domain. The two other phosphorylation sites are located outside the kinase domain in a conserved region on its C-terminal side, the AGC-kinase C-terminal domain. These sites serve as phosphorylation-regulated switches to control both intra- and inter-molecular interactions. Without these priming phosphorylations, the kinases are catalytically inactive (Parker and Parkinson 2001).

AKT exists as multiple isoforms. The three forms (AKT1, AKT2, and AKT3) are coded by three different, highly homologous genes (over 80% at the amino acid level). This allows for ability for the isoforms to compensate for one another. Germ line deletions are non-lethal but reveal isoform-specific traits: AKT1 deletion causes defects in apoptosis and growth regulation (Chen, Xu et al. 2001), AKT2 appears to be an important regulator of insulin sensitivity (Cho,

Mu et al. 2001), and AKT3 deletion causes diminished brain size (Easton, Cho et al. 2005). Their expression patterns are likewise generally overlapping with slight distinctions: AKT1 is ubiquitously expressed, AKT2 is elevated in insulin responsive tissues, and AKT3 is expressed ubiquitously except for skeletal muscle and liver, which have low levels.

AKT regulates several downstream pathways that enhance proliferation and growth, apoptosis evasion, survival, glucose uptake, angiogenesis, and immune cell activation. Cell cycle progression is enhanced through an increase in cyclin D1 and a reduction in CDK inhibitors, allowing the cell to progress into S phase (Liang and Slingerland 2003). Anti-apoptotic effects are mostly through inhibition of caspase 9, release of the anti-apoptotic proteins Bcl-2 and Bcl-X_L, and activation of the anti-apoptotic transcription factor NF-κB (del Peso, Gonzalez-Garcia et al. 1997; Cardone, Roy et al. 1998; Li and Verma 2002).

Mammalian target of rapamycin (mTOR) is another important mediator of PI3K/AKT effects. It is a critical regulator of nutrient availability and controls the cellular response to amino acid levels, growth factors, and other nutrients (Law 2005). This triggers biological responses such as apoptosis inhibition, translation initiation, cell growth and replication (Wullschleger, Loewith et al. 2006). It exists as two different complexes: mTORC1 and mTORC2. These complexes have distinct drug sensitivities, signaling triggers, and downstream pathways with mTORC1 having most of the cell growth, proliferation, and translation regulation roles. mTOR complex 1/regularory-associated protein of mTOR (mTORC1/RAPTOR) is regulated by growth factors and nutrient availability and is inhibited by rapamycin, while mTORC2/ rapamycin-insensitive companion of mTOR (RICTOR) primarily responds to growth factors and is rapamycin insensitive. Care must be taken with mTOR inhibitors since their effect on AKT signaling may vary with cell type and can lead to either upregulation or downregulation depending on context. Inhibition of mTORC1 (by rapamycin) leads to AKT activation, while in other systems; rapamycin exposure inhibits TORC2 assembly and disrupts AKT signaling

PI3K and disease

The PI3K pathway is a tightly controlled balance between activating and suppressing signals. With its wide-ranging effects on cell survival and proliferation pathways, disruptions in PI3K pathway signaling can cause many significant effects on cell and organism function. Disregulation of the PI3K pathway is associated with many diseases.

Cancer

Cancer is one of the more apparent diseases involving PI3K pathway deregulation: it is characterized by the aberrant survival and proliferation of cells. As PI3K is the major survival pathway of cells, it is unsurprising that its signaling is often altered in tumors. In fact, all of the major nodes of the pathway have been found mutated or amplified in cancer (Yuan and Cantley 2008). Constitutive activation allows the cell to evade apoptosis, proliferate unchecked, and undergo structural changes that enhance cell motility and metastasis.

There are two common mechanisms to achieve this activation: mutation of an oncogene and loss of a tumor suppressor. Notable examples from the PI3K pathway include the RTKs, Epidermal growth factor receptors (EGFR, HER2), PI3K itself (especially the p110α and p85α subunits), and AKT (all three isoforms). This usually allows the oncogene to stimulate downstream survival and proliferation pathways in the absence of normal upstream signals. The loss of a tumor suppressor disengages a cell's natural brakes, enabling a cell to bypass regulatory checkpoints and ignore inhibitory and apoptotic signals. It should thus not be surprising that two of the most frequent mutations seen in cancer are p110α and PTEN (Jia, Liu et al. 2008; Zunder, Knight et al. 2008). In fact, the prevalence and necessity of activating mutations suggests that disregulation of the PI3K pathway is an important step in neoplastic progression (Samuels, Wang et al. 2004) along with disregulation of the error-correcting pathway and induction of

telomerase. In particular, AKT is necessary for tumor-induced angiogenesis and tumor growth (Fang, Ding et al. 2007). The necessary activation of the PI3K pathway is also often achieved through upregulation or downregulation of the target protein levels as opposed to the mutation of the target itself. Overexpression of structurally normal PI3K, AKT and RTKs is another common means of cellular transformation. Some cancer progression is isoform-specific: p85α and p85β are essential for androgen receptor (AR) transactivation induced prostate cancer progression (Zhu, Youn et al. 2008). PTEN-deficient cancers are dependent on the p110 beta, not alpha isoform: depletion of p110β, but not p110α, inhibited AKT phosphorylation and cell proliferation. This has been shown in three cell lines that represented the three major cancer types with a high frequency of PTEN mutations: prostate cancer, brain cancer, and breast cancer (Wee, Wiederschain et al. 2008). Also, a p110β specific inhibitor was able to inhibit AKT phosphorylation and cell growth in PTEN-deficient cells but not mutant p110α cell lines. This is supported by a xenograft model where inhibition of p110β was sufficient to suppress AKT phosphorylation and PC3 tumor growth. p110β is necessary for the oncogenic transformation of mouse embryo fibroblasts by RAS or EGFR. Sensitivity to oncogenic transformation is restored by the expression of wild type p110β. It is also partially restored by a kinase dead p110β mutant, implying that p110β has a non-catalytic function. This dependence on the beta isoform is interesting since p110α and PTEN are two of the most frequently mutated targets in cancer though they appear to occur in a mutually exclusive fashion (Saal, Holm et al. 2005; Abubaker, Bavi et al. 2007).

The fact that PI3K pathway activation appears to be so important to cancer progression makes it a particularly good drug target. The switch in isoform use may allow better targeting of the malignant cells. Also, the fact that it is upregulated in the disease state is particularly useful since it is easier to dampen overactivation than to restore tumour-supressor function.

Oncogene addiction

Considering the abundance and variety of genetic mutations in cancer cells, is it even reasonable to believe that targeting a single pathway can significantly affect tumor survival? The theory of oncogene addiction suggests that it might. Oncogene addiction is the concept that cancers with multiple genetic, epigenetic, and chromosomal abnormalities are dependent on or 'addicted' to one or a few genes for both maintenance of the malignant phenotype and cell survival. These mutations greatly changes the signaling pathways or "wiring diagram" of the cell (Weinstein 2000). This may lead them to be more dependant on certain genes than normal cells. Thus, reversal of only one or a few of these abnormalities can inhibit cancer cell growth and in some cases translate to improved survival rates (Weinstein 2000). In other words, while normal cells have multiple redundant systems that can compensate for loss of a particular oncogene, in cancer cells, the oncogene is necessary. If true, a dramatic clinical effect can be achieved by targeting a limited number of systems.

Oncogene addiction was described in reference to the BCR-ABL oncogene in CML (chronic myelogenous leukemia). This is a disease caused by a novel gene fusion of the BCR gene from chromosome 22 to the Abl1 gene on chromosome 9 as a result of a reciprocal translocation. Since this is a product not seen in normal cells there are no endogenous redundant pathways that can replace it. Drugs that target this single oncogene (such as how Gleevec targets BCR-ABL) are highly effective. However, other cancer systems do not seem as thoroughly addicted as the CML model. This is probably due to the presence of redundant mediators that become activated upon inhibition of the target gene. An example is that in glioblastoma cells, it was necessary to inhibit multiple pathways to induce apoptosis (Grant 2008). This adaptive ability is probably more common than the "single target" oncogene addiction model seen in CML. This problem is exacerbated by the inherent genetic instability of cancerous cells.

Normal cells have a distinct set of signaling pathways at their disposal. This limits their available responses to cellular insults: however, cancer cells by their very nature are much less restricted. A key characteristic and contributing factor to tumorigenicity is the ability of the cell to mutate its own signaling pathways. This can be through upregulation or downregulation of the target gene, mutation of the gene itself, or switching between splice isoforms. This would allow the cancer cell to eventually bypass the inhibited oncogene and still activate downstream pathways. In fact, even in CML, the cancer cells eventually become resistant to Gleevec.

It is probably more accurate to say that cancers are addicted to pathways as opposed to specific genes. If the one or two vulnerable pathways of the malignancy are found, they can then be targeted with specific inhibitors. This should kill all of the transformed cells before they have a chance to adapt to the drugs. Since the normal cell is not dependant on these pathways, it is able to compensate for the loss by switching to a redundant pathway, allowing for preferential killing of the neoplastic cells.

Considering the frequency with which PI3K pathway mutations are found in a variety of cancers, the PI3K pathway appears to be a highly addictive pathway. However, successful inhibition will probably be a matter of inhibiting several genes along the pathway simultaneously as opposed to a single "addictive" gene.

Immune system

The PI3K pathway has an important role in immune regulation in both innate and adaptive immunity. Innate immunity is the system that defends the host using non-specific mechanisms. It confers immediate and temporary protection against pathogens. There is mounting evidence that the PI3K pathway is a major regulator of the innate immune system through regulation of cytokines such as IL10 and IL12. Many of the important mediators of innate immunity are also dependant on the PI3K pathway for proper functioning

Inhibition of the PI3K pathway in monocytes, macrophages, and myeloid dendritic cells results in decreased production of the anti-inflammatory cytokine IL10 along with increased synthesis of the pro-inflammatory IL12, IL 1β, IL6, and TNFα. This is mostly through PI3K-mediated regulation of transcription and translocation of the associated transcription factors in these cells (Weichhart and Saemann 2008)

Mast cells are an important mediator of the innate immune system along with their role in allergy and anaphylaxis. When activated (through direct injury, cross-linking of IgE receptors, or by activated complement proteins), they degranulate and release chemical mediators such as histamine, heparin, and cytokines into the surrounding areas. This results in the dilation of blood vessels and recruitment of other inflammatory cells. Gastrointestinal mast cell development is dependant on activation of PI3K, especially the δ isoform. In mice, it is also necessary for IgE-induced hypersensitivity. Inhibition of PI3Kδ leads to deficiencies in proliferation, adhesion, migration, and degranulation. PI3Kγ also plays a role in mast cell function since p110δ-inactivated mice are protected against anaphylactic allergic responses (Weichhart and Saemann 2008).

Other important mediators are the neutrophils. Upon maturation, neutrophils migrate to the site of infection based on the cytokine gradient. There, they can destroy the pathogen through phagocytosis, degranulation, or an oxidative burst (Nathan 2006). Mutation of PI3Kγ results in severe defects in chemotaxis and migration. In addition, PI3K is the main regulator of phagocytosis, respiratory burst, and IL8 production (Weichhart and Saemann 2008).

Dendritic cells are phagocytic cells that are best known for their role in antigen presentation. This is the process by which foreign antigens are processed and displayed on the surface of antigen presenting cells (APCs) such as dendritic cells in order to activate and educate the adaptive immune system. However, they are also important for their ability to produce type 1 interferons (IFNs) in response to viral infection. Viral stimulation of toll-like receptors such as

TLR7 or TLR9 activate the PI3K pathway in plasmacytoid dentritic cells. Interestingly, the production of type I interferons is quite specific; inhibition of PI3Kδ abrogates IFN production, but not other proinflammatory responses such as TNFα and IL6. This specificity results from PI3K's ability to induce translocation of IRF-7 IFN's main transcription in plasmacytoid dendritic cells (Guiducci, Ghirelli et al. 2008).

The PI3K pathway also has an important role in the components of the adaptive immune system. This is the part of the defense that is able to recognize and remember new pathogens for destruction. The PI3K pathway is a critical mediator of B-cell and T-cell activation. Signaling through the B-cell receptor (BCR) and can be counteracted through deletion or chemical inactivation of the PI3K subunits p85α or p110σ. A similar reaction can be achieved through interaction of with the inhibitory receptor FCγRIIB. Activation of FCγRIIB leads to leads to recruitment of the inositol phosphatase SHIP, depletion of the pool of PIP3, downregulation of the downstream pathways, and loss of antigen responsiveness. SHIP-deficient B cells are hyperesponsive to BCR signaling and are more sensitive to BCR-mediated activation. They also develop and mature faster than wild-type cells, and are less sensitive to BCR-induced cell death (Brauweiler, Tamir et al. 2000). PI3K also has major roles in antigen processing and cell motility. Activities such as antigen gathering and migration to the T-cell zone are inhibited upon PI3K block. Class I_A PI3K is a major regulator of B-cell development. Inhibition of PI3K result in developmental blocks, loss of mature B cell maintenance, defects in class switch recombination, and failures in plasma cell differentiation.

In T-cell activation, both the p50α and the p85α PI3K subunits are important for PI3K-mediated T cell receptor signaling. The two subunits are recruited under different contexts, the specifics of which are not yet known. Loss of PI3K isoforms appear to be better tolerated in T-cells than in B-cells. PI3K signaling is essential for proper differentiation of activated T-cells; though there is evidence that class I_A and I_B PI3K play redundant roles in thymocyte

development and survival (Oak, Deane et al. 2006; Swat, Montgrain et al. 2006). Constitutive expression of the PI3K pathway in T-cells results in triggering an invasive lymphoproliferative disease due to the enhanced survival of peripheral T-cells. An autoimmune phenotype also results from a PI3K loss of function mouse model, likely due to the reduction of Treg cells leading to the development of colitis (Okkenhaug, Bilancio et al. 2002). PI3K pathway signaling also has a significant role in the trafficking of T cells into inflamed tissues. Activation of the PI3K pathway reduces the ability of T-cells to re-enter the lymph nodes, promoting migration into peripheral tissues. Both PI3K classes are responsible for the effect: class I_A PI3K (p110δ) mediates TCR/CD28 signaling and class I_B PI3K (p110γ) mediates chemokine signaling (Martin, Schwartz et al. 2008; Thomas, Mitchell et al. 2008).

The immune system is a very promising target of PI3K pathway therapy since it involves PI3K isoforms distinct from the rest of the body. Here, it is the PI3K isoforms PI3Kδ and PI3Kγ that are particularly important in leukocyte signaling (Rommel, Camps et al. 2007). Drugs that directly target these isoforms should limit side effects and improve specificity.

Diabetes and glucose regulation

The PI3K pathway has an important role in regulating glucose tolerance. Activation of the PI3K pathway leads to increased glucose uptake through recruitment of the glucose transporters Glut1 and Glut4 to the plasma membrane and can also regulate the activity of glycolytic enzymes. This is an essential cellular pathway; inhibition of glucose uptake either through PI3K pathway block or through direct impairment has a critical effect on embryo survival (Riley, Carayannopoulos et al. 2006). Normally, blood glucose levels trigger insulin release by the pancreas. Following insulin binding, insulin receptors in responsive tissues phosphorylate IRS-1, IRS-2, and Cbl, which in turn activate and recruit PI3K to the plasma membrane. This then activates further pathways that result in trafficking of the glucose

transporter to the cell surface, allowing import of glucose into the cell. AKT2 is an important mediator in glucose regulation; AKT2 knockout models exhibit reduced insulin sensitivity.

Diabetes mellitus is characterized by insulin-related deficiencies. Type 1 diabetes results from the body's failure to produce sufficient insulin. Type II diabetes results from insulin resistance, reducing uptake of glucose into skeletal muscle, which may also be combined with failures in insulin production. It is highly likely that this insensitivity results from aberrations in PI3K pathway signaling. Genetic screens have revealed that type II diabetics can have mutations of the PI3K pathway that interfere with insulin signal transduction including AKT2 and the PI3K activators IRS-1 and IRS-2. In addition, p110α and p110β double heterozygous mutant mice exhibit glucose intolerance. (Brachmann, Ueki et al. 2005; Luo, Field et al. 2005). The balance between p85 and p110 proteins may be the actual mechanism of regulation. Free p85 blocks IGF1 induced AKT activation, leading to a negative effect on insulin signaling.

PI3K pathway signaling is also the mechanism by which diabetic side effects occur. High glucose impairs PI3K signaling through the accumulation of reactive oxygen species. This results in delayed corneal epithelial wound healing (Xu, Li et al. 2009) and also decreases endothelial cell proliferation in a PI3K/AKT dependant manner. The PI3K pathway may be the source of vascular complications commonly seen in diabetes patients (Varma, Lal et al. 2005). Also, flux through the hexosamine biosynthetic pathway leads to insulin resistance through accumulation of the final product O-linked UDPβ-N-acetylglucosamine (O-GlcNAc) (Vosseller, Wells et al. 2002). High levels of O-GlcNAc lead to inhibition of AKT, modification of the insulin signaling effectors insulin substrate 1 and β-catenin, and impaired insulin responsiveness.

Hematopoietic cells rely on cytokine-mediated glucose uptake via the regulation of the Glut1 transporter. In the hematopoietic myeloid/lymphoid cell line, interlukin-3 stimulation promotes glucose uptake in a PI3K-dependant manner (Wieman, Wofford et al. 2007). This was determined to be through the AKT-mediated trafficking of the glucose transporter Glut1. AKT

activation was sufficient to maintain surface Glut1 levels even in the absence of interleukin-3 signaling. Although mTOR/RAPTOR activity was not necessary for this maintenance, inhibition of mTOR/RAPTOR appeared to inhibit Glut1 activity and diminished glucose uptake.

One important reason for studying the relationship between PI3K and glucose metabolism is to try to find therapies that reverse insulin resistance in type II diabetics. Upregulation of the PI3K pathway in insulin-sensitive tissue should improve insulin sensitivity and thus glucose uptake. An understanding of the PI3K pathway can also be used to counteract PI3K-mediated diabetes symptoms.

Another important reason to study this relationship is to better understand the side effects of PI3K inhibition. Therapeutic inhibition of the PI3K pathway could result in drastic changes glucose regulation in normal cells. While a short-term inhibition should not have drastic effects, long term PI3K pathway inhibition in insulin-sensitive tissues may cause insulin resistance that progress into diabetes. Thus, more study is necessary as to the effects PI3K pathway inhibition has on glucose tolerance.

This research also has implications for cancer therapies. There are many similarities between the glucose pathway in normal cells and the insulin-like growth factor (IGF) pathway that is extremely important in cancer cell progression. Since cancer progression is characterized by the rapid and aberrant proliferation of cells, a high level of glucose uptake is essential for a successful neoplastic transformation. If glucose metabolism is disrupted, cancer cells show enhanced apoptosis. A better understanding of the role of the PI3K pathway in the glucose regulation of multiple cell types can provide new potential targets for cancer treatment.

One final advantage to PI3K pathway inhibitors is their role in drug transport. Inhibition of PI3K blocks drug export from drug resistant colon carcinoma cells. When used in combination with the cytotoxic drug doxorubicin, PI3K inhibition increased the intracellular drug concentration threefold (Abdul-Ghani, Serra et al. 2006). As drug export is an important

mechanism for developing drug resistance, the use of a PI3K pathway inhibitor may be an important step in increasing the effectiveness of current and future drugs.

Current drugs that affect the PI3K pathway

PI3K inhibitors

Considering the roles PI3K plays in various diseases, it is natural to seek inhibitors of the pathway in order to ameliorate these diseases. One approach is to target PI3K directly, thus affecting all downstream signals. The most commonly used PI3K inhibitors in a laboratory setting are Wortmannin and LY294002. These have the advantage of being effective at very low concentrations. However, their non-specific natures prevent them from being used clinically. Wortmannin is an irreversible inhibitor of p110α through a covalent reaction with lysine-802 (Wymann, Bulgarelli-Leva et al. 1996). It is specific to PI3K but affects all PI3K classes, while most of the clinical benefits derive from inhibition of the class 1A (especially the p110α/β isoform). LY294002 has even more non-specific effects; affecting other PI3K related enzymes plus unrelated targets such as CK2, mTOR, and GSK3β, PXDX, and Brd (Davies, Reddy et al. 2000; Gharbi, Zvelebil et al. 2007)

There are new classes of PI3K inhibitors that bind the ATP binding pocket, many of which are in phase I clinical trials (Marone, Cmiljanovic et al. 2008). However, the highly conserved nature of the ATP binding pocket motif may lead to these drugs having non-specific effects on other targets (Folkes, Ahmadi et al. 2008; Garlich, De et al. 2008; Maira, Stauffer et al. 2008). Allosteric inhibitors would be more specific and thus preferable to these, but there are currently few in development (Marone, Cmiljanovic et al. 2008). As all the different PI3Ks have slightly different roles and signaling pathways, one would assume that isoform specific

inhibitors of PI3Ks would be necessary to have the best targeting and reduction of side effects (Jia, Roberts et al. 2009). However, new drugs with broad inhibitions appear to have acceptable toxicities for acute treatments (Maira, Stauffer et al. 2008). It may be that isoform specificities may be only important in treating chronic diseases such as autoimmunity where long-term inhibition would be a problem.

mTOR inhibitors

mTOR inhibitors have a history as immunosuppressive agents. As such, their clinical behavior and toxicities have been well established. Since they function by inhibiting the PI3K pathway, there has been much interest in using them cancer treatment (Teachey, Grupp et al. 2009). The best understood mTOR inhibitor is rapamycin itself (Law 2005). This inhibits protein translation and cell cycle progression, leading to both its immunosuppressive and anti-cancer properties. Considering the importance of the immune system in preventing cancers, it is surprising that an immunosuppressant can be an effective anti-cancer agent. It appears that the anti-cancer effects are dominant over the immunosuppressive effects as it actually can decrease tumor formation in a transplant setting (Luan, Hojo et al. 2002). Nevertheless, there is an interest in developing rapamycin analogs that retain anti-tumor properties while reducing immunosuppressive effects (Raymond, Alexandre et al. 2004). Rapamycin is not equally effective in all targets. In a study of breast cancer cell lines, rapamycin sensitivity was correlated with phosphorylation of AKT, emphasizing the importance of the whole pathway on drug reactions (Noh, Mondesire et al. 2004). There is also evidence that rapamycin sensitivity and effects may differ in different tumor types. While c-myc was not significantly affected by rapamycin treatment in breast cancer cells, in rhabdomyosarcoma cells, c-myc induction was a strong predictor of rapamycin resistance (Hosoi, Dilling et al. 1998). Similarly, cyclin D1 expression is an indicator of rapamycin sensitivity in breast cancer cell lines, but not multiple

myeloma cell lines (Hosoi, Dilling et al. 1998). This highlights how the signaling of even a highly conserved pathway like mTOR can vary between different contexts.

Other mTOR inhibitors have shown promise in a clinical setting with a mTORC1 inhibitor (temsirolimus) in the treatment of renal cell carcinoma (Le Tourneau, Faivre et al. 2008). There is also an interest in developing drugs that can inhibit mTOR in conjunction with other members of the PI3K pathway such as PI3K or the Hypoxia initiation factor HIF-1 (Lohar, Mundada et al. 2008).

AKT inhibitors

The high degree of homology and overlapping roles of the three AKT isoforms creates a challenge when designing inhibitors. On one hand, all three isoforms can regulate downstream survival pathways to such an extent that a single AKT isoform is sufficient to prevent cell death (Liu, Shi et al. 2006). However, isoform-specific effects are also important. Along with the distinct effects on growth, insulin metabolism, and brain size mentioned above, the three isoforms also have different roles in tumor development. In mammary tumors, AKT1 deletion inhibits while AKT2 deletion accelerates development and deletion of AKT3 is phenotypically neutral. This is due to AKT1's important role in normal mammary gland differentiation (Maroulakou, Oemler et al. 2008). Conversely, deregulation of AKT3 and AKT2 are the primary AKT isoform in progression of melanoma and oral squamous cell carcinoma respectively (Stahl, Sharma et al. 2004; Iamaroon and Krisanaprakornkit 2009). Thus both pan-AKT and isoform specific inhibitors are needed.

There are several AKT inhibitors in development. GSK690693 is an ATP-competitive inhibitor that works on all the three AKT isoforms (Rhodes, Heerding et al. 2008). However, it is not completely specific due to the high sequence homology of the ATP binding pocket to other AGC kinases (Manning and Cantley 2007). A few specific inhibitors to other AGC kinases have

been found, providing promise for effective and specific AKT inhibitors (Proud 2007).

Alternatively, perifosine is an alkylphospholipid that targets the pleckstrin homology domain of AKT (Chiarini, Del Sole et al. 2008). It has shown to be an effective anti-cancer drug in a variety of malignancies including prostate cancer and leukemia. In addition it exhibits synergy with other pathway inhibitors and can restore sensitivity to EGFR inhibition in prostate cancer cell lines. (Festuccia, Gravina et al. 2008)

Possible areas for new PI3K pathway antagonists

The growth in the area of PI3K-pathway therapies emphasizes the potential for therapeutic strategies. The regulated expression of PI3K pathway components or isoforms allows for the development of tissue-specific antagonists. There is potential for targeting the PI3K pathway at many levels depending on the desired effects. Upstream targets such as PIP3, or AKT enable complete inhibition of multiple pathways at once while targeting downstream components will be more specific, induce fewer unwanted effects, and may allow for more subtle changes in cell signaling.

In particular, small molecule drugs have many advantages over other therapies. Since they are often orally available they do not incur the high cost and inconvenience associated with other modes of administration. They are easily synthesized; this is in particular contrast with the antibody-based therapies that have a high manufacturing cost. They are also easier to modify since their structures are often much simpler than protein-based drugs. Small molecule drugs are more stable than other drugs and can be lyophilized without damage. High throughput screens of small molecules drugs make it simple to test and optimize inhibitors and/or activators in a fast and cost-effective manner.

In addition to their clinical usefulness, small molecule inhibitors provide great tools for investigating the regulation of the PI3K pathway. Many of their advantages as therapies are also true in a laboratory setting; they provide an unprecedented ability to modulate signaling in a highly controlled manner. Coupled with the right experimental system, the specificity provided by small molecule inhibitors allows us to tease out the subtlest details of PI3K regulation.

In this thesis, I have used three different small molecules drugs to examine the potential of targeting the PI3K pathway in a therapeutic manner. All three have distinct targets. The first (AQX-016A/AQX-MN100) is an activator of SHIP. This targets the PI3K pathway at an initial point in the cascade by promoting dephosphorylation of the PI3K product PIP3 and has the advantage of being specific to hematopoietic cells. The second (QLT0267) is an inhibitor of ILK. This inhibits the PI3K pathway at the level of AKT, farther downstream from PI3K. The third drug (Borrelidin) is an activator of the amino acid deprivation pathway. While it does not target the PI3K pathway directly, the importance of the PI3K pathway in nutrient sensing means that there is a potential for synergistic effects with PI3K pathway inhibitors.

SHIP agonists

One of the major negative regulators of the PI3K pathway is the SH2-containing inositol 5' phosphatase (SHIP). Its key substrate is the second messenger PIP3 where it dephosphorylates the 5' phosphate to form PI(3,4) P2. This depletes the pool of PIP3. PH-containing targets with a high affinity to PIP3 (such as AKT) are no longer attracted to the plasma membrane and become deactivated. This leads to downregulation of the PI3K pathway and its downstream pathways. Disregulation in SHIP signaling causes increased activation sensitivity, faster development and increased resistance to B-cell-receptor-induced death in B-cells (Brauweiler, Tamir et al. 2000). SHIP levels also negatively correlates with histamine release in allergic basophils. Low levels of SHIP appear to cause hyperreleasability of histamine and IL4, resulting

in an allergic reaction in patients (Vonakis, Gibbons et al. 2001). SHIP is also commonly mutated in hematopoietic cell lines and cancer samples (Luo, Liu et al. 2004)

There are several factors that make a SHIP activator a good drug target. It is often preferable to target a natural inhibitor since this will only affect cellular signaling if the pathway is activated in the first place. This should help limit the drug effects to the target, overactive cells. Since SHIP is a hematopoietic -specific phosphatase, it should limit effects to the hematopoietic compartment. This should present fewer side effects than the other lipid phosphatase PTEN.

In light of the advantages of SHIP as a drug target, we developed a small molecule SHIP agonist in collaboration with the Mui, Andersen, and Krystal labs. Dr. Krystal discovered and cloned SHIP in 1996. Once we identified SHIP as an ideal target, Dr. Mui's lab developed a novel *in vitro,* high throughput assay to screen the Andersen invertebrate marine natural product library for a potential SHIP activator. The first analog was AQX-016A (Figure 1B) a more potent structural analog of the parent compound pelerol (Figure 1A) (Yang, Williams et al. 2005). However, AQX-016A contains a catechol structure that may cause non-specific protein inhibition upon metabolism in the liver. Thus the analogue AQX-MN100 (Figure 1C) was developed that lacks this catechol group.

Further investigation showed that this class of compounds is an allosteric agonist of SHIP through binding to the C2 domain (Ong, Ming-Lum et al. 2007), that it was able to inhibit macrophage and mast cell activation, and was a potent anti-inflammatory.

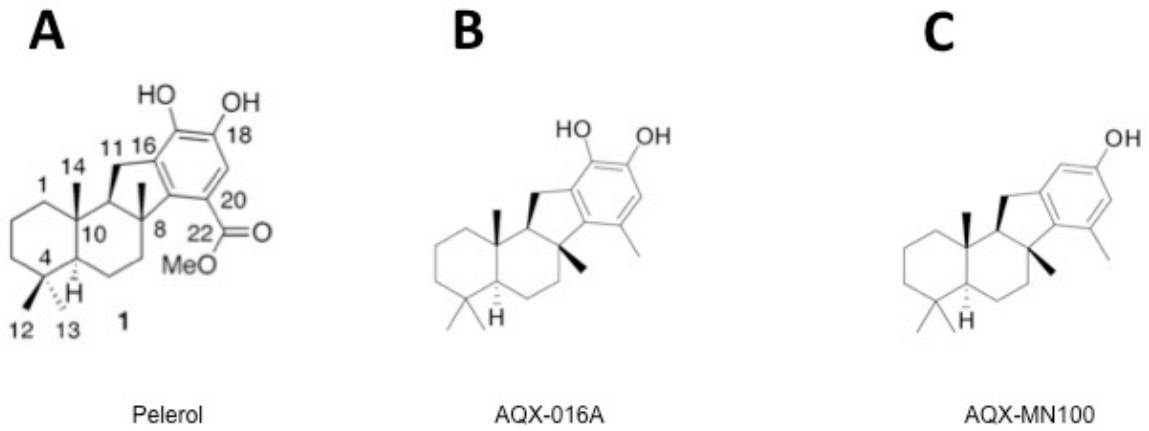


Figure 1. Small molecule SHIP agonists

The compound pelerol was discovered as part of a small molecule screen for SHIP agonists in marine sponge extracts. The compound AQX-016A was then developed as a more potent form of pelerol. AQX-MN100 is the latest version of the drug and lacks the catechol group of AQX-016A.

Potential therapeutic targets for SHIP agonists

Because SHIP appears to have a role in several different hematopoietic cell functions, there are many different diseases in which SHIP activation could be therapeutic. Based on the mechanics of the disease, I picked two disease models to study. Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease where platelets are destroyed by the host's own immune system. It's a particularly interesting disease target for a SHIP agonist because it is not a disease characterized by an aberrant PI3K pathway. However, the current treatment for ITP appears to rely on SHIP to prevent phagocytic cells from destroying the platelets. I hypothesized that a SHIP activator can mimic this effect and the subsequent manipulation of a normal PI3K pathway would prove therapeutic in an ITP model.

Idiopathic Thrombocytopenic Purpura

Idiopathic thrombocytopenic purpura (ITP) is a hematopoietic autoimmune disease characterized by low platelet counts. This is caused by a combination of depressed platelet production and increased platelet clearance (Ballem, Segal et al. 1987). Platelet destruction was widely believed to be the major cause of the thrombocytopenia. Patients with ITP exhibited shortened platelet survival and increased platelet clearance (Branehog, Kutti et al. 1974). Although deficiencies in the platelet production pathway were occasionally observed in early studies (Cohen, Gardner et al. 1961), impaired thrombopoiesis was not accepted as a mechanism of ITP until it was confirmed through platelet labeling studies (Heyns, Lotter et al. 1982). These were succeeded by mechanistic studies that showed binding of antiplatelet antibodies to the megakaryocyte progenitor cells and the subsequent impairment of platelet production (McMillan, Luiken et al. 1978; Mazur, Hoffman et al. 1981; Chang, Nakagawa et al. 2003).

The worldwide incidence of ITP is approximately 2 in 100,000 in adults (Frederiksen and Schmidt 1999; Neylon, Saunders et al. 2003; Fogarty and Segal 2007) and 5 per million in

children (Zeller, Helgestad et al. 2000). There are two forms of the disease: The chronic form is characterized by a high level of circulating autoantibodies. The other form is acute, and often associated with an infectious illness. A wide variety of infectious agents can trigger ITP through production of the proinflammatory cytokine γ interferon. This in turn activates phagocytic cells that are able to locate and destroy opsonized cells.(Musaji, Cormont et al. 2004)

A current treatment for ITP is intravenous administration of immunoglobulins (IVIg) along with platelet transfusions, and immunosuppressants. The platelet transfusions increase the platelet counts while the immunosupression inhibits the immune system sufficiently to prevent autoimmune destruction. In particularly unresponsive cases, a splenectomy is performed. IVIg is a favored treatment due to its effectiveness and milder side effects as compared to corticosteroids and splenectomy (George, Woolf et al. 1996).

However, there are many problems with IVIg as a therapeutic treatment (George, Woolf et al. 1996): First, there are many side effects including increased risk of anaphylactic shock, pulmonary edema, and acute renal failure. Second, since IVIg is made from the pooled IgG immunoglobulins of over one thousand blood donors, there is limited availability, high cost, and a risk of blood borne diseases. Thirdly, it must be administered intravenously, which is both inconvenient and requires specialized personnel. It is therefore desirable to identify alternative treatments that may lower the cost and invasiveness of ITP care and therapy.

Antibody-mediated immunity is dependant upon recognition of infected cells or invading pathogens by antibodies. These bind the antigen with the variable domain leaving the Fc portion of the antibody free to interact with Fc receptorson a variety of immune cells (macrophages, neutrophils, eosinophils, dendritic cells, platelets, etc.). The result of this binding depends on the class of Fc receptor and the kind of cell involved. The activating Fc receptors FcγRI, FcγRIIA, FcγRIIIA, FcγRIIIB stimulate such effects as phagocytosis, cell activation, degranulation, and cytokine release through their intracytoplasmic immunoreceptor tyrosine-based activation motifs

(ITAMs) (Daeron, Latour et al. 1995). This causes destruction of the targeted cell and upregulation of the immune system.

These signals are counteracted by the effects of the FcγRIIB receptors found on B cells, mast cells, macrophages, neutrophils, and eosinophils. Binding of the Fc fragment to the receptor triggers recruitment of SH2-domain-containing phosphatases to its phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM) cytoplasmic tail. These include such inhibitory proteins as the inositol 5-phosphatases SHIP1 and SHIP2, and the protein-tyrosine phosphatase SHP-1. As described above, the SHIP phosphatases downregulate the PI3K pathway through depletion of the PIP3 pool. In B-cells, SHIP is necessary for FcγRIIB-mediated inhibition (Ono, Okada et al. 1997). The protein-tyrosine phosphatases target tyrosyl-phosphorylated proteins. SHP-1 dephosphorylates early targets in the transduction pathway such as inhibitor tyrosine-based activation motifs, protein tyrosine kinases, and adaptor proteins. This inhibits activation of the STAT (signal transducers and activators of transcription) transcription factors, downregulating the signal transduction pathway (You and Zhao 1997; Binstadt, Billadeau et al. 1998).

A murine model of chronic ITP can be produced through administration of platelet-specific polyclonal or monoclonal antibody in mice (Mizutani, Engelman et al. 1993; Alves-Rosa, Stanganelli et al. 2000; Nieswandt, Bergmeier et al. 2000). I.V. administration of a monoclonal antibody (10 μg) caused acute thrombocytopenia within an hour with significant recovery by 24 hours whereas injection of a platelet-unreactive antibody did not alter platelet counts (Mizutani, Engelman et al. 1993). Considering the high rate of platelet clearance, continuous administration of antiplatelet antibody is required for maintenance of the thrombocytopenia. Intravenous or intraperitoneal administration of IVIg successfully ameliorates thrombocytopenia in this model (Crow, Song et al. 2003).

Although the exact mechanism of IVIg is unknown, it is dependant on the inhibitory

FcγRIIB receptor on splenic macrophages (Crow, Song et al. 2003). Loss of the receptor abolishes the protective ability of IVIg and renders it unable to reverse the ITP. This therapeutic activity is probably through FcγRIIB receptor signaling since IVIg does not increase FcγRIIB expression (Ichiyama, Ueno et al. 2005). Since SHIP is a major downstream target of FcγRIIB, and is necessary for FcγRIIB-mediated inhibition in B-cells, it appeared to be a promising target (Ono, Bolland et al. 1996; Bolland, Pearse et al. 1998). The underlying hypothesis of this study is that SHIP activation would mimic IVIg's ability to ameliorate platelet destruction in an ITP model. AQX-MN100 is a small molecule drug that is a potent and selective SHIP activator (Ong, Ming-Lum et al. 2007). I hypothesize that administration of this drug will be as effective as IVIg in the treatment of a murine model of ITP.

Multiple myeloma/ mantle cell lymphoma

The second disease model for SHIP activation is more direct: hematopoietic cancers. Here, a constitutively activated PI3K pathway is critical to maintenance of the disease. Since SHIP activation is able to downregulate the PI3K pathway in hematopoietic cells, I thought that it might be therapeutic in this disease as well.

In cancer, the reactive nature of cell signaling is distorted. The oncogenic cell no longer senses and acts upon external signals. Instead, it manipulates internal signaling to maintain the desired state of growth and proliferation. Inhibitory signals are either ignored through inactivation, or overwhelmed by positive signals. The PI3K pathway is one of the critical pathways for oncogenic mutation. Upon constitutive activation, the cell can maintain growth and proliferation in the absence of external signals. PI3K/AKT pathway activation also activates angiogenesis through upregulation of the vascular endothelial growth factor (VEGF) by activating the hypoxia inducible factor 1 (HIF-1) (Zundel, Schindler et al. 2000). This allows the cell to not only ignore external conditions, but to actually alter them to its benefit.

Multiple myeloma and mantle cell lymphoma are B-cell malignancies. Both are non-Hodgkin's lymphomas, and are caused by chromosomal translocations. This changes the cells such that they no longer undergo normal maturation. Instead, they divide uncontrollably and evade apoptosis, flooding the system with non-useful cells, accumulating in the lymphoid system, and eventually rendering the system non-functional. Neither disease has a known cure with conventional chemotherapy (Geisler, Kolstad et al. 2008). Currently, all that can be provided are treatments that mitigate the symptoms of the disease. As such, the mean survival is two to three years (Kyle, Gertz et al. 2003).

Multiple myeloma is a lymphoma of the plasma cell and makes up approximately 10% of all hematological cancers (Rajkumar and Kyle 2005). As the antibody-producing cells of the immune system, part of plasma cell development involves undergoing reshuffling of the antibody coding sequences known as VDJ recombination. This creates variation in the antigen-binding domain, leading to antibody diversity, a critical part of the adaptive immune system. Occasionally, this genomic instability results in an aberrant chromosomal translocation that allows the cell to undergo unbridled growth. This results in a large population of monoclonal cells, filling the bone marrow, excluding other cell types, and impeding normal functioning. In addition, the disease is exacerbated by the severe kidney damage caused by the malignant B-cells producing vast amounts of protein in the form of whole or partial antibodies (M-protein).

Mantle cell lymphoma is a B-cell lymphoma that makes up about 8% of lymphomas (Foran, Cunningham et al. 2000). It is caused by an abnormal break and translocation in an antigen-naïve pregerminal centre B-cell within the mantle zone. The characteristic translocation is the t(11;14)(q13;q32) [2] although other breaks involving the 11q13 breakpoint have been observed (Vandenberghe, De Wolf Peeters et al. 1992). This is linked to an error in VDJ rearrangement that places the bcl-1- close to the Ig heavy chain enhancer. This gene is the oncogene CCND1 that codes for Cyclin D1, a cell cycle regulatory protein (Withers, Harvey et

al. 1991). This allows the cell to replicate rapidly without experiencing the normal cycle checkpoints.

Both diseases are characterized by constitutive activation of the PI3K pathway (Shi, Gera et al. 2002; Peponi, Drakos et al. 2006; Rudelius, Pittaluga et al. 2006). This is especially true of the more aggressive forms of the disease, usually augmented by loss of PTEN (Rudelius, Pittaluga et al. 2006). Inhibition of PI3K pathway causes cell cycle arrest and apoptosis due to a dependency on AKT expression (Zollinger, Stuhmer et al. 2008; Teachey, Grupp et al. 2009). Since SHIP fulfills many of the same functions as PTEN and is hematopoietic specific, I thought that inhibition of the PI3K pathway through activation of SHIP might be a good strategy for combating this disease. I hypothesized that SHIP activation in cancer cells would decrease the pool of PIP3, leading to downregulation of AKT, and subsequent apoptosis and loss of proliferation. I wished to determine if the SHIP activator AQX-MN100 and AQX-016A could be effective in these diseases.

Integrin-linked kinase (ILK)

Aberrant activation of the PI3K pathway is also implicated in prostate cancer. It has been well established the PTEN loss is associated with more advanced prostate cancer (McMenamin, Soung et al. 1999). Partial or complete PTEN loss is commonly seen in many prostate cancer cell lines as well as primary tumors; ~ 30% of primary prostate cancers and 63% of metastatic prostate tumours exhibit PTEN inactivation (Teng, Hu et al. 1997; Vlietstra, van Alewijk et al. 1998). Early stage tumors usually show a heterozygous loss of PTEN while homozygous loss is associated with late-stage, aggressive, metastatic tumors (Di Cristofano and Pandolfi 2000). This implies that loss of PTEN is a critical mediator of prostate cancer progression.

This is supported by a series of mouse mutants with varying PTEN deletions in prostate epithelia. Trotman et al (2003) developed a “hypomorphic” PTEN mouse model that only

expresses 25%-35% active PTEN ($Pten^{hy}$). With this strain, they were able to develop a series of mice with decreasing levels of prostate PTEN activity ($Pten^{hy/+}$ > $Pten^{+/-}$ > $Pten^{hy/-}$ > Pten prostate conditional knockouts). Tumor progression was correlated with PTEN dose. The most aggressive and invasive tumors were observed in mice with complete PTEN inactivation. $Pten^{hy/-}$ mice developed invasive properties while $Pten^{+/-}$ only developed low-grade cancer. As expected, phosphorylation of AKT was negatively correlated with PTEN dose in these animals.

Another model of prostate-specific PTEN knockout is the ARR2-PB-Cre+ve/PTEN fl/fl mouse line (Wang, Gao et al. 2003). Loss of PTEN in prostatic tissue results high expression of the PI3K pathway and AKT phosphorylation. These mice show the full progression of human prostate cancer development in a predictable manner from hyperplasia at about 4 weeks, to localized neoplasia at 6 weeks, to invasive carcinoma between 9 and 15 weeks, and metastasis occurring at around 15 weeks. In addition, these tumors are androgen sensitive; castration was able to delay the disease. Interestingly, these mice still had residual tumor cells in the prostate when sacrificed 2.5 months after castration, implying that a progression to an androgen-independent stage might be possible. These studies support the importance of PTEN loss in the progression of prostate cancer.

However, since PTEN is a tumour suppressor, its loss PTEN only sensitizes a cell to PI3K pathway stimulation. There still has to be some form of PI3K pathway activation in order for the cell to become oncogenic. One of the critical mediators of PI3K kinase is the integrin linked kinase ILK. ILK was originally identified in a screen for binding partners of the integrin β1 subunit (Hannigan, Leung-Hagesteijn et al. 1996). It is a ubiquitously expressed PI3K dependant kinase with a PH-like domain that binds PIP3 directly and is expressed in most, if not all tissues. ILK binds and is activated by PIP3, then forms a complex with rictor, and directly phosphorylates AKT at serine 473 and activates it (Dedhar, Williams et al. 1999). Rictor is necessary for this activation; inhibition of rictor, either through siRNA or dominant negative

protein, inhibits ILK-associated AKT Ser^{473} phosphorylation (McDonald, Oloumi et al. 2008). Rictor is best known as a component of the mammalian target of rapamycin complex 2 (mTORC2), which is also involved in AKT phosphorylation. AKT activation by ILK leads to cell proliferation, cell cycle progression, evasion of apoptosis, and induction of angiogenesis (Hannigan, Leung-Hagesteijn et al. 1996; Tan, Cruet-Hennequart et al. 2004).

Normal epithelial cells require adhesion to the extracellular matrix to survive and proliferate. Binding to the ECM induces a signaling cascade that leads to expression of the cell cycle protein cyclin D1, activation of Cdk4 and cyclin E associated kinases, phosphorylation of Rb, and down-regulation of the cdk inhibitors p21 and p27. This allows the cell to progress past the checkpoint to the S phase. Lack of a substrate causes the cells to arrest in the G1 phase and undergo apoptosis a process dubbed anoikis (Frisch and Screaton 2001). Adhesion-dependant survival and proliferation is mediated through the interaction of cell surface integrins with ECM ligands in conjunction with other signaling pathways such as MAPK. Many of the signaling effects of integrins are dependent on the β1 subunit and thus presumably ILK.

ILK is often overexpressed or constitutively activated in cancer types such as brain, breast, prostate, pancreatic, colon, ovarian, gastric, and malignant melanomas (Dedhar 2000; Troussard, McDonald et al. 2006). Experimental overexpression of ILK in rat epithelial cells allows them to achieve anchorage independent growth. This is due to the ability of ILK to maintain cyclin D1 and cyclin A levels in the absence of cell adhesion and integrin binding, preventing cell arrest. ILK overexpression also resulted in altered forms of p21 and p27. These novel forms are weaker inhibitors of cdk, enhancing cell-cycle progression (Radeva, Petrocelli et al. 1997). Anchorage-independent growth is a hallmark of oncogenic progression and is part of the epithelial to mesenchymal transformation (EMT). This is partially due to inhibition of E-Cadherin expression and enhanced nuclear localization of Beta-catenin in epithelial cells. The transformed cells thus have mesenchymal properties that enhance aggresivity and metastasis

such as apoptosis and anoikis suppression. These effects are collaborated in breast cancer models where ILK overactivation leads to cell cycle progression, cyclin D/A over-expression, and anchorage-independent cell growth *in vitro*, and hyperplasia and tumor formation *in vivo* (Kalra, Warburton et al. 2009). In prostate cancer, ILK overexpression is correlated with tumor progression (Graff, Deddens et al. 2001). This included tumor grade, proliferation index and five-year survival. ILK thus appears to be upregulated with prostate cancer progression.

ILK is a good example of oncogene addiction: in normal epithelial or mesenchymal cells ILK activity can be inhibited, but pAKT and apoptosis are unaffected, presumably due to alternate pathways; however, inhibition of ILK in cancer cells causes apoptosis and prevents proliferation (Troussard, McDonald et al. 2006). This, in combination with its apparent importance in cancer progression in multiple tumor types makes ILK a very promising therapeutic target.

The ILK inhibitor QLT0267 was developed in a screen for small molecule drugs (Koul, Shen et al. 2005). It is a competitive inhibitor of ATP binding with strong selectivity for the ILK kinase domain and an *in vitro* IC50 of 26 nM (Koul, Shen et al. 2005). In cell-based assays, the IC_{50} of QLT0267 ranged from 5-10 μmol/L. In a glioma cell line with a constitutively active PI3K pathway, QLT0267 inhibited proliferation and colony formation in a dose-dependant manner due to suppression of AKT and GSK-3ß phosphorylation. This then induces cell cycle arrest, decreases cell invasion, downregulated matrix metalloproteinase-2, suppressed mTOR/FKBP12-Rapamycin-Associated Protein, and inhibits VEGF expression.

QLT0267 has been shown to be effective in many diseases including anaplastic thyroid cancer (Younes, Kim et al. 2005), metastatic orthotopic lung-cancer xenografts, and a PC3 xenograft model. It also has activity in several mouse models such as neu-oncogene-induced mammary tumor formation, carcinogen-induced and colitis associated intestinal tumor formation. QLT0267 has synergistic effects in both *in vitro* and *in vivo* models with a variety of

other drugs including Raf and MEK inhibitors; EGFR inhibitors; and the antibody-based drugs erotinib, gefitinib, ceximab.

In the glioblastoma xenograft model, QLT 0267 treatment resulted in a significant delay in tumor growth. There were multiple mechanisms for this result: there was a significant reduction in cell proliferation in the treated mice, presumably due to ILK's role in cell cycle regulation; moreover there was a significant reduction of VEGF and HIF-1α in QLT0267-treated tumors, leading to inhibition of angiogenesis and poorer tumor profusion.(Edwards, Woo et al. 2008).

Since PTEN loss sensitizes the prostate to cancer thorough PI3K activation and ILK is a critical activator of the PI3K pathway, I hypothesized that ILK inactivation would be sufficient to prevent prostate cancer development in a PTEN-deficient prostate cancer model. I tested this through administration of the small molecule ILK inhibitor QLT0267 in the ARR2-PB-Cre+ve/PTEN fl/fl transgenic mouse model of prostate cancer.

Amino acid deprivation pathway: the search for synergy

Since most diseases are complex entities it is unlikely that inhibition of a single target is sufficient. Although inhibition of the PI3K pathways is an important step in disease control, it is important to find other drugs that act in concert with PI3K inhibitors, both to prevent tolerance and to increase effectiveness. Synergy occurs when multiple targets can produce an effect much greater than the sum of their individual effects; a significant effect can be achieved by multiple drugs affecting the same pathway.

The ultimate goal of combination therapy is to achieve synergistic effects. This helps counter compensatory mechanisms in the target cell and limits side effects by reducing doses of the individual drugs. It also allows for better selectivity of the targeted system. Since synergistic effects are dependant on molecular interactions between the targets, treatments can

be designed based on cellular context. An example of this is in a rat asthma model where the combination of sub therapeutic doses of a glucocorticoid and a tricyclic antidepressant (TCA) resulted in synergistic anti-inflammatory effect comparable to a high-dose dexamethasone control. However, unlike the dexamethasone, the combination treatment did not exhibit glucocorticoid-associated toxicity. This is likely due to the TCA target being more highly expressed in lymphocytes (the target of the anti-inflammatory effect) than in liver and pituitary cells (the mediators of glucocorticoid-associated side effects) (Lehar, Krueger et al. 2009).

An example of synergy is evident between mTOR and ERK. mTOR is part of the PI3K/AKT pathway that is frequently deregulated in cancer. ERK is a member of the MAPK pathway that is also important in cellular survival. MEK1/2/ERK1/2 (PD325190) and mTOR (rapymicin) inhibitors in isolation cause significant reduction in tumor growth but they interact synergistically to inhibit prostate cancer cell growth *in vitro* and *in vivo* (Grant 2008). This appears to be particularly effective in androgen-independent prostate cancers, which are notoriously hard to treat. Thus we wanted to find another therapeutic agent that could complement the effects of PI3K pathway inhibition.

The PI3K pathway is key mediator of nutrient responsiveness. Signals such as insulin tell the cell that external resources are high enough to support energetically costly acts such as growth and protein translation. PI3K activation promotes translation through inhibition of the translation inhibitor 4E-BP1 and activation of the p70 S6 kinase through their phosphorylations (Chung, Grammer et al. 1994; Weng, Andrabi et al. 1995; Brunn, Williams et al. 1996).

Since amino acids are a required substrate for protein translation, amino acid withdrawal leads to quick deactivation of the translational machinery, resulting in cell cycle arrest. There are multiple pathways that mediate this effect. In CHO-IR or HEK293 cells (Hara, Yonezawa et al. 1998), amino acid withdrawal prevents insulin-stimulated phosphorylation of p70 S6 kinase and eIF-4E BP1 in an mTOR-dependant manner. Increasing exogenous amino acid

concentrations mimics insulin signaling to an extent that cannot be increased upon by insulin itself. This modulation is not through tyrosine phosphorylation, PI3K, AKT, or MAPK activity but is dependant on mTOR. This is supported by the fact that mTOR is able to phosphorylate 4E-BP1 *in vitro* at sites corresponding to insulin stimulation *in vivo* (Fadden, Haystead et al. 1997).

Another amino acid-dependant regulation pathway is found in immune cells. Immune cells are acutely sensitive to nutrient deprivation. This pathway is used as a natural form of immunosupression. The catabolic enzymes IDO and Arginase are expressed by regulatory antigen presenting cells (APCs), which deplete the microenvironment of arginase and tryptophan. This deficiency leads to a build up of uncharged tRNA that binds and activates the eIF2 kinase GCN2. This phosphorylates and inactivates eIF2α, inhibiting translation and leading to downstream effects such as T-cell anergy and apoptosis, regulatory T cell differentiation in naïve T-cells, and suppressor function in Treg cells. An example of this is in pregnancy: the maternal immune system must not attack and reject the fetal tissue even through it is foreign.

The amino acid deprivation pathway may also have a role in cancer treatment. In the LNCaP prostate cancer cell line, autocrine Fas induced death only occurs in serum free media, perhaps due to growth factor induced Fas inhibition. However, cyclohexamide (a translation inhibitor) counteracts this inhibition and sensitizes LNCaP cells to fas induced death. Since the amino acid deprivation pathway also inhibits translation, it may also induce Fas induced apoptosis in cancer cells.

Borrelidin is a drug that activates this amino acid deprivation pathway by inhibiting tRNA synthetase. This leads to a build up of uncharged tRNAs and activation of GCN2 and downstream pathways. Since translation inhibition can cause cell cycle arrest and sensitize cells to apoptosis, I hypothesized that activation of the amino acid deprivation pathway would make

cancer cells more susceptible to PI3K pathway inhibitors. In order to test this, I evaluated the ability of a tRNA synthetase inhibitor Borrelidin, and two PI3K pathway inhibitors (QLT0267 and LY294002) to inhibit proliferation and induce apoptosis both independently and in combination.

The PI3K pathway is a critical mediator of cell homeostasis. In normal cells, it pays an important role in enabling the cell to act on external stimuli. When the PI3K pathway is perturbed, it can lead to a variety of diseases. Sometimes, (like the loss of SHIP in allergy patients), the PI3K aberration is a primary mutation that initiates the disease. In other diseases, (such as cancer), PI3K modification is an adaptation that allows the cell to survive and proliferate in ignorance of external signaling. While there is a lot known about the regulation of PI3K in a variety of cell types and disease states, there is still a lot left to discover.

Hypothesis

Due to the importance of the PI3K pathway in all cell types, I hypothesized that manipulation of the PI3K pathway is a potential therapy for a variety of diseases. In this thesis, I will describe several novel approaches to targeting the PI3K pathway. First, I will determine PI3K pathway effects on insulin signaling in normal hematopoietic cells. Then, I will investigate how manipulation of normal PI3K pathway signaling with the SHIP activator pathway inhibitor can be therapeutic in a murine model of the autoimmune disease idiopathic thrombocytopenic purpura. Next, I will examine the ability of PI3K pathway inhibitors to counteract aberrant PI3K pathway activation in cancer. Finally, I will outline a strategy to potentiate these PI3K pathway inhibitors using another small molecule drug that's part of the nutrient-sensing pathway.

Materials and Methods

Experimental Animals

Animals were housed in the Jack Bell Research Centre animal facility. All animal studies were used according to the guidelines of the Canadian Council on Animal Care. Three mouse lines were used in this study. *Pten*$^{+/-}$ mice were generated by R. Parsons (Podsypanina, Ellenson et al. 1999). These mice were backcrossed with C57BL6 mice for more than ten generations. The genotypes of the mice were determined as described (Podsypanina, Ellenson et al. 1999). ARR2-PB-Cre+ve/PTEN fl/fl mice were generated by crossing PTEN(flox/flox) mice (Dr. Tak Mak, UHN), with the ARR2Probasin-Cre transgenic line (Dr. Pradip Roy Burman, USC). We used male ARR2-PB-Cre+ve/PTEN fl/fl mice that were 8 weeks old.

Primary Myocyte culture and insulin challenge

Primary myocytes from haploinsufficient (*Pten*$^{+/l}$) and wild-type mice were harvested and cultured as described in Wong et al. (Wong, Kim et al. 2007). The cells were serum starved for 12 hours followed by incubation with 1 μmol/l insulin for the indicated time points.

Western blotting

Rabbit polyclonal anti PKB, anti-phospho-PKB (Ser473), anti-phospho-GSK-α/β (Ser21/9), and anti-cleaved parp, were obtained from Cell Signaling Technology (Beverly, MS, USA). Mouse monoclonal anti-vinculin and rabbit anti-actin antibody were obtained from Sigma (St Louis, MO, USA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Dako, Samples for western blotting were washed with PBS and lysed using a NP40-based lysis buffer. Protein concentration was determined using a bicinchronic acid kit (Thermo Fisher Scientific, IL, USA) and 20 micrograms of protein per sample was analyzed by SDS-PAGE, then

transferred to nitrocellulose. Blots were blocked with 5% milk in tris-buffered saline (TBS) solution containing 0.1% Tween-20 for 1 hour before overnight incubation with primary antibodies. Following this, the blots were incubated with a Horseradish peroxidase-conjugated secondary antibody for 1 hour. Protein bands were visualized using an enhanced chemiluminescence system in conjunction with either x-ray film or a Dyversity camera system (Syngene, MD, USA).

Chemicals

AQX-MN100 and AQX-016A were dissolved in EtOH and stored at -20 C. The drug was then dissolved in either cyclodextrin (Cyclodes Technologies, High Springs, FL) or cremaphore EL (Sigma-Aldrich Canada, Oakville, ON, Canada) carrier in order to improve aqueous solubility. QLT0267 was obtained from QLT Inc and has been described previously. It inhibits the kinase activity of ILK in cell-free assay at 26 nmol/L. It was also able to induce tumour growth inhibition of a thyroid cancer xenograft *in vivo* at 50 mg/kg (Younes, Kim et al. 2005). For the *in vitro* studies, it was dissolved in DMSO. For oral administration, QLT0267 was carried in a PTE solution instead (66.6% polyethylene glycol 300/8.2% Tween80/25% ethanol [95%]/0.2% citric acid [w/w]). The *in vivo* dosing schedule was based on previous *in vivo* studies with QLT0267 (Younes, Kim et al. 2005). LY294002 was purchased from Sigma-Aldrich Inc. (St. Louis, MO).

Cells and cell culture conditions

The MM cell lines RPMI8226 and U266 from American Type Culture Collection (Rockville, MD), OPM1 and OPM2 from Dr, Jonathan Keats (Mayo Clinic; Scottsdale, AZ), and MM.1s from Dr. Steven Rosen (Northwestern University; Chicago IL), Primary murine B-lymphocytes were obtained from wild type and SHIP-/- C57Bl/6J spleens kindly provided by Dr. Laura Sly

(University of British Columbia; Vancouver, Canada) and purified as previously described (Kennah 2007). MM cell lines and primary murine B-lymphocytes were maintained in RPMI-1640 medium containing 10% FBS and 2 mM L-glutamine. The experiments involving MM cell lines were carried out in RPMI-1640 medium containing 5% FBS and 2 mM L-glutamine. All cells were propagated at 37°C and 5% CO2 (95% air).

Propidium iodide apoptosis assay

For the AQX-016A apoptosis assay (Granta, Z138, NCEB, RPMI 8226, U266), cells were seeded in a 24-well flat bottom plate at a density of 1 x 10^6 cells/mL in 250 µl medium containing the appropriate concentration of AQX-016A.

For the AQX-MN100 apoptosis assay, (MM.1S, OPM1, OPM2, RPMI 8226) cells were seeded in a 24-well-flat bottom plate at a density of 5 x 10^5 cells/mL in 500 mL with the appropriate concentration of AQX-MN100 (serial dilution in cyclodextrin).

Cells were cultured for 24 hours. They were then washed once with phosphate buffered saline (PBS) to remove any residual media and then fixed with cold 80% ethanol for 24 hours. The cells were then washed twice with staining solution (1x PBS, 0.1% Triton X-100, 0.1 mM EDTA), then stained with 200 µl staining solution with 50 µg/mL propidium iodide and 50 µg/mL DNase-free RNase (Roche Diagnostics; Montreal, Canada) for 30 minutes at room temperature. The stained cells were then analyzed by flow cytometry using the Epics XL cytometer (Coulter Immunology; Hialeah, FL). All sub G_0 cells were gated as apoptotic. Each experiment was repeated three times.

Washout assay

MM.1S and OPM2 were subjected to an AQX-MN100 washout assay to determine the minimal exposure that committed the cells to apoptosis. Cells were seeded as described above for the

determined time, and then washed and resuspended in fresh medium for the remainder of a 24 hour period. They were then fixed and analyzed as described above.

Proliferation assay

LNCaP cells were seeded in 6-well plates in 1 ml of media containing full serum and allowed to adhere overnight. 1 mL of media containing the appropriate concentration of drug was then added and the cells allowed to incubate for 24 hours. 1 μCi of tritiated thymidine was then added to each well of the proliferation assay and left for 16 hours after which they were frozen at -80°C to stop incorporation and lyse the cells. The lysed material was then transferred to a 96-well plate for harvesting and counting.

Chronic ITP Model

The murine ITP model was based on that described by Crow et. al (Crow, Song et al. 2003). Briefly, 20 Balb/C mice were split into four age-matched groups: 80 mg/kg AQX-MN100, 5 mg/kg AQX-MN100, 2 g/kg IVIg (positive control), and a cremaphore control group. ITP was induced in the mice through daily i.p injections of 2 μg rat α-mouse CD41 (Integrin α_{IIb}) antibody (BD Pharmigen, San Jose, CA) in 200 μl PBS. 10 μl of blood was collected daily using a needle puncture of the tail vein and EDTA-precoated pipette tips. Platelets counts were determined by electrical impedance using the scil-vet animal blood counter (Scil animal care company, Gurnee, IL) according to the manufacturer's instructions. Thrombocytopenia was allowed to develop for two days and then 2 g/kg IVIg was administered as a single 1 ml i.p. injection in the positive control group. 200 μl of either AQX-MN100 or control vehicle was administered as a daily oral gavage to the other groups. In addition, the drinking water was replaced with either 200 mg/kg AQX-MN100 (in 2%ETOH/2%Cremaphore), or a 2% ETOH/2% cremaphore solution in the treatment and control groups respectively. This was

supplemented with 8% sucrose and 0.3% NaCl to prevent weight loss from the high ethanol concentration. The IVIg group had normal drinking water throughout the while study. Mice were weighed daily in order to monitor weight loss.

LPS-mediated ITP model

Mice were split into three age-matched groups: 8- mg/kg AQX-MN100, 5 mg/kg AQX-MN100, and cremaphore control. Baseline blood samples were taken and then the mice were dosed with 200 μl of the appropriate solution as an oral gavage. After 30 minutes, 10 EU LPS and 0.25 μg α-CD40 antibody was administered as 200 μl i.p. injection. After 5 hours, a second blood sample was taken and compared to the first. Platelet counts were obtained using the scil-vet animal blood counter.

AQX-MN100 Clotting assay

Bleeding times were determined using a modification of the protocol described by Hirsch et al (Hirsch, Bosco et al. 2001) Briefly, anesthesia was induced using isoflurane vapor and maintained during the procedure. The distal 0.5 cm of tail (measured with a ruler) was cut off and immediately inserted into PBS at 37ºC. Tail bleeding time was defined as time required for bleeding to stop. In cases where bleeding did not stop spontaneously at 500 seconds, bleeding was stopped at that time using applied pressure and time was indicated as 500 seconds.

QLT0267 Prostate cancer model

Eight week old mice were gavaged daily with 300 μl of either the QLT0267 PTE solution (200 mg/kg) or the PTE vehicle for 28 days. Mice were weighed every three days to ensure that no major weight loss was caused by drug toxicity in the experiment. The mice were then euthanized, prostates were harvested, wet weights determined, and slides for histopathology

were made.

Histopathology and immunohistochemical staining

Tissue sections were stained with H&E for histopathology and then examined blindly by a pathologist (Ladan Fazili) to determine the incidence of normal, hyperplasia, PIN, or carcinoma. Tissue sections were also analyzed by immunohistochemisrty for pAKT activity using an anti-pAKT antibody (Cell Signaling Technology, Beverly, MS, USA). All histopathology and immunochemical staining was performed by the Prostate Centre pathology core.

Statistical analysis

Statistical significance (p-value <0.05) is determined using student's t-test or ANOVA as appropriate. Data are presented as mean ± SD.

Results

PTEN haploinsufficiency increases insulin sensitivity in primary myocytes

Before investigating the role of PI3K in disease, it is important to understand its role in normal physiology. It should not be forgotten that there is much that is yet to be elucidated about the mechanisms by which PI3K affects its diverse downstream pathways. It is useful to determine the degree of response of the PI3K pathway to exogenous manipulation. The optimal therapeutic target is one that is sensitive to subtle modulation. This allows a level of control that can maximize target effects while minimizing side effects. A better understanding of PI3K pathway signaling can also help identify novel targets for new drugs or markers for diagnosis. In addition, it helps predict the outcomes of inhibiting different targets in the cascade. This is critical for determining side effects, especially for such a conserved cellular pathway as PI3K. Thus, these mechanistic studies are a critical part of PI3K-related therapies.

First, I wished to determine if it was possible to modulate the PI3K pathway in normal cells through manipulation of the PTEN phosphatase. One important normal function of PI3K signaling is insulin stimulated glucose uptake. In insulin-sensitive cells such as myocytes, insulin receptor binding activates PI3K and causes glucose to be taken up from the blood stream. Since PTEN antagonizes the activity of PI3K, it would follow that loss of PTEN would upregulate insulin-mediated PI3K pathways signaling in primary myocytes. As systemic PTEN knockout is an embryonic lethal mutation, haploinsufficient ($Pten^{+/l}$) mice were used. We hypothesized that PTEN haploinsufficiency would be sufficient to increase insulin sensitivity in primary myocytes through increased activation of the PI3K pathway (Wong, Kim et al. 2007).

The role of PTEN in insulin signaling was established by analyzing phosphorylation

levels of AKT and its downstream effector GSK3ß during an insulin challenge in haploinsufficient (*Pten*$^{+/l}$) and wild type mice. As expected, haploinsufficient mice exhibited more AKT S473 and GSK3ß phosphorylation as compared to the wild type (Figure 2). Not only were phosphorylation levels higher at a given time point, but the phosphorylation was also sustained for much longer. Both AKT S473 and GSK3ß phosphorylation were maintained for the full duration of the experiment (6 hours) in the haploinsufficient mice while wild type myocytes showed decreased phosphorylation after 4 hours. This supports the theory that even a 50% reduction in PTEN activity is sufficient to affect PI3K pathway activation and insulin response in primary cells. I contributed in the western immunoblot analysis seen in figure 5 in the paper (Wong, Kim et al. 2007).

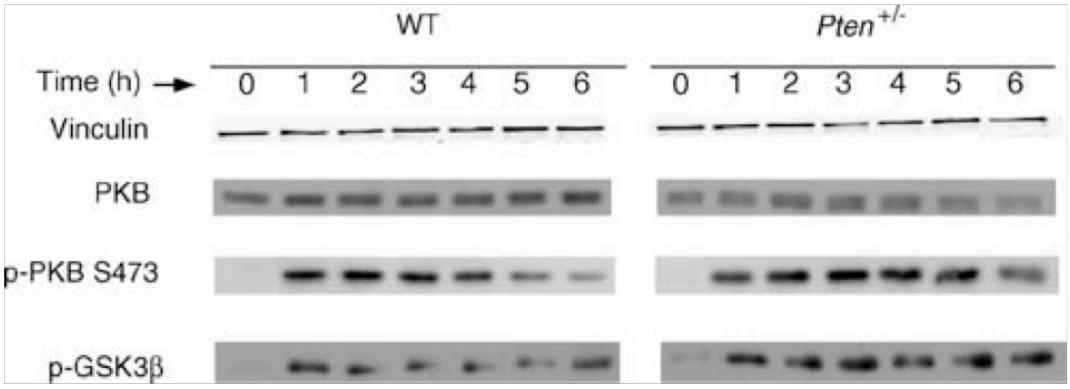


Figure 2. Phosphorylation of PKB and GSK3ß

Myocytes were serum-starved for 12 h prior to incubation with 1 μmol/l insulin for the indicated times. Cell lysates were prepared and proteins were separated by PAGE followed by western immunoblot analysis for vinculin, total PKB, phospho-PKB (p-PKB S473) and phospho- GSK3ß (p- GSK3ß) as indicated. (Wong, Kim et al. 2007)

SHIP activation is not sufficient to induce amelioration of ITP in a murine model

Since loss of a single copy of PTEN was sufficient to affect PI3K signaling in murine myocytes, I wished to determine if manipulation of the PI3K pathway in a normal cell could be therapeutic in a disease model. However, there is as yet no known activator of PTEN. PI3K pathway inhibitors (LY294002 and wortmannin) exist, but their extreme toxicities prevent their use *in vivo*. Attention then shifted to the other negative regulator of PI3K, the SH2-containing inositol 5' phosphatase (SHIP). We have a specific molecular small molecule activator of SHIP that is able to dampen the PI3K pathway both *in vitro* and *in vivo*. I chose idiopathic thrombocytopenic purpura (ITP) as a disease model. ITP is an autoimmune disease where the body produces a high level of anti-platelet antibodies. These then target platelets for destruction by the immune system. This is modeled in mice through administration of anti-platelet antibody.

A current therapy for treating ITP is the intravenous administration of immunoglobulins (IVIg) (Crow, Song et al. 2003). In a murine model, a single 2g/kg IVIg administration after the induction of thrombocytopenia is sufficient to reverse the disease (Crow, Song et al. 2003). Since IVIg action is dependant on the FcγRIIB receptor and FcγRIIB-mediated inhibition is through recruitment of SHIP, we hypothesized that administration of the SHIP activator AQX-MN100 would mimic the efficacy of IVIg, and ameliorate platelet destruction in a murine model of ITP.

First, I needed to determine the kinetics of thrombocytopenia induction. Based on previous studies (Crow, Song et al. 2003), I hypothesized that a 2 μg dose of anti-platelet antibody should render the mice maximally thrombocytopenic within 24 hours. After 2 days the platelet count reached a minimal level and did not decrease even with the administration of additional antibody (Figure 3). The induced thrombocytopenia remains for at least 48 hours

after the antibody administration.

Once the effects of the anti-platelet antibody alone had been established, I hypothesized that administration of AQX-MN100 would reverse thrombocytopenia and increase platelet numbers. Platelet destruction was induced through i.p. injection of an anti-platelet antibody and mice were allowed to become thrombocytopenic over two days. They were then treated from day 2 to day 4. The first treatment group received daily gavages of an 80 mg/kg AQX-MN100 solution and 200 mg/kg AQX-MN100 in the drinking water. The second treatment group received no gavage, but received 500 mg/kg AQX-MN100 in the drinking water. These doses were proven to be effective in the endotoxemia model (Ong, Ming-Lum et al. 2007). A third group received daily gavages of a 5% cremaphore/EtOH solution with 2% cremaphore/EtOH in the drinking water to control for cremaphore/EtOH effects. Finally, an IVIg group was included as a curative therapy against which to test the possible effects of SHIP inhibition. Thrombocytopenia was induced as expected (Figure 4). However, none of the treatment groups were able to recover platelet counts during the 5 day experiment and there was no statistically significant difference between any of the treatment groups. This was particularly troublesome since this dose of IVIg has been curative in the same mouse model. In addition, the AQX-MN100-treated mice exhibited extreme weight loss (Figure 5). This was probably due to the presence of the cremaphore or EtOH in the water weakening them to the point that they were unable to feed. According to animal care protocols, mice must be euthanized if they lose more than 20% of their body weight. All of the treatments with the treated water were approaching that limit within 4 days. In contrast, there was no weight loss in the IVIg treated mice (which had normal water). To prevent weight loss in future experiments, 8% sucrose and 0.3% NaCl was added to the drinking water along with any treatment as adapted from colitis models (Khan, Blennerhasset et al. 2002). This was able to abrogate the weight loss caused by the cremaphore/EtOH solution in the drinking water.

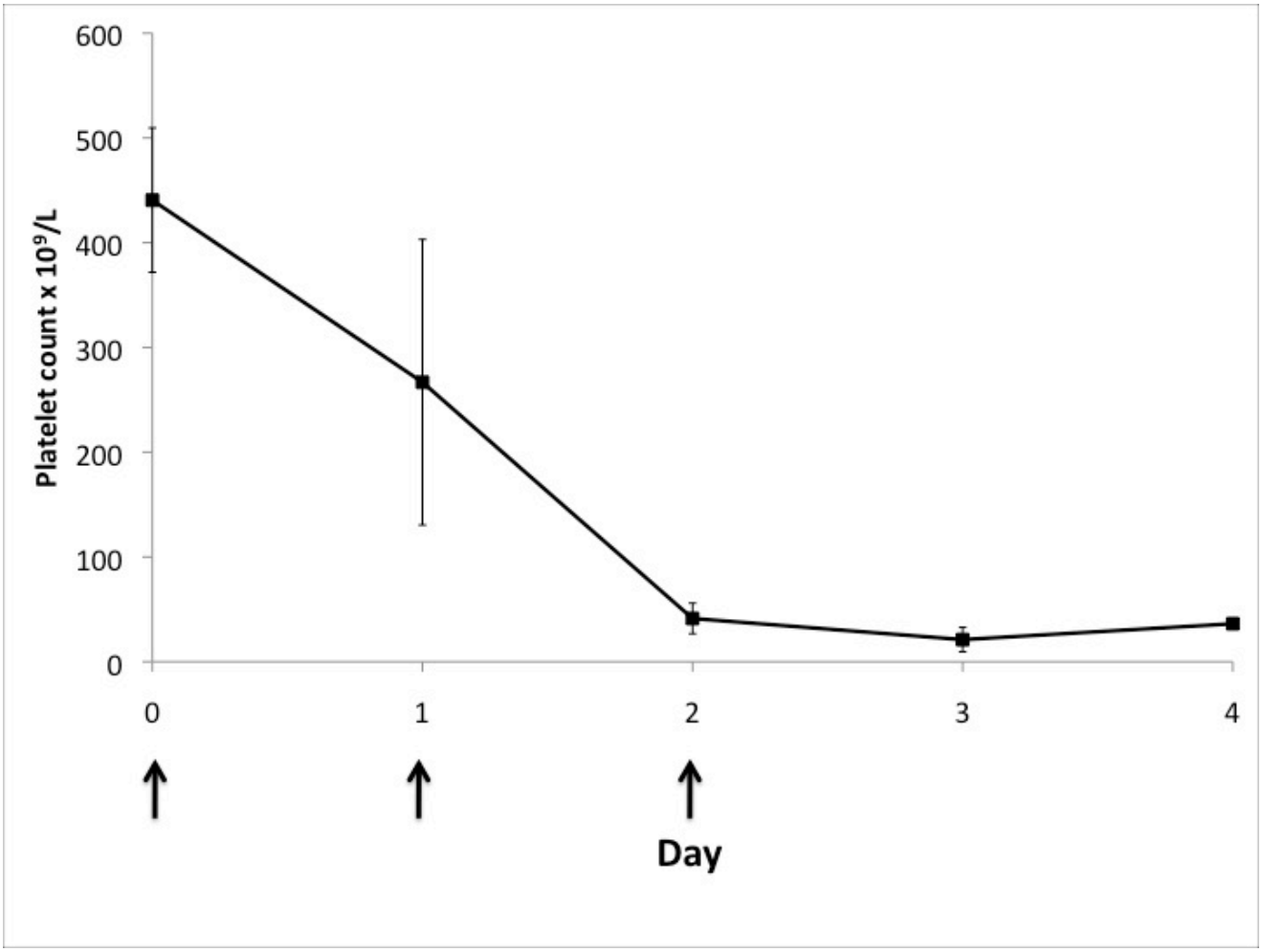


Figure 3. Induction of thrombocytopenia by i.p. injection of anti-platelet antibody

Wild Type BALB/c mice (n=10) were injected with 2 μg anti-integrin αIIb antibody on days 0-2. The arrow (↑) denotes injection of anti-integrin αIIb antibody. Mice were bled daily for platelet enumeration. Data is shown as an average ± SD.

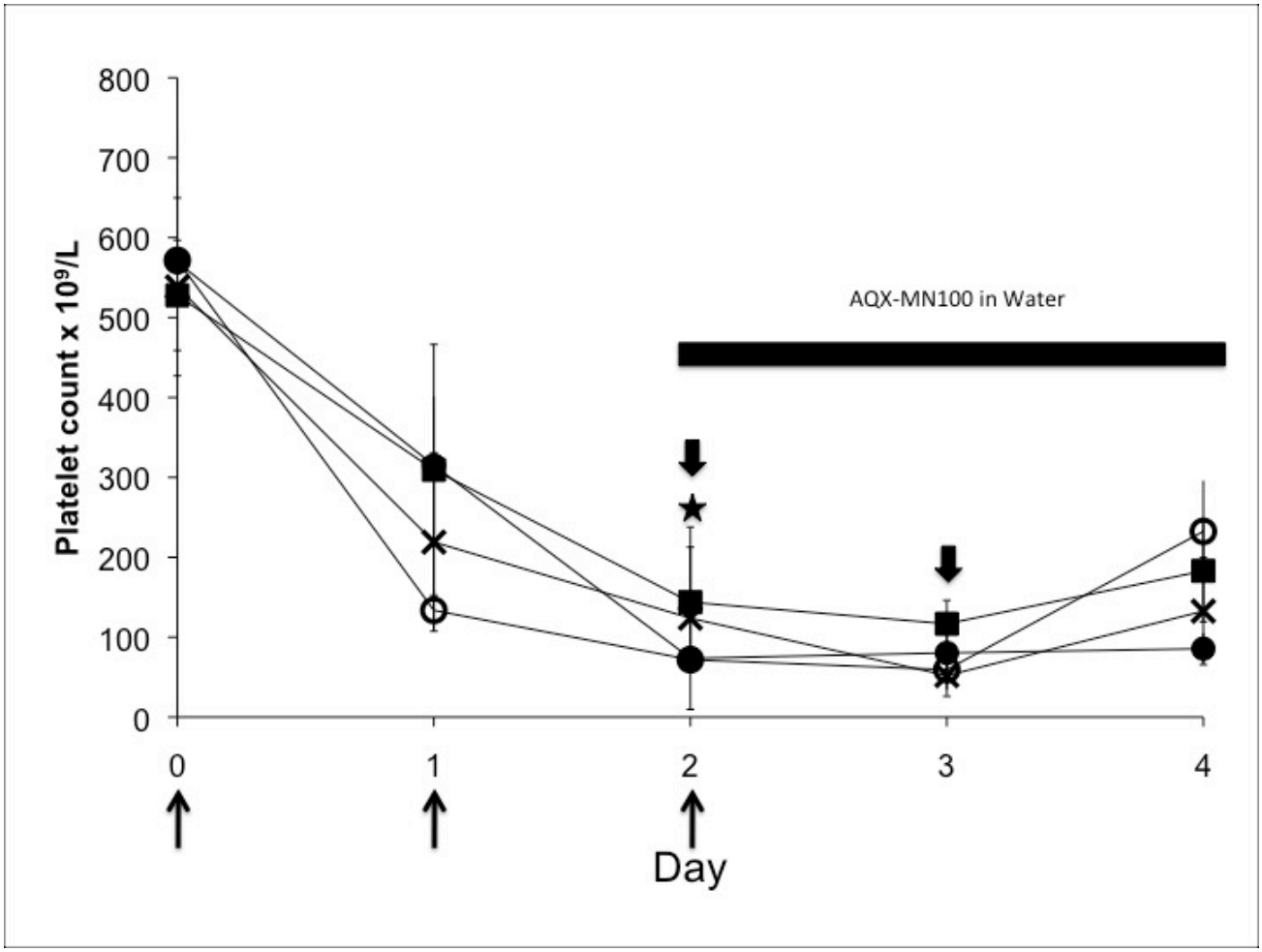


Figure 4. AQX-MN100 does not rescue experimental idiopathic thrombocytopenia

Wild-type BALB/c mice were injected with 2 μg anti-integrin αIIb antibody on days 0-3. The arrow (↑) denotes injection of anti-integrin αIIb antibody. The IVIg-treated group (closed square) received a single injection of 2 g/kg IVIg on day 2. (★ denotes injection of IVIg). The AQX-MN100 treated groups received either daily gavages (denoted by ↓) of 80 mg/kg AQX-MN100 solution along with 200 mg/kg AQX-MN100 in the drinking water (open circle) or no gavages and 500 mg/kg AQX-MN100 in the drinking water (closed circle). Control mice (**X**) received daily gavages of 5%Cremaphore/5%EtOH and 2%EtOH/2% cremaphore in the drinking water. Mice were bled daily for platelet enumeration. Data is shown as an average of five mice per group.

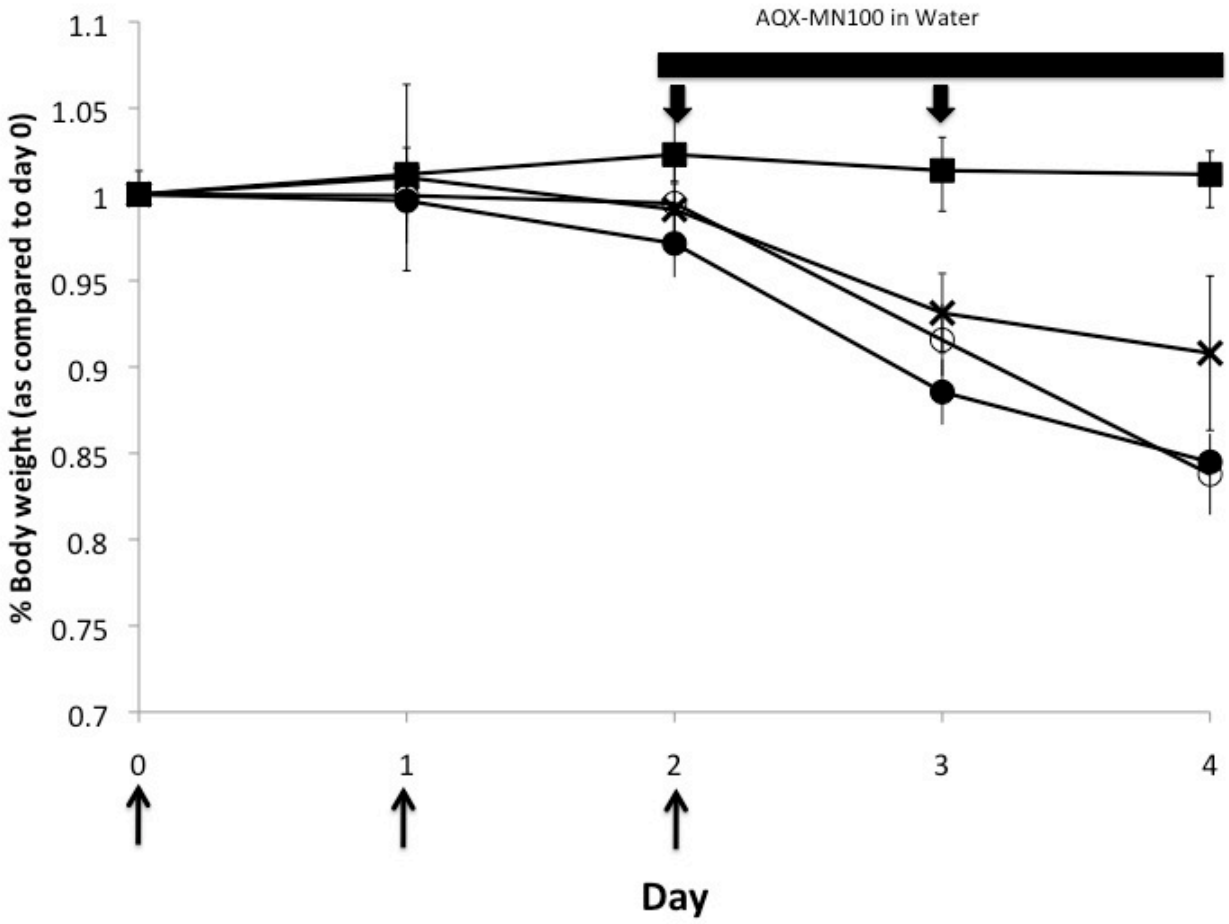


Figure 5. Weight loss as a result of AQX-MN100 treatment

Wild-type BALB/c mice were injected with 2 μg anti-integrin αIIb antibody on days 0-3. The arrow (↑) denotes injection of anti-integrin αIIb antibody. The IVIg-treated group (closed square) received a single injection of 2 g/kg IVIg on day 2. (★ denotes injection of IVIg). The AQX-MN100 treated groups received either daily gavages (denoted by ↓) of 80 mg/kg AQX-MN100 solution along with 200 mg/kg AQX-MN100 in the drinking water (open circle) or no gavages and 500 mg/kg AQX-MN100 in the drinking water (closed circle). Control mice (**X**) received daily gavages of 5%Cremaphore/5%EtOH and 2%EtOH/2% cremaphore in the drinking water. Mice were weighed daily. Data is shown as an average of five mice per group.

Once the weight loss problem had been solved, the experiment was repeated again and extended so that the IVIg treatment group would have sufficient time to recover platelet numbers. Thrombocytopenia was induced and maintained through the experiment using daily i.p. injections of anti-platelet antibody. The mice were then treated with daily gavages of either a 5 mg/kg or 80 mg/kg AQX-MN100 solution, while the control group was gavaged with a cremaphore-only solution. In addition, the drinking water was replaced with either 200 mg/kg AQX-MN100 solution (treatment groups), or a cremaphore control solution in order to provide a constant supply of the drug. In a mouse model of endotoxemia, an oral administration of 20 mg/kg AQX-016A was sufficient to inhibit inflammation (Ong, Ming-Lum et al. 2007) showing that the drug was in circulation and efficacious at this dose.

An IVIg treatment (2 g/kg IVIg with normal water) was included as a curative therapy against which to compare the possible effects of SHIP inhibition. Blood samples for platelet level monitoring were obtained using a needle and micropipette in order to minimize blood loss in the experimental animals. Platelet enumeration was determined by electrical impedance using the Scil-vet animal blood counter, an automated hematology analyzer. Mouse weights were monitored daily.

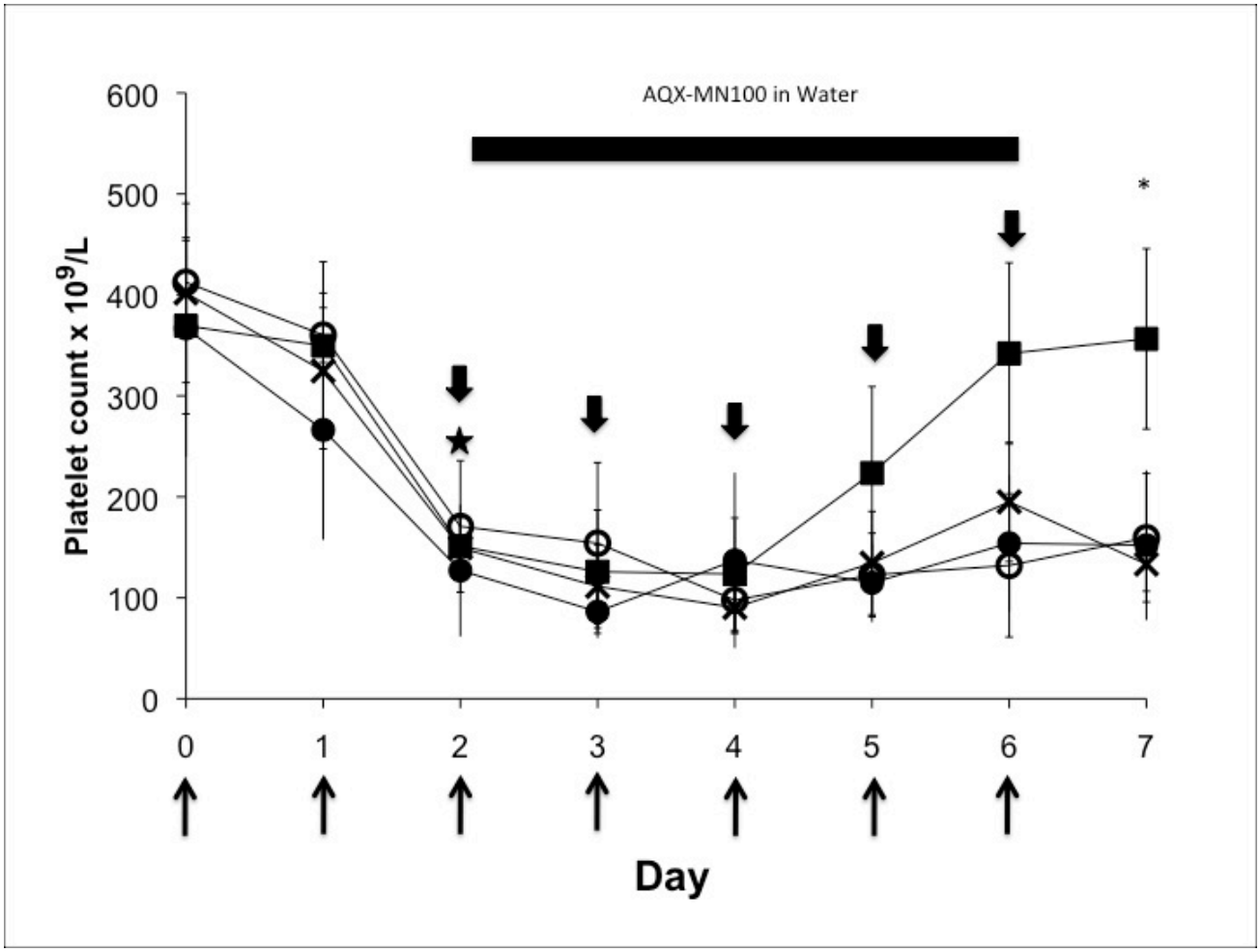


Figure 6. AQX-MN100 does not rescue experimental idiopathic thrombocytopenia

Wild-type BALB/c mice were injected with 2 μg anti-integrin αIIb antibody on days 0-6. The arrow (↑) denotes injection of anti-integrin αIIb antibody. The IVIg-treated group (closed square) received a single injection of 2 g/kg IVIg on day 2. (★ denotes injection of IVIg). The AQX-MN100 treated groups received daily gavages (denoted by ↓) of either 5 mg/kg (open circle) or 80 mg/kg (closed circle) AQX-MN100 solution along with 200 mg/kg AQX-MN100 in the drinking water from days 2 to 6. The carrier control group (X) received daily gavages of 5%Cremaphore/5%EtOH and 2%EtOH/2% cremaphore in the drinking water during the same period. Mice were bled daily for platelet enumeration. Data is shown as an average of five mice per group (* $p<0.05$).

Maximal thrombocytopenia was observed after approximately 48 hours of antibody treatment. As expected, the IVIg positive control was able to recover platelet levels 5 days after treatment (Figure 6). Neither the 5 mg/kg, nor the 80 mg/kg dose of AQX-MN100 was able to induce recovery of platelet levels as compared to the cremaphore control.

There are many possible reasons why AQX-MN100 proved unsuccessful at ITP recovery in this model. The pharmacokinetics and pharmacodynamics of the drug may not be sufficient to achieve systemic continuous inhibition of immune cells. AQX-MN100 has a half-life of 2 hours in mice and is most active 30 minutes after administration (Unpublished data, A. Mui, personal communication, March 12, 2010). Macrophages and neutrophils have an extremely high turnover rate, so there is a constant stream of new cells that have not been exposed to the drug and are able to phagocytose the antibody-coated platelets. IVIg also has FcγRIIB-independent effects. Perhaps those are the mechanisms that are responsible for the IVIg-mediated rescue of ITP. It may also be possible that the targets of IVIg effects are not the macrophages and neutrophils themselves. Instead, IVIg action may be through activating FcγR receptors on dendritic cells (Siragam, Crow et al. 2006) which then downregulate the phagocytic macrophages. Even though FcγRIIB on the macrophages is necessary for the inhibition to occur, activation of the FcγRIIB pathway in isolation from the other DC-mediated signaling may not be sufficient to prevent immune platelet destruction (Siragam, Crow et al. 2006).

A more interesting explanation is that inhibition of the PI3K pathway blocks late phagocytosis and not platelet binding. There is evidence that the effects of SHIP activation are in this phase (Kamen, Levinsohn et al. 2007). Thus, the drug is effective at preventing platelet destruction, but this is not recognized by our assay, which is size dependant. Additional experiments would need to be done to see if this is so such as incubation of treated and untreated macrophages with a labeled anti-platelet antibody. If phagocytosis is being blocked, you would

expect to see the AQX-MN100 treated macrophages coated with platelets while the untreated cells would be relatively bare. Analysis can be done using either FACS or microscopy.

In the light of this experiment, we wished to determine if AQX-MN100 was effective in treating the more acute form of ITP. In these cases, ITP is not chronic, but is associated with either a bacterial or viral infection. Here, the patient has a low level of autoantibody that is insufficient to trigger thrombocytopenia in a healthy state. However, upon immune system stimulation (as in infection), there are sufficient activated macrophages and neutrophils to exploit and destroy the platelets. This is simulated in a mouse model where concentrations of LPS and anti-platelet antibody that are individually too low to cause platelet destruction synergize in combination to induce thrombocytopenia (Tremblay, Aubin et al. 2007).

Macrophage activation is characterized by secretion of inflammatory cytokines and the enhanced ability to kill intracellular pathogens (Mosser 2003). This is dependant on the PI3K pathway (Rommel, Camps et al. 2007). PI3K pathway inhibitors limit infection-mediated induction of an inflammatory response (Guha and Mackman 2002). SHIP upregulation is equally effective at preventing an immune response (Sly, Hamilton et al. 2009) The SHIP activator AQX-MN100 can inhibit macrophage activation as assessed by TNFα production (Ong, Ming-Lum et al. 2007). Since immune activation is an essential step in acute ITP, it follows that prophylactic administration of AQX-MN100 may be effective at treating the acute form of ITP in a murine model.

A 0 hour blood sample was taken to determine baseline platelet levels. Mice were pre-treated with an oral gavage of a 5 mg/kg AQX-MN100 (in 0.5% EtOH/cremaphore), 80 mg/kg AQX-MN100 (in 8% EtOH/cremaphore), or a cremaphore control solution (8% EtOH/Cremaphore). 30 minutes later an i.p. injection of a low dose of LPS and anti-platelet antibody was administered to induce thrombocytopenia. These doses and the 30-minute time point were based on proprietary empirical data and were previously shown to be effective in an

endotoxemia model (Ong, Ming-Lum et al. 2007). A second blood sample was taken 5 hours later and platelet levels compared to the baseline.

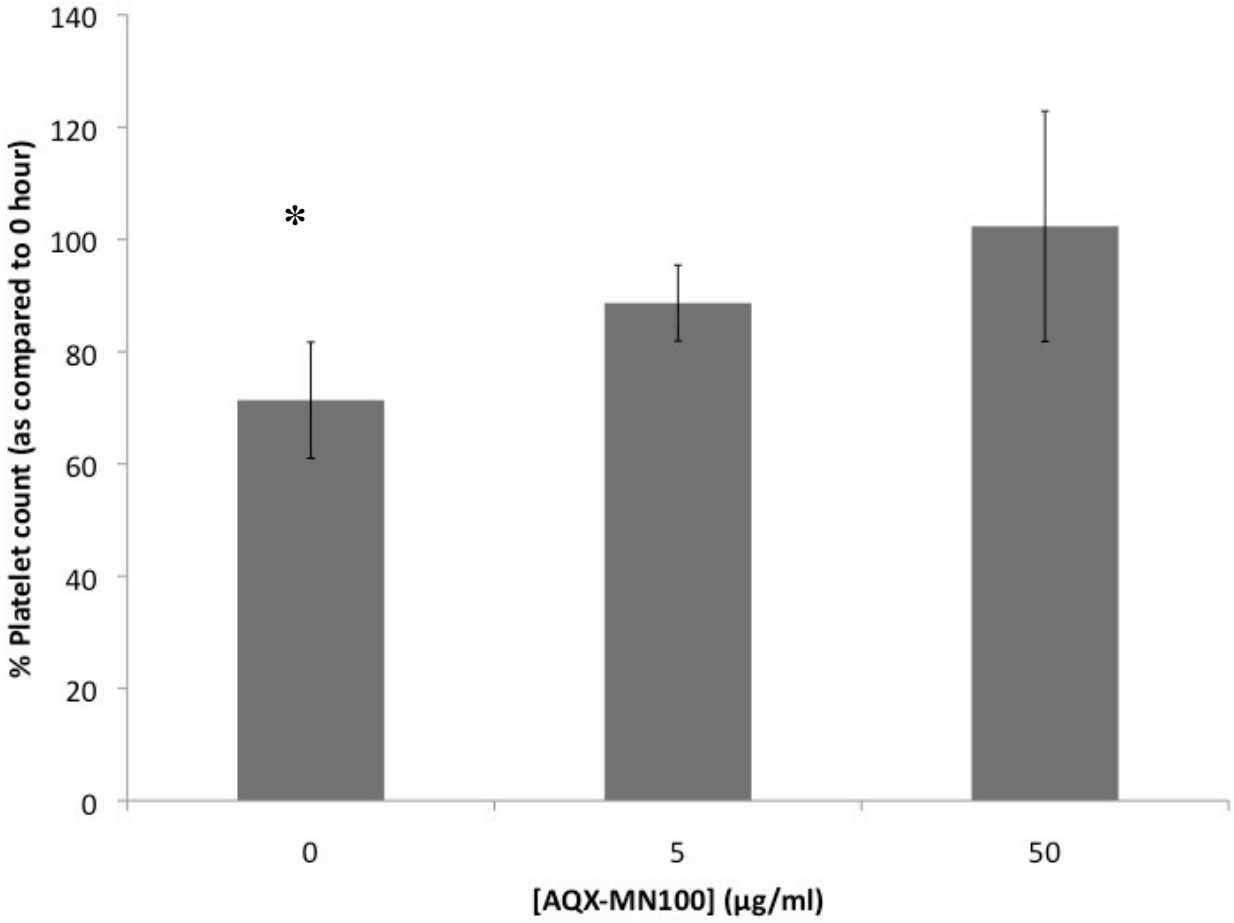


Figure 7. AQX-MN100 protects against LPS-mediated ITP

Balb C mice (n= per treatment) were gavaged with 200 µl of an 80mg/kg, 5 mg/kg, or 8% EtOH/Crem control solution. Half an hour later, 0.25 µg MW Reg30 Rat anti-mouse CD41 antibody and 10 EU (1 ng) LPS in 200 µl PBS pH 7.2 was administered to induce platelet loss. Blood was taken five hours later and platelet counts compared to baseline. Results are reported as the average value +/- SE. Significance of the reported effects was calculated by comparing the 5 hour values to the 0 hour values with a paired t-test (* $P<0.05$)

Only the cremaphore control group showed a significant drop in platelet numbers (Figure 7), though the decrease was not as greatly reduced as reported by other groups with the same dose of LPS and anti-platelet antibody (Tremblay, Aubin et al. 2007). However, the different treatments were not significantly different from each other as determined from an ANOVA (p=0.32). This implies that even though only the cremaphore control showed a significant drop, I cannot conclude that the AQX-MN100 treatments were prophylactic. A future experiment with several time points to elucidate the optimal time for an AQX-MN100 effect is probably called for.

Aside from AQX-MN100's potential effects on platelet numbers, a SHIP agonist might also affect platelet function. Inactive circulating platelets are recruited to wound sites and activated thorough binding of cell surface receptors. This in turn leads to cytoskeletal reorganization and aggregation of the platelets. This activation is mediated by PI3K. Platelets from PI3Kγ-null mice show impaired aggregation and spreading after agonist stimulation (Hirsch, Bosco et al. 2001; Lian, Wang et al. 2005). This is likely due to the role of the PI3K pathway on calcium influx in platelets. An impaired calcium response is seen both in PI3Kγ knockout platelets and normal platelets in the presence of a PI3K inhibitor (Lian, Wang et al. 2005). Since AQX-MN100 inhibits the PI3K pathway, there is a potential that it will inhibit clotting. This would be a significant side effect from a toxicology point of view. To determine whether AQX-MN100 could impair clotting *in vivo,* I determined the bleeding times of treated and untreated mice after amputation of the tail tip. Mice were pre-treated with a gavage of the AQX-MN100 solution 30 minutes before tail bleeding times were assayed. These doses and the 30-minute time point were based on proprietary empirical data and were previously shown to be effective in an endotoxemia model (Ong, Ming-Lum et al. 2007). Outliers were identified and removed using a Dixon test. There was no difference between the treatment groups when analyzed using ANOVA (p=0.30)(Figure 8). This is notable since even the low 5 mg/kg dose

was shown to be effective in LPS-mediated ITP and endotoxemia models. The fact that even the high 80 mg/kg dose does not appear to affect clotting suggests that AQX-MN100 does not have a significant effect on blood coagulation.

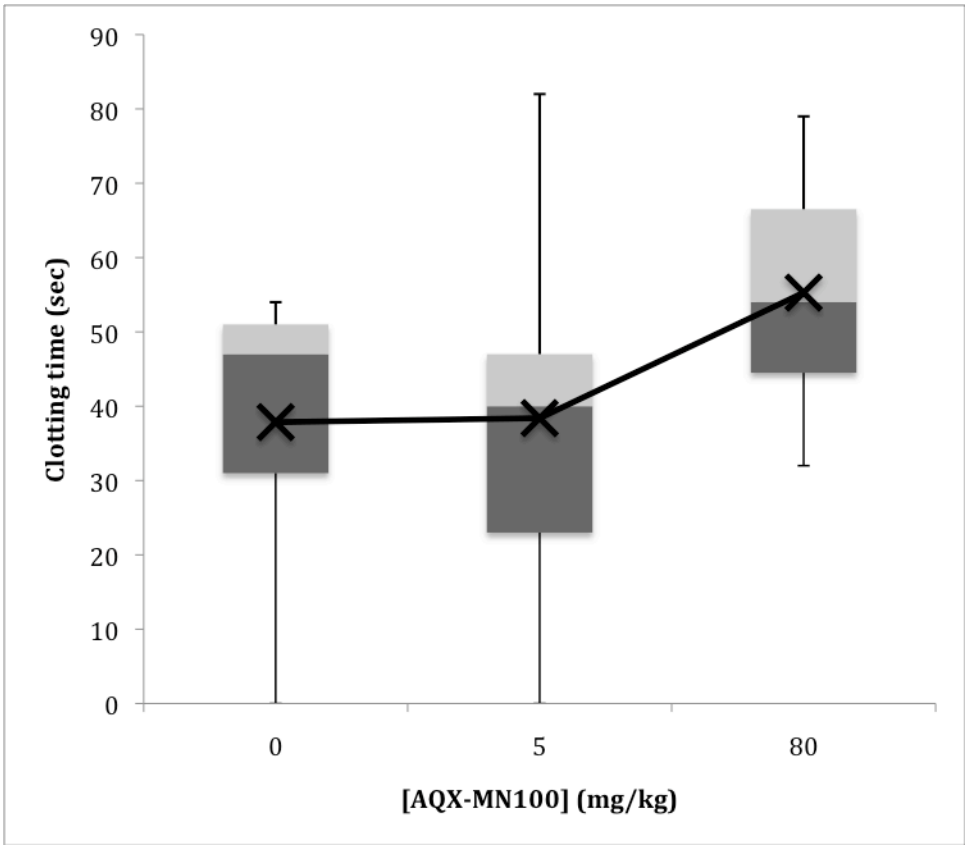


Figure 8. AQX-MN100 does not affect bleeding times

Tail bleeding times were assayed 30 minutes after drug administration. Data is presented as a box and whisker plot to represent the range and distribution of the data. In addition, the means of each dose is represented as a trend line (**X**). Outliers were identified and removed using a Dixon test. No differences in bleeding time were observed (p=0.30 using ANOVA).

SHIP activation induces apoptosis in hematopoietic cancers

After the limited therapeutic success of the SHIP activator in the treatment of ITP, I wanted to find a model in which I would expect a stronger therapeutic effect. Since the hematopoietic cancers multiple myeloma and mantle cell lymphoma have constitutively activated signaling through the PI3K pathway, I hypothesized that they would respond strongly to the inhibitory effects of a SHIP activator.

In order to determine the efficacy of the SHIP agonists AQX-016A and AQX-MN100, we exposed several hematopoietic cancer cell lines to increasing doses of the drug. We used multiple myeloma and mantle cell lymphoma cell lines since they are hematopoietic cancers with a constitutively active PI3K/AKT pathway. Apoptosis was determined using propidium iodide staining and flow cytometry analysis.

AQX-016A was the first pelerol derivative tested. Three mantle cell (Granta, Z138, and NCEB), and two multiple myeloma cell lines (RPMI 8226, U266) were incubated with decreasing concentrations of AQX-016A and then analyzed for apoptosis. AQX-016A caused dose-dependant apoptosis in all five cancer cell lines (Figure 9). RPMI 8226 cells had an extremely high basal apoptotic index (40%) and achieved a maximal apoptotic index of 62%. The IC_{50} for the observed concentrations was 5 μM. U266 had a basal apoptotic index of 15%, a maximal apoptotic of 36%, and an IC_{50} of 4 μM. Granta cells had a basal apoptotic index of 18%, a maximal apoptotic index of 44% and an IC_{50} of 2.5 μM. NCEB cells had a basal apoptotic index of 16%, a maximal apoptotic index of 32% and an IC_{50} of 6 μM. Z138 cells had the lowest basal apoptotic index of 10% and a maximal apoptotic index of 52% with an IC_{50} of 2.5 μM

The mantle cell lymphoma line Z138 appeared to be the most sensitive to the drug with

the lowest IC_{50}, while the other two appeared more resistant, requiring 2.5-5 μM of AQX-016A to induce a significant increase in apoptosis and having much higher IC_{50}s. The two multiple myeloma lines appeared more sensitive, exhibiting dose-dependant effects from 0.625-1.25 μM AQX-016A and IC_{50}s of 4-5 μM.

This result is not surprising since all the mantle cell lymphoma cell lines are characterized by constitutive activation of AKT. This is sustained even in the absence of serum suggesting that this activity is not dependant on exogenous signaling (Rudelius, Pittaluga et al. 2006). This activation is counteracted by PI3K and AKT inhibitors such as LY294002, wortmannin, and the Calbiochem AKT inhibitor (IL-6-hydroxymethyl-chiro-inositol-2-[R]-2-o-methyl-3-o-octadecylcarbonate) (Rudelius, Pittaluga et al. 2006). As expected, the SHIP agonist AQX-016A has the same effect as these inhibitors (Figure 9). These cell lines vary in PTEN status: Z138 expresses PTEN while NCEB and Granta are both PTEN null (Rudelius, Pittaluga et al. 2006). As for the multiple myeloma cell lines, RPMI8226 expresses PTEN (Shi, Gera et al. 2002), but the PTEN status of U266 is less sure. A FISH analysis shows PTEN deletion (Mazars, Portier et al. 1992; Chang, Qi et al. 2006), but another study shows protein expression of PTEN via western blot (Descamps, Pellat-Deceunynck et al. 2004). Basal AKT phosphorylation is similarly uncertain with some studies showing basal phosphorylation (Hecht, von Metzler et al. 2008) while others do not (Hideshima, Catley et al. 2006). Thus, it is difficult to say whether PTEN status affects AQX-016A sensitivity in multiple myeloma cell lines. However the PTEN-expressing mantle cell lymphoma line (Z138) appears more sensitive than the PTEN-null ones.

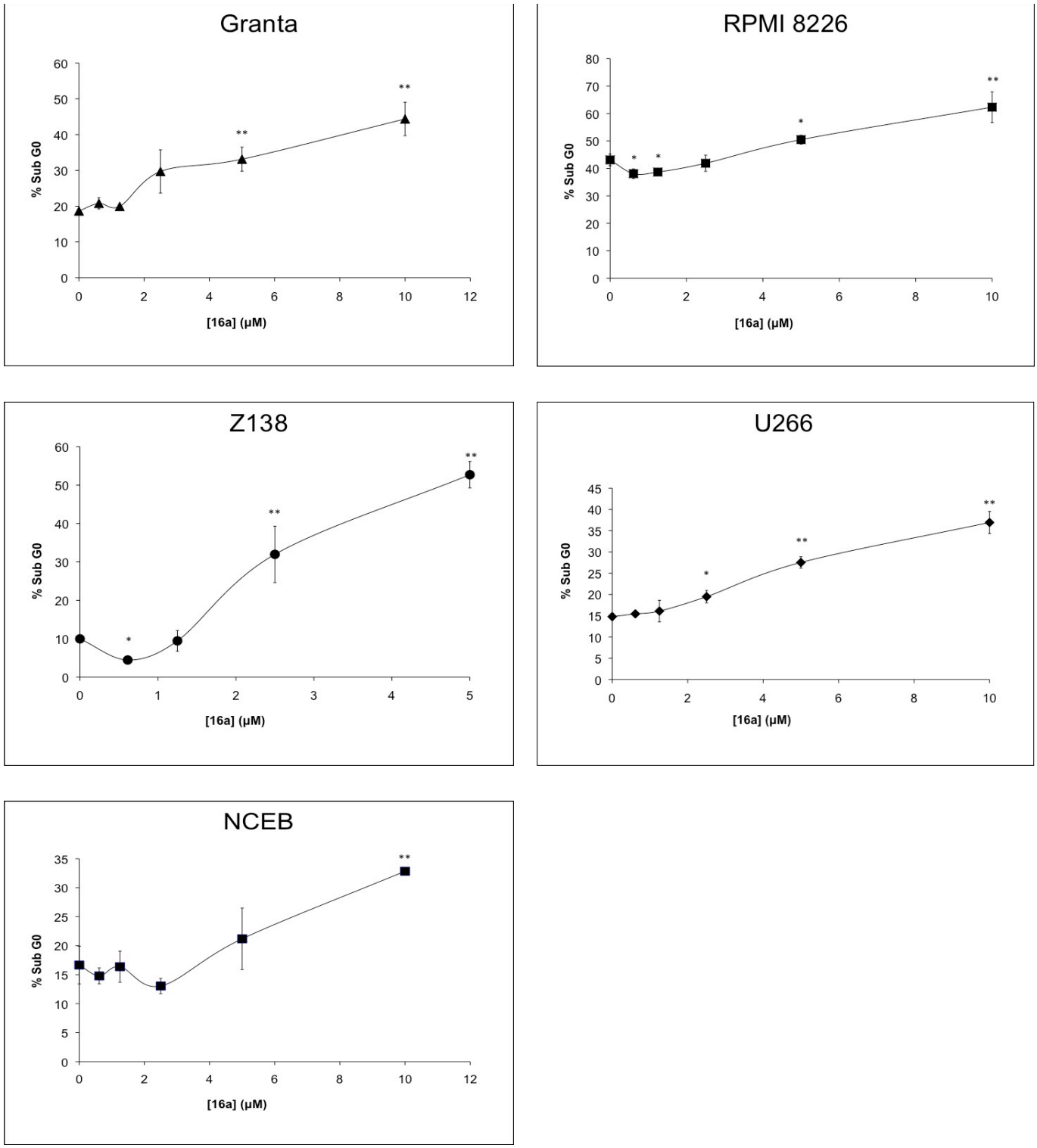


Figure 9. AQX-016A induces apoptosis in a variety of MM and MCL cell lines

Cells from three mantle cell (Granta, Z138, and NCEB), and two multiple myeloma cell lines (RPMI 8226, U266) were incubated with the indicated concentration of AQX-016A for 24 hours, then stained with PI and analyzed by flow cytometry. Data represent the mean percentage of cells in the sub-G0 (apoptotic) phase ± SD. (* $p<0.05$, ** $p<0.005$)

Following the success of AQX-016A as an inhibitor of the PI3K pathway, chemical modifications were made to AQX-016A in response to concerns about the catechol structure. This structure has a potential to cause non-specific protein inhibition after being metabolized by the liver. The resulting drug was named AQX-MN100 and was used in all subsequent experiments. AQX-MN100 has the same biologic activity as AQX-016A as determined by an *in vitro* phosphatase assay. It also inhibited TNFα production in macrophages with an EC_{50} of 0.3 to 0.5 μM (Ong, Ming-Lum et al. 2007).

AQX-MN100 was tested on four multiple myeloma cell lines: RPMI8226 from the AQX-016A experiments, OPM1, OPM2, and MM.1S. All of these cell lines showed a marked sensitivity to AQX-MN100 treatment (Figure 10). RPMI8226 showed the greatest sensitivity, especially as compared to AQX-016A. The other three cell lines required a greater (2.5 μM) concentration of AQX-MN100 to increase apoptosis above basal levels. This may be due to the constitutive activation that is shared by MM.1S, OPM1 and OPM2, but not RPMI 8226 (Hideshima, Catley et al. 2006).

Interestingly, this is not related to PTEN status as OPM1, OPM2 are PTEN null, but MM.1S and RPMI8226 express wild type PTEN (Hyun, Yam et al. 2000; Hideshima, Nakamura et al. 2001; Shi, Yan et al. 2005). However, in both of these cell lines, AKT is universally phosphorylated at Ser^{380}. This increases PTEN stability but results in loss of activity. (Vazquez, Ramaswamy et al. 2000; Bahlis, Starovic et al. 2005). In the absence of a PTEN phosphatase, this may result in constitutively an activated PI3K pathway in spite of their PTEN status.

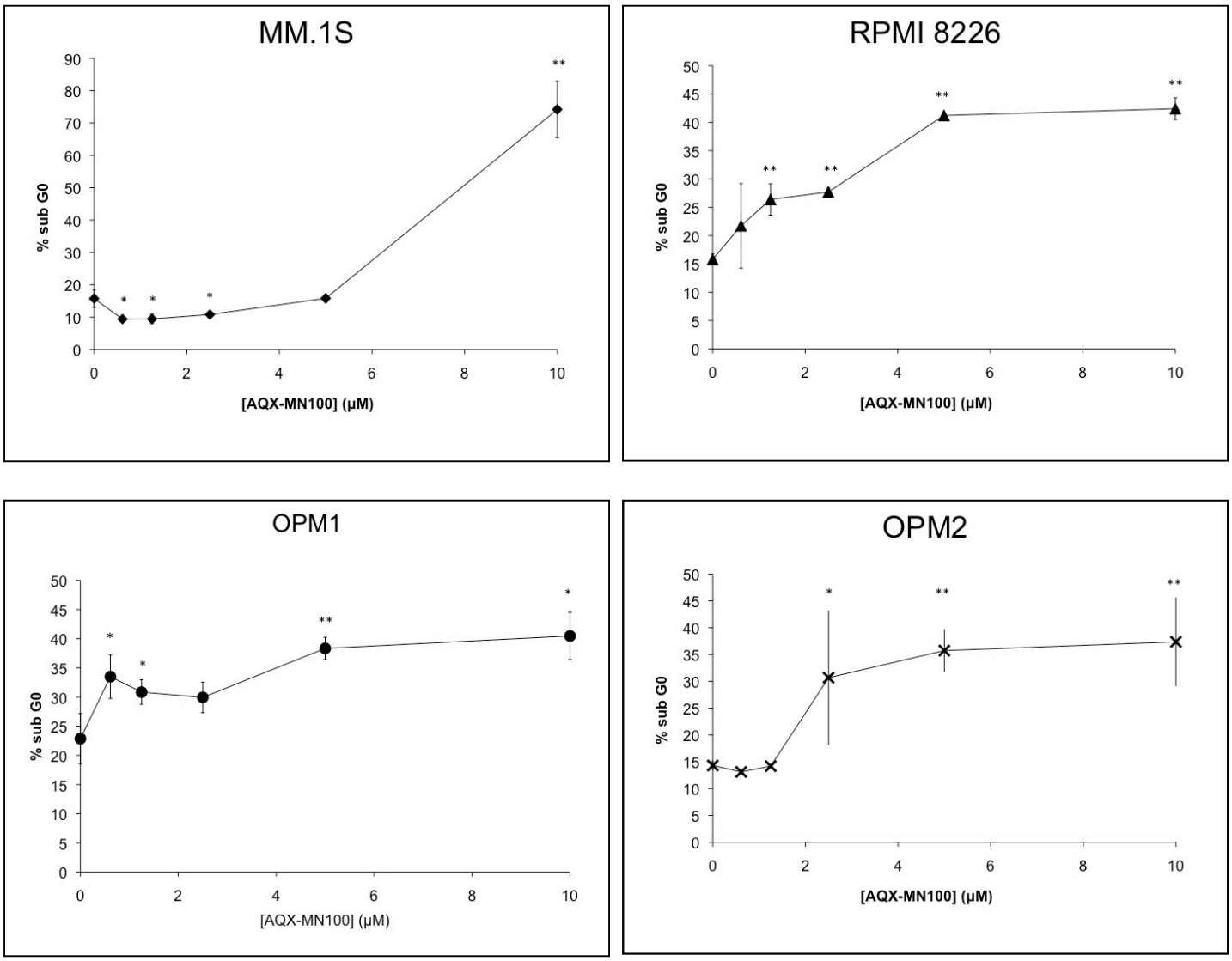


Figure 10. AQX MN100 induces apoptosis in MM cell lines

Cells were incubated with the indicated concentration of AQX MN100 for 24 hours, then stained with PI and analyzed by flow cytometry. Data represent the mean percentage of cells in the sub-G0 (apoptotic) phase ± SD. * $p<0.05$, ** $p<0.005$

Finally, we wished to determine the minimum exposure necessary to commit MM cells to apoptosis. This was done with two cell lines (OPM2 and MM.1S) and was conducted in both a time and dose dependant manner. The cells were incubated with the drug for the specified amount of time after which they were washed and the media replaced with drug-free media for the remainder of the 24-hour assay. When viewed from a dependant manner, it is apparent that 5 μM is sufficient to induce apoptosis within a 6-hour period (Figure 11A). As would be expected, apoptosis is enhanced through increase of either drug concentration or exposure time. When viewed as a function of concentration, 2 hours at a 5 μM dose is sufficient to commit OPM2 cells to apoptosis, while MM.1S does not exhibit significant apoptosis until the 4 hour time point (Figure 11B).

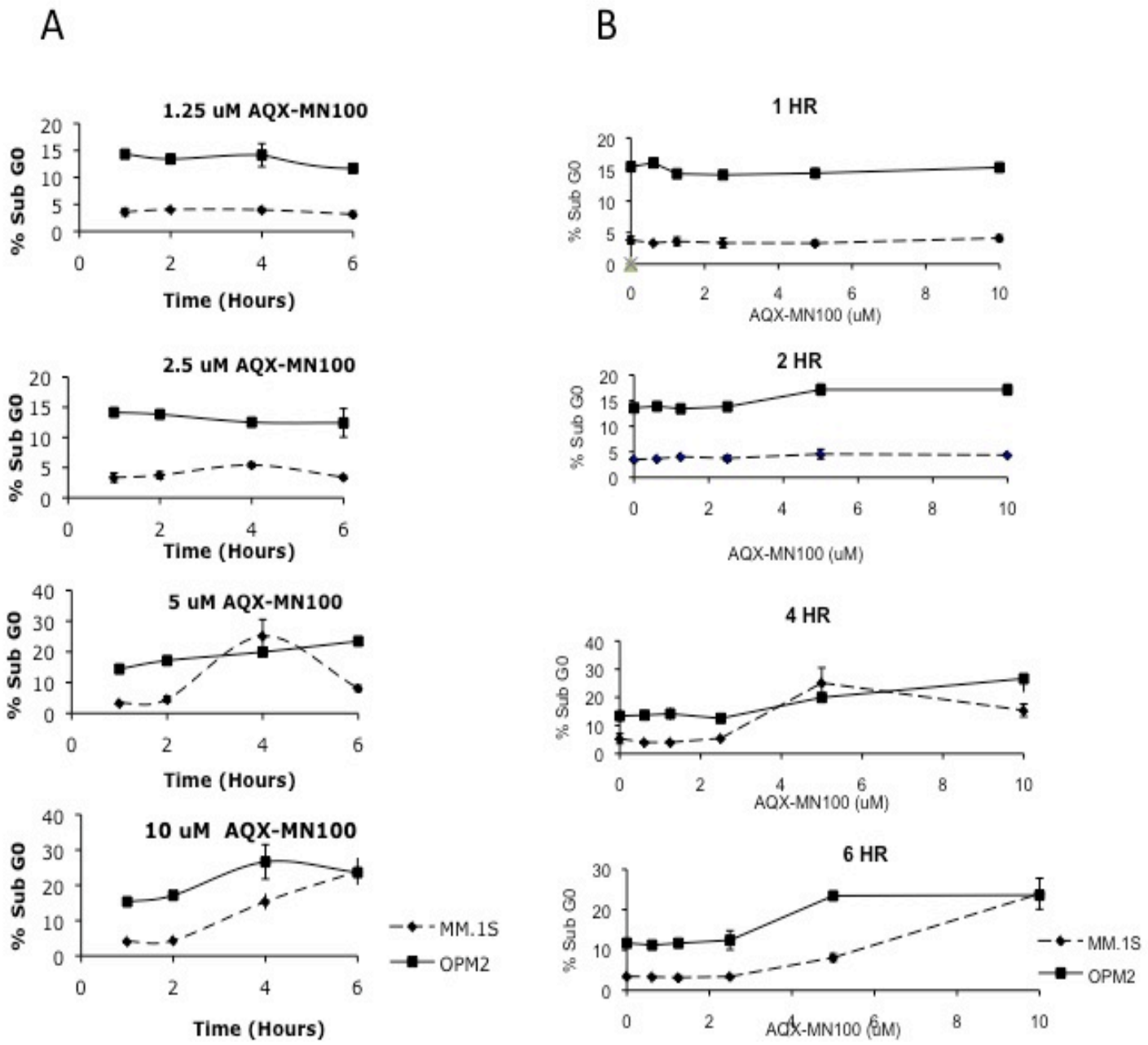


Figure 11. AQX-MN100 induces apoptosis in a time-dependant manner

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 as a function of time (A) or concentration of AQX-MN100 (B).

The ILK inhibitor QLT0267 does not inhibit prostatic carcinogenesis in a prostate specific PTEN knockout mouse model

Since chemical inhibition of a constitutively activated PI3K pathway proved to be therapeutic in the hematopoietic cancers multiple myeloma and mantle cell lymphoma, I wanted to know if it could also be useful in the treatment of another form of cancer. Prostate cancer is characterized by the constitutive activation of the PI3K pathway, usually through loss of the tumor suppressor PTEN and activation of the oncogenic ILK kinase. The ARR2-PB-Cre+ve/PTEN fl/fl mouse line develops prostate cancer according to a well-established timeline that mirrors human prostate cancer progression. We had an inhibitor of ILK that has shown efficacy in subcutaneous and *in vitro* models of cancer. We predicted that ILK inhibition would be sufficient to inhibit prostate cancer progression in this murine model. To test if QLT0267 can inhibit the progression of prostate cancer in ARR2-PB-Cre+ve/PTEN fl/fl transgenic mice, we treated 8-week-old mice with daily gavages of either the QLT0267 PTE (200 mg/kg) solution or the PTE vehicle. This dosing schedule was based on previous *in vivo* experiments with QLT0267 (Edwards, Woo et al. 2008). Prostates were harvested after 24 days, weighed, and sent for histology.

There was no difference in the wet weights of the prostate between the QLT0267 and the control treatments ($p = 0.35$) (Figure 12B). The control had a mean prostate weight of 0.59 % of body weight (SD = 0.012), while the QLT0267 mice had a mean prostate weight of 0.65% of body weight (SD = 0.102). (Figure 12B). There was also no difference in histology or pAKT staining (Figure 12A). Both the QLT0267-treated and the PTE control mice showed the same degree of cancer progression as determined by H&E staining (Figure 13). All of the p-AKT staining was membrane located and both treatments showed the same, moderate intensity of p-

AKT staining (Figure 12A). Complete histology data is included in Appendix 1.

A

Pathology:

	Mild Hyperplasia/Displasia +	Moderate Hyperplasia/Displasia ++
QLT0267	5	5
Control	4	6

B

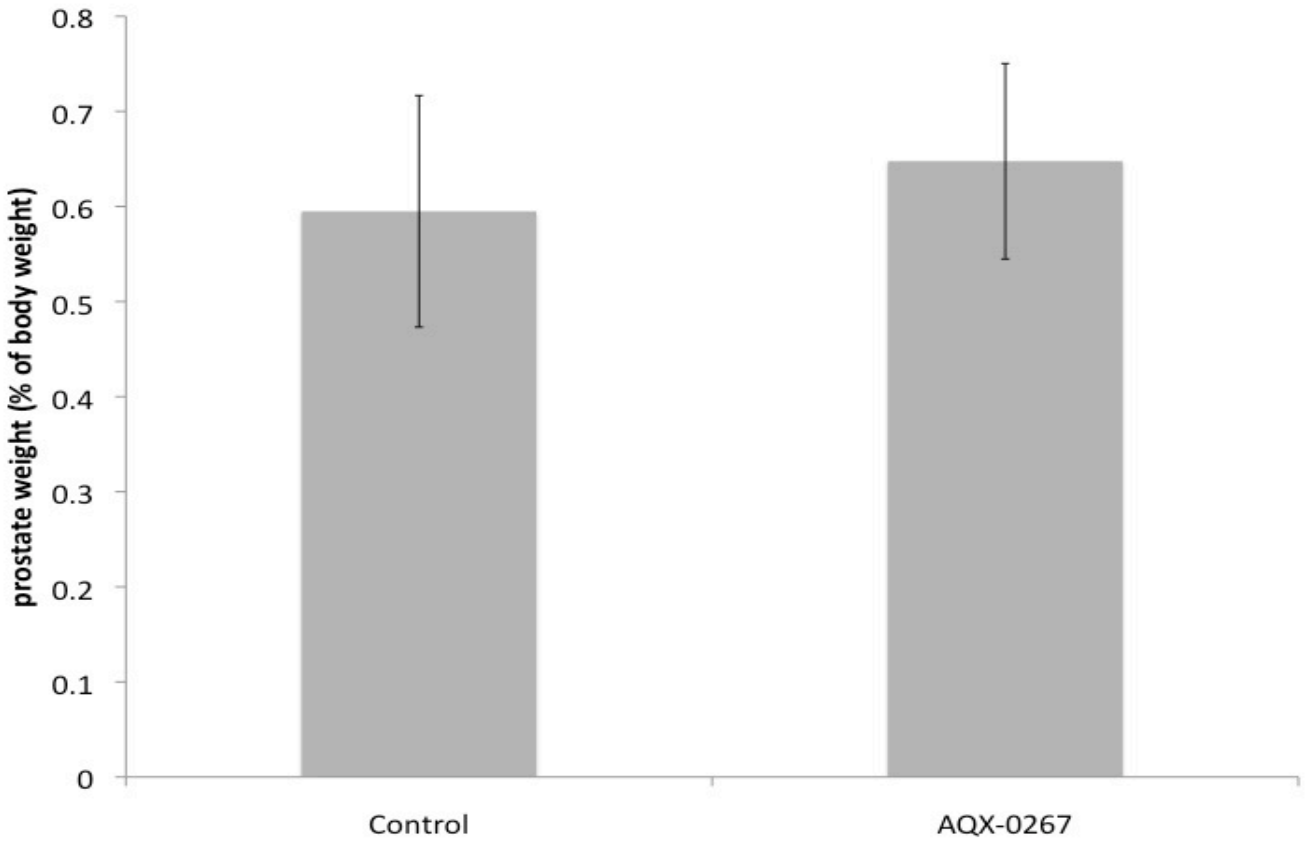
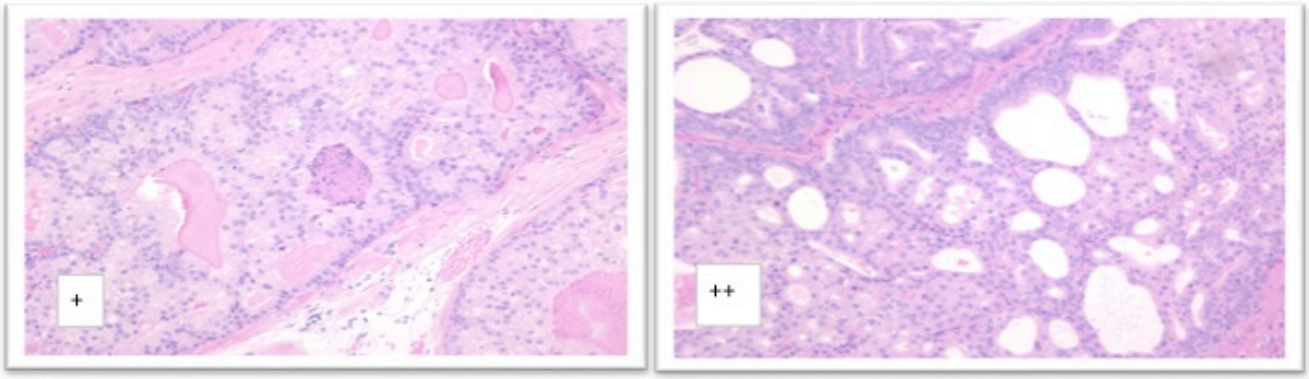


Figure 12. QLT0267 does not inhibit prostatic carcinogenesis in a prostate specific PTEN-knockout mouse model

(A) Prostate grade as determined from hematoxylin and eosin-stained slides and p-AKT location and intensity. No significant difference was seen between the treatment and control group. (B) Wet weights of QLT0267-treated, and PTE control prostate tissue after 28 days in ARR2PB-Cre+ve/PTEN fl/fl transgenic mice. Bars represent mean weights +/- SD. No difference in prostate weight was observed between QLT0267-treated and control mice (p=0.35).

A



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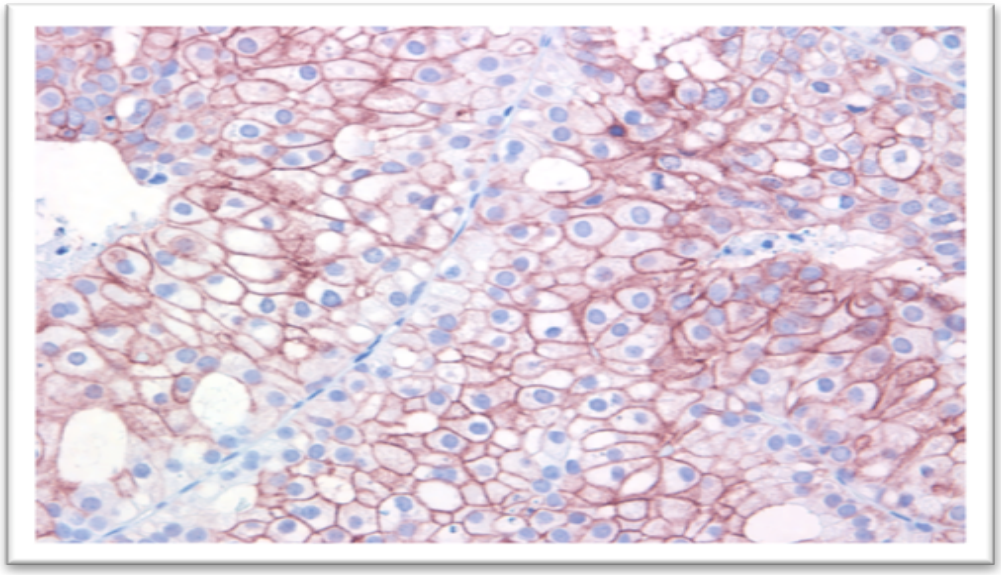


Figure 13. Representative histology

(A) Representative H&E stained slides showing mild (+) and moderate (++) hyperplasia/displasia. (B) Representative immunohistochemisty staining showing pAKT expression. Both the treatment and control groups had the same, moderate, intensity of membrane located p-AKT staining.

PI3K inhibitors are capable of synergy with inhibitors of the amino acid pathway

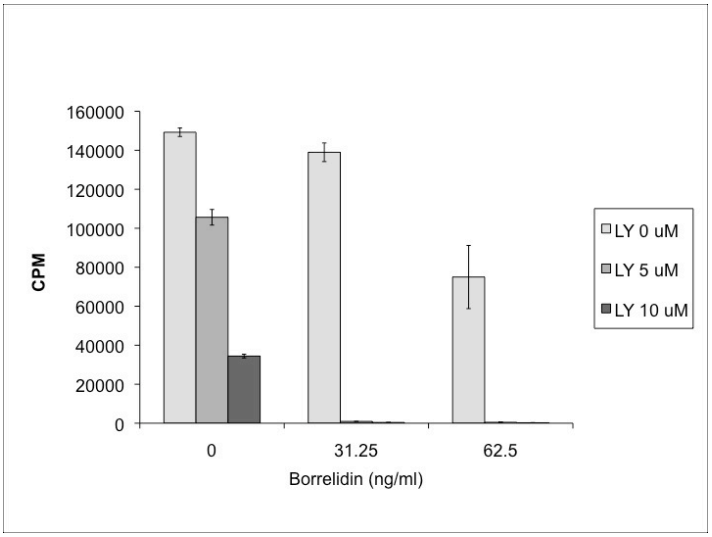
Given that inhibition of the PI3K pathway is an effective means of antagonizing cancer progression, I wanted to see if it was possible to enhance this effect. The PI3K has a well-established role in nutrient sensing. Consequently, it seemed plausible that inhibition of the amino acid deprivation pathway could enhance the therapeutic effects of PI3K pathway inhibition.

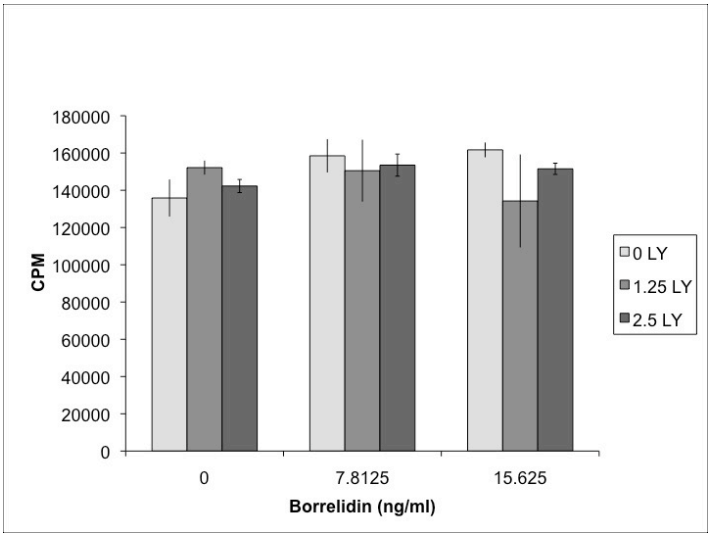
We wanted to see if amino acid deprivation pathway could have an effect on the apoptosis of LNCaP cells in combination with a PI3K pathway inhibitor. The effect of QLT0267, Borrelidin, and LY-294002 were determined using a tritiated thymidine proliferation assay on a dose curve of the drug in LNCaP cells. The cells were then incubated with a combination of either Borrelidin/LY-294002 or Borrelidin/QLT0267 to determine if the drugs had any synergistic effects on proliferation. The presence or absence of synergy was assessed by combination index (CI) analysis using the computer program CalcuSyn (Biosoft, Manchester, UK). CI values equal to one indicates additive effects, CI values less than one indicate synergism while CI values less than one indicate antagonism. 31.25 ng/ml of borrelidin alone resulted in a minimal 15% drop in proliferation while 62.5 ng/mL of Borrelidin resulted in a 60% drop in proliferation (Figure 14A). LY-294002 treatment alone resulted in a 30% (5 μM LY-294002), and 70%(10 μM) drop. However, the combination of LY-294002 and Borrelidin together resulted in a synergistic effect producing almost a 100% inhibition of proliferation in all cases (CI < 0.38) (Figure 14C). Lower concentrations of LY-294002 and borrelidin did not significantly inhibit proliferation, either singly or in combination (Figure 14A). This inhibition of proliferation is correlated with the increase of apoptosis as seen by a western blot for parp

cleavage (Figure 14B). Parp cleavage (indicating apoptosis), is much more apparent in the LY-294002/Borrelidin sample as opposed to either LY-294002 or Borrelidin alone.

QLT0267 in isolation showed a similar inhibition to LY-294002 producing a 30% (2.5 μM) and 60% (5 μM) decrease of proliferation (Figure 15A). However, in combination with Borrelidin, there was an additive effect as opposed to a synergistic one, with a 20-40% inhibition of proliferation (Figure 15C). The QLT0267/Borrelidin does not have this increase in apoptosis, consistent with the results from the proliferation assay (Figure 15B).

A





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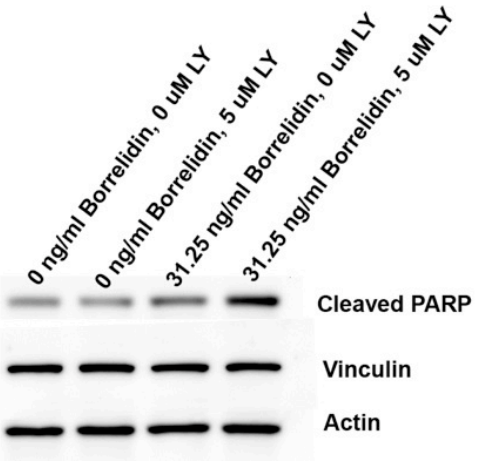


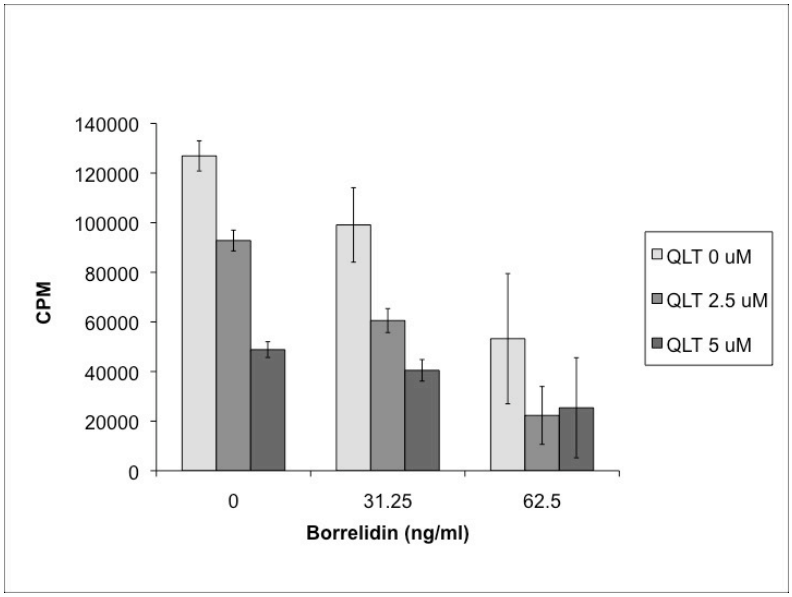
C

[LY] (μM)	[Borrelidin] (ng/ml)	CI	Significance
5	31.25	0.267	Strong Synergysm
5	62.5	0.326	Synergysm
10	31.25	0.308	Synergysm
10	62.5	0.380	Synergysm

Figure 13. The tRNA synthetase inhibitor borrelidin exhibits synergy with the PI3K pathway inhibitor LY-294002 to promote apoptosis of LNCaP cells

(A) The synergistic potential of borrelidin with LY-294002 was determined using tritiated thymidine incorporation in LNCaP cells. (B) Western blot showing parp cleavage as a marker of apoptosis. (C) Determination of synergy based on combination index (CI)

A



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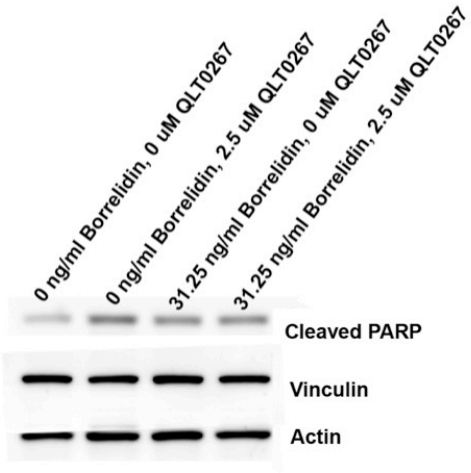

C

[QLT0267] (μM)	[Borrelidin] (ng/ml)	CI	Significance
2.5	31.25	1.152	Nearly additive
2.5	62.5	0.889	Moderate Synergysm
5	31.25	1.286	Moderate antagonism
5	62.5	1.280	Moderate antagonism

Figure 14. The tRNA synthetase inhibitor borrelidin exhibits additivity with the PI3K pathway inhibit QLT0267 to promote apoptosis of LNCaP cells

(A) The synergistic potential of borrelidin with QLT0267 was determined using tritiated thymidine incorporation in LNCaP cells. (B) Western blot showing parp cleavage as a marker of apoptosis. (C) Determination of synergy based on combination index (CI)

Discussion

There is a continual search for novel treatments with better efficacy and fewer side effects. Small molecule drugs are a particularly interesting form of treatment due to their low cost, ease of synthesis, and ease of administration. High throughput screens of natural and synthetic molecule libraries have made it possible to find agonists and inhibitors in a highly efficient manner.

A good target for molecular intervention of disease is the PI3K pathway. It is an integral survival pathway of the cell and changes in its signaling are implicated in many diseases. In fact, it may be a necessary step in some disorders such as cancer. These changes may be reversed using targeted therapies. Alternatively, it may be desired to downregulate normal signaling pathways such as those involve in the immune response. This is also achievable through PI3K pathway inhibitors. Finally it would be useful to find other pathways and drug targets that can act in synergy with PI3K pathway drugs.

The breadth and complexity of the PI3K pathway provides an opportunity for tissue specific therapies. Many components of the PI3K pathway have limited expression. For example SHIP, PI3Kδ, and PI3Kγ are all limited to the hematopoietic compartment. Tissue specificity can also be achieved using synergy between multiple drugs. In this case, only tissues that have the right activated pathways will be affected to a significant degree.

There are multiple advantages to having several approaches to targeting a single pathway such as PI3K. An array of drugs allows customization of treatment to specific situations, such as different diseases, tissues, or genetic backgrounds. Multiple drugs can be used in combination to provide synergistic results while limiting side effects. A wide choice of therapies allow for greater insurance against developing drug resistance in disease. This is especially important considering the genomic instability of tumours. This instability allows the cancer cells to rapidly

mutate, selecting for changes that may confer greater resistance to drugs.

Care must be taken as to what level of the signaling cascade we wish to target. Targeting upstream products have the advantage of having more widely spread effects but also increase the probability of off-target effects, either in the cell or even in other cell types. Downstream effectors should be more specific, but the potential for feedback loops and cross-talk between pathways may limit this specificity.

Further studies in the mechanics of PI3K pathway signaling will allow better targeting of new drugs in the PI3K pathway. It is important to know how normal signaling works so that we can determine which pathways get stimulated under which conditions. It also teaches us what goes wrong when disease disturbs PI3K pathway signaling. Despite the complexity of signal transduction, it is possible that there are a few significant points in the pathway where maximal effect can be had by minimal interference.

In this thesis, I have outlined some novel approaches to disease management. There is some basic elucidation of the mechanisms of PI3K pathway regulation in normal cell, two new small molecule drugs with proven effectiveness in diseases such as cancer and autoimmunity, and a description of a third drug that exhibits synergy with PI3K pathway inhibitors.

While a great amount of attention is focused on the aberrations of the PI3K pathway in disease, there is as yet much to be learnt from the PI3K pathway in normal cells. It should not be forgotten that there is much that is yet to be elucidated about the mechanisms by which PI3K affects its diverse downstream pathways. This is important for several reasons. A better understanding of PI3K pathway signaling can help identify novel targets for new drugs or markers for diagnosis. Also, it helps predict the outcomes of inhibiting different targets in the cascade. This is critical for determining side effects, especially for such a conserved cellular pathway as PI3K. Thus, these mechanistic studies are a critical part of PI3K-related therapies.

One important normal function of PI3K signaling is insulin stimulated glucose uptake.

Insulin receptor binding activates PI3K and causes glucose to be taken up from the blood stream. My first study was to determine if PTEN status had a significant effect on this process (Wong, Kim et al. 2007).

As systemic PTEN knockout is an embryonic lethal mutation, haploinsufficient mice were used. The role of AKT/PKB in insulin signaling was interrogated by analyzing phosphorylation levels of AKT/PKB and its downstream effector GSK3ß during an insulin challenge. Haploinsufficient mice exhibited more PKB S473 and GSK3ß phosphorylation as compared to the wild type. Not only were phosphorylation levels higher at a certain time point, but it was also sustained for much longer. I contributed in the western immunoblot analysis seen in figure 5 in the paper.

This fits in well with the data found by others in the group. Haploinsufficient mice have a lower blood glucose level during both ad libitum feeding and during fasting (Wong, Kim et al. 2007). They also exhibit greater insulin sensitivity as compared to wild type mice. In an insulin challenge, an i.p. injection of insulin depressed the blood glucose level for up to 120 minutes in the haploinsufficient mice while the wild type mice were able to recover back to fasting levels within that time (Wong, Kim et al. 2007). A glucose tolerance test mirrored these results. Haploinsufficient mice blood glucose levels were able to return to fasting levels in half the time of wild type mice. In order to determine if this difference was due to increased insulin production or to greater insulin sensitivity, the plasma insulin levels were measured. The haploinsufficient mice had a lower insulin level as compared to the wild type mice. A glucose challenge induced the same change in insulin level, suggesting that there is no difference in insulin production between the two groups. There was also no difference in beta cell mass, islet morphology or islet function as determined from cultured islets. This implies that the difference seen between the haploinsufficient and wild type mice are due to differences in insulin sensitivity as opposed to differences in insulin levels or production.

Glucose uptake rates were then studied using skeletal muscle. Real-type glucose uptake was monitored in mice using a glucose uptake marker (^{18}FDG) with PET. Glucose uptake rates were higher in the haploinsufficient mice in all time points. Western blot analysis showed that haploinsufficient mice had less PTEN protein as expected. To determine the mechanism, primary myocytes were cultured from hind limb muscles of haploinsufficient and wild type mice). Haploinsufficient mice exhibited a higher 2-deoxy[^{3}H]glucose uptake as compared to the wild type mice both in the presence and absence of insulin. This is consistent with a constitutively activate PI3K/AKT pathway

Western blot analysis was used to determine if this difference in insulin sensitivity was a result of differential glucose transporter proteins. PI3K/PIP3 controls the translocation of GLUT4 from intracellular vesicles to the cell surface, possibly in both an AKT dependant and independent manner while GLUT1 is not PI3K regulated. As expected, the haploinsufficient mice had increased GLUT4 expression on the cell surface while there was no difference in GLUT1 levels between the two groups.

This study is significant in many respects. First of all, insulin insensitivity is a hallmark of type 2 diabetes. Since loss of PTEN improves insulin sensitivity, this reveals a potential strategy for combating this disease. It may be that diabetes is another disease that can be treated with a small molecule drug, either through tissue-specific PTEN inhibition or PI3K activation. Secondly, this is a sober reminder of the potential for side effects when inhibiting a major cellular pathway. Most of the small molecule drugs discussed in this thesis are PI3K pathway inhibitors. If they are used *in vivo* and affect insulin-sensitive cells, they are likely to cause insulin insensitivity in the patient. While this may be a minor symptom as compared to high-grade cancer, it could be a significant factor in a milder or more chronic disease. Long-term disruption of insulin signaling as seen in diabetes has wide ranging effects from blindness to loss of limbs. Obviously this is an important consideration to keep in mind and test for when

considering new drugs for clinical use.

Thirdly, an understanding the glucose regulation may be an important step in combating cancer. Cancer cells preferentially use glucose as an energy source and thus maintain a high level of glycolysis. The insulin-like growth factor system is composed of three ligands (Insulin, IGF-1 and IGF-2), two receptors (insulin receptor, IGF-1R and IGF-IIR) and a variety of binding proteins (IGFBP 1-6). Most of the effects of IGFs on growth and differentiation are through the IGF1 receptor that is highly similar to the insulin receptor. Both receptors exist as preformed dimers. Ligand binding leads to autophosphorylation and induction of tyrosine kinase activity. This causes recruitment and phosphorylation of SH2 domain-containing docking proteins, stimulating downstream pathways such as PI3K and MAPK(Werner and Le Roith 2000). In addition to their roles in normal cellular regulation, IGFs are important in the progression of a variety of cancers. Primary tumors and cell lines from a variety of cancers overexpress IGF-1 and IGF-2, and IGF-1R which act in an autocrine manner (Werner and LeRoith 1996). This leads to upregualtion of the PI3K pathway and associated effects such as antiapoptotic activity, drug resistance, metastasis and increased proliferation. IGF-1R appears to be necessary for some types of tumorigenic progression. Abrogation of IGF-1R results in cells that are more resistant to transformation by oncogenes (Morrione, DeAngelis et al. 1995). These features make the IGF system a promising one for further study. New drugs are being made to target members of the pathway. The IGF pathway may also be a good source of cancer molecular markers; IGF-1 levels may be a predictor of cancer risk (Chan, Stampfer et al. 1998). The investigation of the role PTEN has on insulin signaling casts further light on related IGF signaling. Further elucidation of these pathways could lead to the development of a more thorough understanding of the disease and better therapies.

Once I had established the feasibility of manipulating the PI3K pathway in normal cells, I wanted to see if this could be an effective method of treatment. As a disease model, I picked

idiopathic thrombocytopenic purpura. This is characterized by the destruction of platelets by the body's own immune system. Since an established treatment for ITP may act through SHIP, I hypothesized that SHIP activation (using the SHIP activator AQX-MN100) would also be therapeutic.

Unfortunately, AQX-MN100 was not able to protect or recover platelet numbers in a murine model of classic ITP. This is despite the fact that SHIP is activated by FcγRIIB, a necessary mediator of IVIg-induced platelet recovery in the same murine ITP model.

Crow *et al* found that SHIP1 (along with SHP-1, and BTK) was not necessary for this FcγRIIB-mediated IVIg treatment (Crow, Song et al. 2003). This is notable since the known FcγRIIB signaling pathways (based on B-cells), are through these signaling mediators. Nevertheless, SHIP activation remained a good potential IVIg mimetic. It may be that the FcγRIIB signaling pathway in B cells does not hold for other cell types. Pathway redundancies may also mean that SHIP is not necessary, but may still be sufficient. In addition, there is evidence that SHIP may inhibit monocyte function independently from FcγRIIB (Cox, Dale et al. 2001). Thus, it was hoped that SHIP activation would be sufficient to mimic IVIg results. Apparently this is not so. It may be that the FcγRIIB-independent effects of IVIg such as competitive binding, small immune complexes, or IVIg-primed dendritic cells (Crow and Lazarus 2008) are more important to ITP protection than was previously thought. This is supported by data that shows that macrophages and neutrophils may not be the primary target of IVIg effects (Siragam, Crow et al. 2006). Though the FcγRIIB receptors on these cells are necessary for IVIg to have effect, activation of these pathways may not be sufficient in the absence of these other signals such as dendritic cell mediated anergy.

Even if SHIP activation was sufficient to prevent phagocytosis of the platelets, it might not prevent platelet binding. Although the activation of SHIP inhibits the late stages of phagocytosis, this is not useful as a therapy as the platelets are still unavailable for their normal

role in clotting.

In spite of its inability to rescue mice in the classic ITP model, the ability of AQX-MN100 to prevent macrophage activation allowed it to be a very effective agent in the prevention of LPS-mediated ITP. This is significant since infections render ITP more resistant to ITP treatment. Also, a majority of ITP are acute infection-related cases as opposed to chronic. As such, AQX-MN100 treatment may have the potential to replace IVIg in these cases. This would bring many advantages in regards to cost, safety, and convenience. If it is not suitable as a complete replacement, it may be used in conjunction with IVIg, increasing effectiveness and lowering the required dosage of IVIg.

Since the inactivation of the PI3K pathway in a normal cell was not sufficient for treatment, we next investigated inactivation of a constitutively activated PI3K pathway. The hematopoietic cancers multiple myeloma and mantle cell lymphoma provided an excellent model for this. The pelerol derivatives AQX-016A and AQX-MN100 are highly effective inhibitor of the PI3K pathway as shown by its ability to inhibit proliferation and induce apoptosis in multiple myeloma and mantle cell lymphoma cell lines. It is also specific to SHIP containing cells, thus limiting its side effects to the hematopoietic compartment. As such it has much promise as a novel anti-cancer drug.

After this success, I investigated the use of a PI3K pathway inhibitor in another cancer model, prostate cancer. Due to the importance of ILK activation in prostate cancer development, I predicted that the ILK inhibitor QLT0267 would be able to delay prostate cancer progression in a mouse model. However, QLT0267 failed to inhibit prostate cancer progression in an ARR_2-PB-Cre^{+ve}/PTEN *fl/f* transgenic mouse model. This drug has previously been shown to be effective in both in-vitro and xenograft prostate cancer models. However, our study is the first to attempt to stop tumor development in a whole organ using this drug. This model presents a novel problem of tumor vascularization and drug delivery. In the *in vitro* models, the tumor cells

are readily exposed to a high concentration of the drug and thus one would expect a high level of uptake and efficacy. Even the *in vivo* models such as the glioblastoma, anaplastic thyroid cancer, and pancreatic cancer (Yau, Wheeler et al. 2005; Younes, Kim et al. 2005; Younes, Yigitbasi et al. 2007; Edwards, Woo et al. 2008) were only done as a xenograft. While this is a better clinical model, it is still an unnatural simulation of how a real cancer progresses. Instead of a solid tumor, the xenograft is a loose ball of cells. Therefore, once in place it is bathed in the bloodstream, exposing most of the cells to any drug. In contrast, our prostate cancer model occurs in the mouse's own organ as opposed to an ectopic tumor. This is a much better model of how prostate cancer develops naturally in humans. Consistent with the human model, delivery of drug to the developing tumor is limited by the vascularization of the prostate. The murine prostate is a poorly vascularized organ. It is likely that the lack of inhibition in this case is due to the inability of the drug to reach the prostate tissue. Our future experiments involve an orthotopic administration directly into the prostate gland, ensuring exposure of the tissue to the drug. If this is functional, then further optimization will need to be done to see if similar results can be achieved orally. There are still unanswered questions as to the pharmacodynamics, tissue bioavailability, and drug biodistribution of QLT0267 in a whole organ model. Inhibition of prostate cancer development in a whole organ model has been achieved using diet modulation, giving credence to the possibility that drugs may also work (Berquin, Min et al. 2007).

The theory of oncogene addiction provides an alternate explanation for these results. This states that oncogenic cells are more dependant on deregulated pathways than normal cells. Inhibition of these pathways that cause cell cycle arrest and apoptosis in cancer cells, will not affect normal cells since the normal cells can compensate using alternative pathways. This occurs with ILK where QLT0267 causes inhibition of AKT phosphorylation, apoptosis, and decreased mTOR expression in human breast cancer lines. However, administration of the ILK inhibitor in normal human breast epithelial cells did not affect AKT phosphorylation or

apoptosis (Troussard, McDonald et al. 2006). It is possible that in our mouse prostate cancer models, the epithelial cells were not yet addicted to ILK expression. It is therefore understandable why ILK inhibition may not have been able to delay prostatic carcinogenesis in this experiment. Importantly, it must be noted that this was a single experiment. Without further study, one cannot draw any reliable conclusions about the feasibility and efficacy of ILK inhibition in prostate cancer.

The use of compounds with synergistic effects is an important tool in the fight against diseases. In the final part of my thesis, I investigated the ability of PI3K pathway inhibitors to synergize with an activator of the amino acid deprivation pathway. Here, we showed that a PI3K pathway inhibitor LY-294002 has synergistic effects in combination with an amino acid deprivation pathway activator (borrelidin) for inhibiting proliferation and inducing apoptosis is a cancer cell line (LNCaP). Another PI3K pathway inhibitor (QLT0267) produced additive effects instead. This highlights the need for several different inhibitors of the same pathway. Even though LY-294002 and QLT0267 inhibit the same PI3K pathway, they had different interactions with Borrelidin. It is not clear why this occurs. It might be that the different targets of the drugs (PI3K and ILK respectively) have different interactions with the amino acid deprivation pathway targeted by borrelidin. LY-294002 acts further upstream by inhibiting the production of the potent second messenger PIP3 while QLT0267 inhibits ILK and its downstream target AKT. This implies that the synergistic effect of LY-294002 is due to its inhibition of an AKT independent pathway. It appears that inhibition of ILK alone produces a lesser, but still additive effect. The difference in synergistic potential may also be a result of the difference in off-target effects between the two PI3K inhibitors. It is likely that the subtleties of PI3K pathway signaling require the use of several different compounds, either singly or in combination, to achieve the desired result.

Even though QLT0267 did not have a synergistic effect with borrelidin, this may still be

a clinically important effect. This ability to increase effect without increasing individual drug doses fuels the search for drug combinations with synergistic effects. As long as the *in vivo* side effects of the two drugs are sufficiently mild, an additive effect may be sufficient to increase efficacy while limiting dose-dependant side effects.

Targeting a pathway such as PI3K can have variable results. In this thesis, modulation of the PI3K pathway proved effective in many instances. PTEN haploinsufficient mice exhibited changed glucose tolerance. SHIP activation caused apoptosis in multiple myeloma and mantle cell lymphoma cell lines and prevented platelet destruction in an infection-mediated activation model. There were also instances of synergy between PI3K pathway inhibitors and an activator of the amino acid deprivation pathway in a prostate cancer cell line.

Equally important were the instances when PI3K pathway modulation failed to achieve the desired results. This highlights the complexity of developing clinical therapies. While many treatments are effective in an *in vitro* model, there are many added factors to consider when dealing with animal models. This is well illustrated by the ILK inhibition experiment. QLT0267 was very effective in a wide range of *in vitro* and xenograft prostate cancer models, but failed to inhibit prostate cancer progression in the *in vivo* model. It has yet to be determined whether this is due to drug metabolism, poor drug targeting, aggressiveness of the murine model, other unconsidered factors, or (as is likely), a combination of complications. The failure of PI3K pathway inactivation in the treatment of chronic idiopathic thrombocytopenic purpura highlights another problem with therapies in an *in vivo* model. It is likely that an insufficient understanding of the mechanics of the disease was the major factor in the lack of therapeutic success. I hypothesized that since FcγRIIB-mediated SHIP activation in macrophages and neutrophils were necessary for IVIg effects, SHIP activation would be a sufficient mimetic. However, autoimmunity is a complex phenomenon involving multiple cell types. An attempt to mimic an empirically derived therapy such as IVIg without a full understanding of the disease or the

mechanisms of the therapy is risky. This is not to say that it is without value as it resulted in a deeper understanding of the mechanisms of how IVIg mediates its effects on ITP. Since AQX-MN100 activates SHIP in both macrophages and neutrophils *in vivo* (as shown by TNFα and myeloperoxidase activity), this strongly suggests that FcγRIIB-mediated SHIP activation is not the sole effector of IVIg's curative effects. It seems likely that other pathways (or even cell types) are involved in mediating IVIg's effects on ITP.

It is factors like these that one must keep in mind when considering therapeutic targets such as PI3K and emphasizes the need for further study in the area. A wider range of therapies that are effective *in vitro* increases the possibility of finding a variety that are also effective *in vivo*, and ultimately in a clinical setting. This is necessary since multiple drugs that target different parts of the pathway provides the potential for synergistic effects, increasing effectiveness and specificity as compared to a single agent. Ultimately, this thesis provides a justification of the PI3K pathway as a therapeutic target in a variety of diseases, as well as some of the potential obstacles of applying the research to clinical contexts. Most of all, it emphasizes how much there is left to discover about this central cellular pathway.

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Appendix 1: Histology and Immunochemistry of H&E Stained Slides.

PTEN KN			p-AKT		
Mouse ID		Hyperplasia/Dysplasia	Location/prostate	Location/Cellular	Intensity
21	1C	Éä	Anterior- Ventro lateral	Membrane	Moderate
21	2C	ÉÉää	Anterior- Ventro lateral	Membrane	Moderate
22	1C	É	Anterior- Ventro lateral	Membrane	Moderate
22	2C	É	Anterior- Ventro lateral	Membrane	Moderate
22	3C	ÉÉ	Anterior- Ventro lateral	Membrane	Moderate
22	4C	ÉÉ	Anterior- Ventro lateral	Membrane	Moderate
23	1C	ÉÉ	Anterior- Ventro lateral	Membrane	Moderate
25	1C	ÉÉ	Anterior- Ventro lateral	Membrane	Moderate
25	2C	ÉÉ	Anterior- Ventro lateral	Membrane	Moderate
26	1C	É	Anterior- Ventro lateral	Membrane	Moderate
21	1T	ÉÉ	Anterior- Ventro lateral	Membrane	Moderate
22	1T	É	Anterior- Ventro lateral	Membrane	Moderate
22	2T	É	Anterior- Ventro lateral	Membrane	Moderate
22	3T	ÉÉ	Anterior- Ventro lateral	Membrane	Moderate
22	4T	É	Anterior- Ventro lateral	Membrane	Moderate
22	5T	ÉÉ	Anterior- Ventro lateral	Membrane	Moderate
25	1T	ÉÉ	Anterior- Ventro lateral	Membrane	Moderate
25	2T	ÉÉ	Anterior- Ventro lateral	Membrane	Moderate
25	3T	É	Anterior- Ventro lateral	Membrane	Moderate
26	1T	É	Anterior- Ventro lateral	Membrane	Moderate

* Mild to Moderate Luminal epithelial cell Hyperplasia/Mild to moderate dysplasia with rare patches of **necrotic** cells

** Moderate Luminal epithelial cell Hyperplasia/Moderate Dysplasia and cribriform growth pattern

Appendix 2: Animal Care Certificates.

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-0434

Investigator or Course Director: Shoukat Dedhar

Department: Biochemistry & Molecular Biology

Animals:

Mice C57BL/6, CD1 500

Start Date: **September 1, 2006** **Approval Date:** **April 14, 2009**

Funding Sources:

Funding Agency: National Cancer Institute of Canada
Funding Title: Role of Integrin-Linked Kinase in Prostate Cancer Progression

Funding Agency: National Cancer Institute of Canada
Funding Title: Cell-extracellular matrix interactions in differentiation and oncognenesis

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Regulation of E-cadherin expression and wnt signalling by integrin-linked kinase

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-0344

Investigator or Course Director: Alice L.F. Mui

Department: Surgery

Animals:

Mice CD1 105
Mice Balb/C, C57BL/6 210

Start Date: **July 1, 2006** **Approval Date:** **August 27, 2009**

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: SHIP agonists for the treatment of inflammatory disease and hemopoietic malignancies

Funding Agency: Aquinox Pharmaceuticals Inc.
Funding Title: Synthesis of SHIP activating analogues of Pelorol

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: IL - 10 signal transduction in macrophages

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Negative regulators of immune cell function

Unfunded title: N/A