Pharmacological Targeting of the Mitochondrial Phosphatase PTPMT1

by

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Department of Biochemistry
Duke University

Date: May 1st 2009

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Dr. John D. York

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in the Graduate School of Duke University

2009
ABSTRACT

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Abstract

The dual specificity protein tyrosine phosphatases comprise the largest and most diverse group of protein tyrosine phosphatases and play integral roles in the regulation of cell signaling events. The dual specificity protein tyrosine phosphatases impact multiple cellular processes including mitogenesis, differentiation, adhesion, migration, insulin secretion and programmed cell death. Thus, the dysregulation of these enzymes has been implicated in a myriad of human disease states. While the large volume of genetic data that has become available following genome sequencing efforts over the last decade has led to the rapid identification of many new dual specificity protein tyrosine phosphatases, the elucidation of the cellular function and substrates of these enzymes has been much slower. Hence, there is a need for new tools to study the dual specificity protein tyrosine phosphatases and the identification of inhibitors of these enzymes is regarded as an attractive prospect, potentially affording not only new means of studying these enzymes, but also possible therapeutics for the treatment of diseases caused by their dysregulation. However, the identification of potent, selective inhibitors of the dual specificity protein tyrosine phosphatases has proven somewhat difficult.
PTPMT1, Protein Tyrosine Phosphatase Localized to the Mitochondrion 1 is a recently discovered, mitochondrion-localized, dual specificity phosphatase which has been implicated in the regulation of insulin secretion. However, the details of the mechanism by which PTPMT1 impacts insulin secretion, as well as its substrate in the pancreatic β-cell, have yet to be uncovered. Thus, the identification of a potent, selective inhibitor of the enzyme would aid in further study of PTPMT1. This work describes the identification of such an inhibitor of PTPMT1 following an *in vitro* screen of small molecule, chemical compounds using an artificial substrate. Following the screen, the lead compound emerged as a potent and potentially selective inhibitor of PTPMT1 both *in vitro* and in cells. Studies using this compound have shown that the compound induces increased secretion of insulin in a dose-dependent manner and thus support the notion that PTPMT1 may serve as a potential target for the treatment of Type II diabetes.
Dedication

To my parents,

for giving me the courage to pursue my dreams.
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1 Introduction

1.1 The biological significance of protein phosphorylation

Over half a century ago, scientists first began to speculate that reversible protein phosphorylation might be of some physiological importance, but they were at a loss as to what the significance might be. The study of reversible protein phosphorylation intensified in the early 1950’s as evidence began to emerge for the rapid turnover of phosphate groups in both tumors and normal tissue and for the chemical association of phosphate with cellular proteins (Davidson, Frazer et al. 1951; Johnson and Albert 1953). It was soon demonstrated that this binding of phosphate to protein could be attributed to the covalent modification of specific amino acid residues on the protein (Kennedy and Smith 1954), and that the phosphorylation of these residues occurred in intact protein and utilized ATP (Burnett and Kennedy 1954).

In retrospect, clues to the significance of protein phosphorylation could have been drawn from the extensive studies of the enzyme glycogen phosphorylase which were occurring at that time. In the 1940s, Corgi and Green had demonstrated that glycogen phosphorylase existed in two forms, phosphorylase a and phosphorylase b, which possessed different activities and could be interconverted by an enzyme in the cell which added a prosthetic group to one of the forms (Cori and Green 1943; Green and Cori 1943). Over ten years later, at about the same time that the rapid and
reversible phosphorylation of cellular proteins was being discovered, the true nature of the prosthetic group which converted phosphorylase b to phosphorylase a would finally become clear. Studies revealed that this conversion required ATP (Fischer and Krebs 1955) and resulted in the binding of phosphate to phosphorylase b (Krebs and Fischer 1956), while the inactivation of phosphorylase a and its conversion to phosphorylase b was associated with the release of inorganic phosphate (Sutherland and Wosilait 1955). The reversible phosphorylation of glycogen phosphorylase, which resulted in interconversion between the two forms and regulated activity, was later shown to occur specifically on a serine residue of the protein (Fischer, Graves et al. 1959). In spite of this specific example of reversible phosphorylation of a particular protein as a means of controlling enzymatic activity, the true significance and broad applicability of reversible protein phosphorylation as a means of regulation would not be recognized for more than a decade (Cohen 2002).

Today, the reversible phosphorylation of proteins, which is thought to occur in all organisms (Moorhead, De Wever et al. 2009), is widely recognized as one of the most common and perhaps the most important means of regulating cell signaling events. The addition or removal of a phosphate moiety on a particular protein residue does not simply change the amino acid structure, but often impacts both local and wider protein conformation, which may in turn influence the catalytic capacity of the protein, its
cellular localization, or its ability to interact with other molecules in the cell. In humans, a significant proportion of cellular proteins appear to be phosphorylated in a regulated manner. It has been estimated that about 50% of all phosphoproteins are modified on more than one site, and greater than 75% of these multi-site phosphorylated proteins exhibit differential regulation of the individual phospho-sites (Olsen, Blagoev et al. 2006). There is further evidence that phosphorylation of one site on a protein may impact the ability of additional sites on the protein to be phosphorylated or even undergo other post-translational modifications such as acetylation or methylation, which may in turn further influence protein function (Hunter 2007). Thus, it is not surprising that the reversible phosphorylation of proteins, often referred to as a molecular switch, has been shown to greatly influence a vast array of reactions and signals in the cell.
1.2 Protein tyrosine phosphatases and the dual specificity PTPs

The so-called molecular switch of reversible protein phosphorylation would remain static without the opposing actions of two very important groups of enzymes, protein kinases and protein phosphatases. The former is responsible for the addition of phosphate moieties, while the latter is responsible for their removal. Thus, for any particular phosphorylation–regulated reaction in the cell, it is the concerted action of both the involved kinases and phosphatases that ensures proper regulation. While the dysregulation of protein kinases has long been recognized as a causative factor in disease, and thus protein kinases have often been targeted for therapeutic intervention (Pytel, Sliwinski et al. 2009), over the last decade, protein phosphatases have risen to prominence (Stephens, Han et al. 2005; Pulido and Hooft van Huijsduijnen 2008). With the vast volume of genetic information that has become available as the result of genome sequencing efforts, the rate of discovery of new phosphatases has grown rapidly (Alonso, Sasin et al. 2004). Today protein phosphatases have been identified in each of the three domains of life: archaea, bacteria and eukaryotes (Kennelly 2003), and in almost every cellular organelle.

In human cells, most protein phosphorylation occurs on serine, threonine and tyrosine residues, and it has recently been estimated that this accounts for about 86%, 12% and 2% of protein phosphorylation events, respectively (Olsen, Blagoev et al. 2006).
The protein phosphatases responsible for the dephosphorylation of these residues can be divided into four groups that appear to have evolved independently (Moorhead, De Wever et al. 2009). These four protein phosphatase families consist of the phosphoprotein phosphatases, which are defined by a catalytic domain containing the amino acid sequences GDXHG, GDXVDRG and GNHE; the metallo-dependent protein phosphatases, which require two metal ions in their active site for catalysis; the aspartate-based phosphatases, which are defined by an aspartate-containing catalytic motif (DXDXT/V) and the protein tyrosine phosphatases (PTPs), which are defined by a cysteine- and arginine-containing catalytic motif, CX_{5}R (Moorhead, De Wever et al. 2009). Figure 1. Twenty five years ago, when the first protein tyrosine phosphatases were discovered (Brautigan, Bornstein et al. 1981; Foulkes, Howard et al. 1981), selective dephosphorylation of phosphotyrosine residues was considered novel. Today, 107 PTPs have been identified within the human genome, and PTPs represent the largest group of human protein phosphatases (Alonso, Sasin et al. 2004).

While the many members of the protein tyrosine phosphatase family have little overall amino acid sequence homology, they generally share the CX_{5}R catalytic motif (Guan and Dixon 1990) as well as some similar structural features in the catalytic domain. These features include a phosphate-binding loop in which the residues of the catalytic motif are flanked by a β-sheet and alpha helix (Wang, Sun et al. 2003). Within
the catalytic sequence, the cysteine, generally has a low pKₐ (Zhang and Dixon 1993), and serves as the phosphorus-attacking nucleophile to form a phospho-cysteinyl intermediate during catalysis, while the arginine forms two hydrogen bonds with the phosphoryl group of the substrate and helps to stabilize the transition state. The cysteine residue is absolutely essential for catalysis, and abrogation of protein tyrosine phosphatase activity can be accomplished by its removal/substitution (Streuli, Krueger et al. 1989; Guan and Dixon 1990).

Although protein tyrosine phosphatases share a similar catalytic mechanism and sequence and were originally defined by their ability to catalyze the dephosphorylation of phosphotyrosine residues (Brautigan, Bornstein et al. 1981; Foulkes, Howard et al. 1981), within ten years of their discovery it was revealed that not all members of the PTP family preferentially target phosphorylated tyrosine (Guan, Broyles et al. 1991; Charles, Abler et al. 1992; Keyse and Emslie 1992). Furthermore, while most protein tyrosine phosphatases catalyze the dephosphorylation of proteins, about 20% of PTPs catalyze the dephosphorylation of other molecules, including carbohydrates, mRNA and phosphoinositides (Moorhead, De Wever et al. 2009). Thus, the protein tyrosine phosphatases can be divided into four families based on shared amino acid sequence homology within the catalytic domain, and further divided into subfamilies based on domain structure and substrate specificity. Figure 1 The four protein tyrosine
phosphatase families are defined as the Class I, Class II and Class III cysteine-based PTPs and the Class IV aspartate-based PTPs (Alonso, Sasin et al. 2004), with Class I cysteine-based PTPs consisting of the classical PTPs and VH1-like dual specificity PTPs, Class II represented solely by the low molecular weight PTP (LMPTP), Class III containing the CDC25 PTPs and the final family, the aspartate-based PTPs, consisting of the EyA (eyes absent) PTPs (Alonso, Sasin et al. 2004). Figure 1.

Of the four PTP families, the Class I cysteine-based PTPs contain 99 of the 107 known protein tyrosine phosphatases. Class I is further divided into 38 classical PTPs and 61 dual specificity or VH1-like PTPs. Figure 1. The classical PTPs are subdivided into transmembrane classical PTPs and non-receptor PTPs, while the dual specificity or VH1-like PTPs are subdivided into MAP kinase phosphatases, atypical dual specificity phosphatases, slingshot phosphatases, PRLs (phosphatases of regenerating liver), CDC14 phosphatases (cell division cycle phosphatases 14), PTENs (phosphatase and tensin homologues deleted on chromosome 10) and myotubularins (Alonso, Sasin et al. 2004). Figure 1. While the classical PTPs preferentially dephosphorylate phosphotyrosine residues, the dual specificity PTPs were so called because of their ability to dephosphorylate phosphoserine and phosphothreonine residues in addition to phosphotyrosine.
The catalytic mechanism of the dual specificity PTPs is akin to that described for the entire PTP family where the catalysis of substrate dephosphorylation is mediated by the cysteine of the phosphate-binding loop of the enzyme. The cysteine is absolutely essential for catalysis and acts as a nucleophile in the dephosphorylation reaction, forming a thiol-phosphate intermediate with the phosphate group of the substrate (Zhou, Denu et al. 1994). The thiol-phosphate intermediate is stabilized by hydrogen bonding with the arginine residue of the catalytic phosphate-binding loop (Zhang, Wang et al. 1994). An acidic residue, which is conserved in the loop adjacent to the catalytic phosphate-binding loop among the dual specificity PTPs, the so-called WPD loop, functions as a general acid and protonates the leaving group of the serine-, threonine- or tyrosine-phosphorylated substrate (Denu, Zhou et al. 1995). In many dual specificity PTPs, this WPD loop undergoes a significant positional change upon substrate binding and closes over the active site like a flap (Wang, Sun et al. 2003). In the final step of the catalytic mechanism, the serine residue of the catalytic phosphate-binding loop facilitates hydrolysis of the phosphocysteine intermediate (Denu and Dixon 1995).

Today, it is clear that the substrate specificity of the dual specificity PTPs goes far beyond tyrosine, serine and threonine residues, and selected dual specificity phosphatases have been shown to utilize phosphoinositides and mRNA as substrates (Takagi, Taylor et al. 1998; Maehama, Taylor et al. 2001). The variation in substrate
selectivity of the dual specificity PTPs compared to the classical PTPs has been partially attributed to the difference in shape and depth of the active site. The active site of the dual specificity phosphatases is shallower than that of the classical PTPs by about 3 Å (Yuvaniyama, Denu et al. 1996), while in those dual specificity PTPs such as the PTENs which utilize phosphoinoside as substrates, the active site is both wider and shallower than the classical PTPs (Lee, Yang et al. 1999). Interestingly, a small subset of dual specificity phosphatases appear to be catalytically inactive (Alonso, Sasin et al. 2004), but have been postulated to act as regulators of other subfamily members.

Figure 1: The protein phosphatases
1.3 The biological significance of the dual specificity PTPs

The dual specificity PTPs have a myriad of biological substrates and cellular roles which have recently been reviewed extensively (Pulido and Hooft van Huijsduijnen 2008; Patterson, Brummer et al. 2009), thus only a brief overview of the function of the various dual specificity PTPs and their involvement in disease will be considered here. The dual specificity PTPs, through their regulation of cellular signaling events, have been implicated in diverse cellular processes ranging from mitogenesis to cell differentiation, cell adhesion and migration, and programmed cell death. Overexpression, deletion and/or mutation of various dual specificity PTPs have all been associated with various disease states including cancer, Type II diabetes, immune disease, and fertility disorders (Pulido and Hooft van Huijsduijnen 2008).

1.3.1 The MAPK phosphatases

One of the most extensively studied subfamilies of the dual specificity PTPs is the MAPK phosphatase subfamily (the MKPs). There are 11 classical MAPK phosphatases encoded in the human genome: DUSP1/MKP-1, DUSP2, DUSP4/MKP-2, DUSP5, DUSP6/MKP-3, DUSP7/MKP-X, DUSP8, DUSP9/MKP-4, DUSP10/MKP-5, DUSP16/MKP-7 and MK-STYX. The MKPs are involved in the regulation of the activity of the MAP kinases, ERK, JNK and p38 MAP kinase. The selectivity of the different MKPs for their substrate MAPKs is mediated by a three-part, modular docking surface, referred to as a kinase interaction motif, in the N-terminus of the enzyme, with charged residues within
the docking surface contributing to selectivity (Tanoue, Yamamoto et al. 2002). These dual specificity phosphatases bind their cognate substrate MAPKs and are activated by interaction of specific residues in their kinase interaction motif with the C-terminal domains of their substrate MAPK (Camps, Nichols et al. 1998; Nichols, Camps et al. 2000). The activated MKPs then selectively dephosphorylate phosphotyrosine and phosphothreonine residues on the MAPKs, causing inactivation. Thus, the MKPs, by regulation of the duration of MAP kinase activity, are involved in numerous, diverse signaling pathways including growth, development, apoptosis and the immune response (Jeffrey, Camps et al. 2007). The MKPs localize to the cytosol or nucleus and show different substrate selectivities towards ERK, JNK and p38 MAPK, with several MKPs being capable of inactivating multiple members of the MAPK family (Jeffrey, Camps et al. 2007).

Dysregulation of MKP activity has been shown to be involved in many human cancers, with DUSP1/MKP-1 levels being increased in breast cancer and decreased in prostate cancer, while DUSP2 levels are increased in ovarian cancer and leukemia. In addition, DUSP6/MKP-3 is hypermethylated in pancreatic cancer, and DUSP7/MKP-X levels are elevated in acute myeloid leukemia (Jeffrey, Camps et al. 2007). Interestingly, DUSP6 seems to be a tumor suppressor in the development of pancreatic cancer (Furukawa, Fujisaki et al. 2005).
1.3.2 The atypical dual specificity PTPs

There are 19 genes encoding atypical dual specificity PTPs in the human genome. They include the human ortholog of the first dual specificity PTP identified, VHR, atypical MAPK phosphatases such as DUSP14/MKP-6, the mRNA capping enzyme RNGTT and laforin. Thus, the atypical dual specificity PTPs have diverse cellular substrates and roles, and these have been extensively reviewed recently (Patterson, Brummer et al. 2009).

Several atypical dual specificity PTPs have been shown to be involved in various disease states. For example, overexpression of DUSP26, which utilizes p38 MAPK as a substrate is associated with anaplastic thyroid cancer cell growth due to inhibition of p38 MAPK-mediated apoptosis (Yu, Imoto et al. 2007). In addition, DUSP26 is downregulated in malignant glioma and implicated in decreased β-catenin-N-cadherin-mediated cell-to-cell adhesion. This is thought to occur via reduced dephosphorylation by DUSP26 of Kap3, a subunit of the KIF3 complex protein motor that directs subcellular transport of the β-catenin-cadherin complex (Tanuma, Nomura et al. 2009).

Mutations of the dual specificity PTP, laforin, which dephosphorylates glycogen, are associated with the autosomal recessive neurogenerative disorder, Lafora Disease. Lafora Disease is characterized by buildup of insoluble phosphorylated glucose polymers in neurons and leads to progressive myoclonus epilepsy. Symptoms of the disease begin in late childhood and death occurs within 10 years. (Ganesh, Tsurutani et al. 2004).
Laforin has also been reported to utilize GSK3β as a substrate in thymocytes and primary embryonic fibroblasts, causing selective dephosphorylation of Ser\(^9\) (Wang, Liu et al. 2006). This dephosphorylation of GSK3β is associated with phosphorylation of cyclin D1 and negative regulation of the cell cycle, while inhibition of laforin is associated with increased cell growth and increased phosphorylation of retinoblastoma protein. Thus, in this context, laforin has been shown to function as a tumor suppressor (Liu, Wang et al. 2008).

The atypical dual specificity PTP, VHR, has been shown to utilize both ERK and JNK as cellular substrates, thereby dephosphorylating and inactivating these MAPKs (Todd, Tanner et al. 1999; Todd, Rigas et al. 2002). Through its dephosphorylation of ERK and JNK, VHR thus plays a role in cell cycle progression, cell differentiation and cell growth (Cerignoli, Rahmouni et al. 2006). Interestingly, gene expression profiling in prostate cancer tissue samples shows increased levels of VHR in metastatic prostate cancer compared to normal prostate (Arnoldussen, Lorenzo et al. 2008). In addition, in the androgen-responsive prostate cancer cell line, LNCaP, exposure to androgen protects LNCaP cells from apoptosis via inactivation of JNK. This inactivation of JNK is due to overexpression of VHR, and knockdown of VHR protein levels using RNA interference results in increased apoptosis (Arnoldussen, Lorenzo et al. 2008). Similarly, in the human, androgen-dependent, prostate cancer xenograft model, CWR22,
deprivation of androgen by castration leads to increased apoptosis and tumor regression, with a concomitant decrease in expression of VHR and increase in the levels of phosphorylated JNK (Arnoldussen, Lorenzo et al. 2008).

1.3.3 The slingshot phosphatases

There are 3 slingshot phosphatases encoded by the human genome; SSH1, SSH2 and SSH3. The slingshot phosphatases are involved in the regulation of actin dynamics and directed cell migration. Slingshot-mediated directed cell migration is important in neurite extension (Endo, Ohashi et al. 2007), as well as vascular smooth muscle cell migration and vascular healing (San Martin, Lee et al. 2008). SSH1 has been shown to directly bind to and dephosphorylate/activate phospho-cofilin, a phospho-actin depolymerizing factor (ADF) (Kurita, Watanabe et al. 2008). SSH1 is activated by its interaction with actin, and its ability to dephosphorylate phospho-cofilin increases more than 1000-fold in the presence of F-actin (Kurita, Watanabe et al. 2008). As a result of its involvement in the regulation of actin dynamics, SSH1 contributes to the regulation of directed cell migration. SSH1 is essential for confining the formation of lamellipodia to the leading edge of the cell, thus allowing directional migration of the cell in response to stimulation by chemokine (Nishita, Tomizawa et al. 2005).

Most recently, it has been shown that protein kinase D1, binds to and phosphorylates SSH1 on Ser$^{978}$ which is located in the actin binding site of SSH1. The
phosphorylation of this serine residue on SSH1 creates a 14-3-3 binding motif which facilitates binding of 14-3-3β and blocks F-actin binding, thus resulting in sequestration of SSH1 in the cytoplasm and prevention of its relocalization to the lamellipodia (Eiseler, Doppler et al. 2009). This sequestration impinges upon the ability of SSH1 to engage, dephosphorylate and activate cofilin, thus resulting in decreased actin polymerization and directed cell migration. Interestingly, knockdown of cellular slingshot levels by RNA interference has been associated with reduced cancer cell invasion and migration (Horita, Ohashi et al. 2008)

### 1.3.4 The PRLs

There are 3 phosphatases of regenerating liver (PRLs) encoded by the human genome: PRL-1, PRL-2, PRL-3. The PRLs are often membrane-associated by means of a farnesyl group at their C-terminus. In cells, the PRLs are generally associated with the plasma membrane or the endoplasmic reticulum, but PRL-1 may translocate to the nucleus during specific parts of the cell cycle. Interestingly, PRL-1 has been shown to bind the centrosomes of the spindle of mitotic cells and abrogation of this interaction results in mitotic defects (Wang, Kirby et al. 2002). While PRL-1 and PRL-2 are rather ubiquitously expressed in various tissues, PRL-3 shows highest expression in heart and muscle (Dumaual, Sandusky et al. 2006). PRL-3 has been found to be expressed in the fetal heart, developing blood vessels and pre-erythrocytes, and hence has been
theorized to be involved blood vessel formation (Guo, Li et al. 2006). PRL-3 has also been shown to play a role in tumor angiogenensis (Guo, Li et al. 2006).

PRLs have also been linked to the development of various cancers. Both PRL-1 and PRL-3 have been shown to play a role in migration and invasion, with the promotion of invasion by the PRLs being linked to regulation of Rho proteins (Fiordalisi, Keller et al. 2006). Importantly, overexpression of PRL-3 is associated with liver and lymph node metastasis of colorectal cancer (Saha, Bardelli et al. 2001; Bardelli, Saha et al. 2003; Kato, Semba et al. 2004; Wang, Li et al. 2007), and high expression of PRL-3 is considered to be predictive of decreased long term survival in patients with colorectal cancer, gastric cancer and breast cancer (Peng, Ning et al. 2004; Wang, Peng et al. 2006; Miskad, Semba et al. 2007).

### 1.3.5 The CDC14 phosphatases

There are 4 CDC14 genes encoded by the human genome; CDC14A, CDC14C, CDKN3 and PTP9Q22. The CDC14 phosphatases are involved in exit from mitosis, meiosis, and DNA damage checkpoint control (Alonso, Sasin et al. 2004; Bassermann, Frescas et al. 2008; Schindler and Schultz 2009; Waples, Chahwan et al. 2009). CDC14A and CDC14B have been shown to be involved in oocyte meiosis (Schindler and Schultz 2009; Schindler and Schultz 2009). For example, inhibition of CDC14A results in delayed exit from Meiosis I and increased incidence of aneuploidy (Schindler and Schultz 2009).
In addition, CDC14A has been shown to utilize Erk5 as a substrate, and thus is implicated in regulation of cell proliferation and differentiation (Hansen, Bartek et al. 2008). RN-tre (a GTPase activating protein for Rab 5) has been implicated as a substrate for CDC14A during mitosis (Lanzetti, Margaria et al. 2007).

Although CDKN3 (KAP) has been shown to inhibit cell cycle progression, it is overexpressed in glioblastoma, with this overexpression being correlated with decreased long term survival of patients with glioblastoma. This apparent paradox is explained by the fact that the overexpressed form of CDKN3 in glioblastoma is a splice variant that acts as a dominant-negative to promote cell proliferation. The dominant-negative variant also decreases Cdc2 expression and promotes cell migration. Thus, CDKN3 is associated with increased migration and proliferation leading to a more severe cancer phenotype in glioblastoma (Yu, Jiang et al. 2007).

1.3.6 The PTENs

There are 5 traditional PTENs encoded by the human genome: PTEN, TPIP, TPTE, TNS and TENC1. The PTENs are phosphoinositide phosphatases which selectively cause dephosphorylation of phosphatidylinositol 3,4,5-triphosphate (PIP$_3$) at the D3 position. PIP$_3$ plays an important role in signaling as it binds to, and activates, proteins such as Akt, which contain pleckstrin homology domains (Keniry and Parsons 2008). In general,
these proteins are involved in mitogenic signaling pathways which promote cell growth and survival.

Mutation of PTEN results in diseases such as Cowden’s Syndrome which are associated with developmental defects and increased risk of the development of cancer (Yin and Shen 2008). PTEN has been shown to function as a tumor suppressor and mutation of PTEN has been observed in numerous cancers (Cairns, Okami et al. 1997; Tashiro, Blazes et al. 1997; Duerr, Rollbrocker et al. 1998). The deleterious effects of mutation of PTEN have been linked to the accumulation of phosphatidylinositol 3,4,5-triphasphate in the cell, where constitutively high cellular levels of PIP\(_3\) are associated with cell cycle progression and resistance to apoptosis (Keniry and Parsons 2008). The role of PTEN in the development of cancer has recently been reviewed extensively (Keniry and Parsons 2008).

1.3.7 The myotubularins

There are 16 myotubularins (MTMs) encoded by the human genome; MTM1 and MTMR1 through MTMR15. The MTMs are phosphoinositide phosphatases which dephosphorylate phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5-bisphosphate at the D3 position and are generally localized to the cytoplasm (Laporte, Bedez et al. 2003). Dephosphorylation of the latter substrate results in the generation of phosphatidylinositol 5-phosphate. Because of their impact on the concentration and
species of phosphoinositides in the cell, the MTMs are involved in endocytosis-related membrane trafficking (Clague and Lorenzo 2005).

Interestingly, not all members of the MTM subfamily of dual specificity PTPs are catalytically active. The catalytically inactive MTMs possess critical mutations in the phosphate-binding loop of the active site which render them unable to engage in dephosphorylation of substrate (Laporte, Bedez et al. 2003). Importantly, these catalytically inactive MTMs are believed to act as regulators of the catalytically active MTMs through the formation of heterodimers. The formation of these heterodimers is associated in specific cases with targeting of the enzyme to selected subcellular locations and increased phosphatase activity of the catalytically competent myotubularin in the heterodimer (Mochizuki and Majerus 2003; Nandurkar, Layton et al. 2003).

Mutation of the MTMs has been associated with several human diseases involving muscular dystrophy. For example, mutations of MTM1 are associated with X-linked myotubular myopathy which is characterized by muscular dystrophy and results in early death, while mutations of MTMR2 are associated with Charcot-Marie Tooth Syndrome which is characterized by neuropathy and muscular dystrophy (Pulido and Hooft van Huijsduijnen 2008). Interestingly, MTMR2 has been shown to heterodimerize with the catalytically inactive MTMR13, and loss of MTMR13 has also been associated
with the development of symptoms of the neuropathy of Charcot-Marie Tooth Syndrome (Senderek, Bergmann et al. 2003).

In summary, since dual specificity protein tyrosine phosphatases play such an integral part in normal cell signaling, aberrant regulation or expression of these enzymes has been associated with many disease states. In particular, the association of aberrant expression of many dual specificity PTPs with development of cancer marks these enzymes as potential therapeutic targets. However, in order to properly develop strategies for therapeutic intervention, an understanding of the means by which these enzymes are normally regulated is necessary.
1.4 Post-translational mechanisms of control of the dual specificity PTPs

Although the PTPs have been extensively studied as dephosphorylators and hence post-translational modifiers and regulators of other proteins, there is limited information regarding their own post-translational modification and regulation. Most of our knowledge has come from study of the post-translational regulation of classical PTPs, particularly PTP1B and the RPTPs (den Hertog, Ostman et al. 2008), but information regarding the post-translational control of the dual specificity PTPs is more scarce. However, our knowledgebase of the post-translational modification and regulation of the dual specificity PTPs has been growing within recent years, and along with this has come an increased appreciation for the importance of the roles of these post-translational regulatory events in the proper functioning of the dual specificity PTPs.

1.4.1 Phosphorylation

It is perhaps not surprising that the most prevalent type of post-translational modification, phosphorylation, has emerged as an important post-translational regulator of the activity of the dual specificity PTPs. The majority of the evidence for this regulation has come from studies of the phosphorylation of the MAP kinase phosphatases by ERK1/2 in a regulatory feedback mechanism. Experiments have shown that activated/phosphorylated ERK1 (p44MAPK), possibly in concert with activated ERK2
(p42$^{\text{MAPK}}$), induces phosphorylation of DUSP1/MKP-1 at Ser$^{359}$ and Ser$^{364}$ leading to protein stabilization in the absence of any effect on intrinsic enzyme activity (Brondello, Pouyssegur et al. 1999). In addition, in *Xenopus* oocytes, ERK2 was found to induce phosphorylation of both serine and threonine residues in DUSP1. Threonine phosphorylation occurs on Thr$^{168}$, and leads to stabilization of DUSP1 without affecting its phosphatase ability or its ability to bind substrate (Sohaskey and Ferrell 2002). Thus, there appears to be a role for the phosphorylation of specific residues of DUSP1 by ERK1/2 in stabilization of the enzyme.

Other studies have shown that activated ERK2 induces phosphorylation of DUSP1 at Ser$^{296}$ and Ser$^{323}$, marking it for ubiquitination and thus leading to protein degradation in the absence of any effect on intrinsic enzyme activity (Lin, Chuang et al. 2003; Lin and Yang 2006). Activated ERK2 has also been shown to induce phosphorylation of DUSP6 /MKP-3 at Ser$^{159}$ and Ser$^{197}$, marking it for ubiquitination and degradation by the proteasome in the absence of any effect on the enzyme’s catalytic activity (Marchetti, Gimond et al. 2005). In this system, there is also evidence that mTOR works in concert with the ERK pathway to contribute to the phosphorylation of DUSP6 at serine$^{159}$ (Bermudez, Marchetti et al. 2008), while Mek has been shown to be upstream of phosphorylation of DUSP6 at Ser$^{174}$ and Ser$^{300}$ which also promotes proteasomal degradation of DUSP6 (Jurek, Amagasaki et al. 2009). Hence,
phosphorylation of particular serine residues on DUSP1 and DUSP6 by ERK1/2 appear to mark the enzyme for degradation.

Although the details of the conditions under which ERK1/2 causes stability-enhancing phosphorylation of the MKPs versus stability-diminishing phosphorylation of the MKPs remain to be fully elucidated, this evidence for the regulation of the MAP kinase phosphatases by ERK1/2 has generated much excitement. ERK1/2 phosphorylation of the MAP kinase phosphatases represents an important means of post-translational regulation of the MKPs as it is part of a feedback mechanism where ERK1/2 is negatively regulated by MKPs via dephosphorylation which causes inactivation. However, at the same time ERK1/2 regulates its own negative regulators, the MKPs, via phosphorylation, causing either increased or decreased stability and thus either curtailing or prolonging its own activity.

The atypical, dual specificity PTP, VHR, which dephosphorylates ERK1/2 and JNK1/2 in T cells, has also been reported to undergo phosphorylation as a means of regulation. VHR has been reported to be phosphorylated on Tyr^{138} by both ZAP-70 (Alonso, Rahmouni et al. 2003) and the Jak kinase family member, Tyk2 (Hoyt, Zhu et al. 2007). This tyrosine phosphorylation of VHR in T-cells is necessary for the inhibition of ERK2 by VHR but does not seem to affect the intrinsic phosphatase activity of VHR. Since Tyr^{138} is located away from the active site of VHR, it has been suggested that
phosphorylation of Tyr\textsuperscript{138} may be important for interaction of VHR with its substrates or other proteins in the cell that influence its ability to interact with and dephosphorylate substrate. Thus, phosphorylation may also prove to be a significant means of post-translationally regulating the activity of the dual specificity PTPs by impacting the ability of these enzymes to interact with their substrates.

1.4.2 Ubiquitination

As suggested by the preceding description of the phosphorylation of the MKPs by ERK1/2, the dual specificity PTPs are also subject to post-translational modification by ubiquitination. Ubiquitination is the process by which the protein ubiquitin is covalently linked to lysine residues on target proteins via its C-terminal glycine residue by the enzyme E3 ligase. Proteins which are polyubiquitinated in the cell are usually subject to degradation by the proteasome (Hershko and Ciechanover 1998).

Ubiquitination of the dual specificity PTPs has been shown to be triggered, in some cases, by phosphorylation of selected serine residues. Amongst the MKPs shown to be ubiquitinated following targeted phosphorylation of serine residues are DUSP1 and DUSP6. Apart from the MAP kinases phosphatases, laforin, one of the atypical dual specificity PTPs has also been shown to be ubiquitinated by the E3 ligase malin, leading to degradation of laforin by the proteasome (GentryMS-2005-8501). In addition, PTEN has also been shown to be regulated by ubiquitination (Wang and Jiang 2008). PTEN is
ubiquitinated by the E3 ubiquitin ligase NEDD4-1 (neural precursor-expressed, developmentally downregulated 4-1). NEDD4-1-catalyzed ubiquitination of PTEN is negatively regulated by the C-terminal tail of PTEN which intramolecularly interacts with the C2 domain of PTEN, inhibiting binding of NEDD4-1 and resulting in stabilization of PTEN (Wang and Jiang 2008). Thus, ubiquitination represents another important means of post-translational control of dual specificity PTPs as it allows regulation of activity through the control of cellular protein levels.

1.4.3 Acetylation

Recent evidence suggests that the dual specificity PTPs may also be subject to acetylation as a means of post-translational regulation. Acetylation of proteins as a regulatory mechanism was first demonstrated with the acetylation of the MAP kinase kinases by the *Yersinia*-encoded protein YopJ. YopJ was shown to acetylate MAP kinase, kinases on serine residues in the activation loop thus preventing their phosphorylation/activation by upstream kinases (Mukherjee, Hao et al. 2007).

Acetylation of at least one of the PTPs acting on MAP kinases, MKP-1, has been very recently demonstrated, with this acetylation ultimately affecting the action of MKP-1. The acetylation of MKP-1 was mediated by the histone acetylase p300 following stimulation of Toll-like receptors. This acetylation occurred on Lys$^57$, which is located in the substrate binding domain of MKP-1, and promoted increased affinity of MKP-1 for
its substrate, p38 MAPK, without affecting its intrinsic enzymatic activity (Cao, Bao et al. 2008). This enhanced binding nevertheless promoted increased MKP-1-mediated dephosphorylation of p38 MAPK and hence downregulation of signaling. Thus, acetylation potentially represents a noteworthy means of post-translational regulation of the activity of the dual specificity PTPs as a result of its impact on the ability of the enzyme to bind substrate and thus effect dephosphorylation and influence cell signaling.

1.4.4 Oxidation

Another potentially important post-translational modification of the dual specificity PTPs which regulates their activity is oxidation of the catalytic cysteine. Recognition of the generation of reactive oxygen species in response to stress or other external stimuli as an important mediator of signaling in cells is growing, and reactive oxygen species themselves have been recognized as important intracellular messengers in cell signaling (Forman, Fukuto et al. 2008). Since the catalytic cysteine of PTPs has a low pKₐ, at neutral pH it is particularly susceptible to oxidation which causes inactivation of the enzyme. The susceptibility to oxidation differs amongst the dual specificity PTPs, with myotubularins being the most resistant to oxidation (Ross, Lindsay et al. 2007). In vitro, selective oxidation of the catalytic cysteine of the atypical dual specificity PTP, VHR, by hydrogen peroxide has been demonstrated, leading to complete abrogation of catalytic activity. This oxidation led to the formation of a cysteine sulfenic acid
intermediate and was found to be reversible (Denu and Tanner 1998). Cu$^{2+}$ has also been found to inhibit VHR activity \textit{in vitro} via induction of oxidation of the catalytic cysteine (Kim, Cho et al. 2000). In a more physiologically relevant model, in cells containing activation-impaired NF-κB (Ikkβ$^{-/-}$), treatment with TNFα was shown to induce the accumulation of reactive oxygen species which led to the selective oxidation of the catalytic cysteine of the MAP kinase phosphatases MKP-1, MKP-3 and MKP-5, leading to inhibition of activity and aggregation of the proteins in the cell (Kamata, Honda et al. 2005). In addition, the generation of reactive oxygen species in cells by platelet-derived growth factor signaling was shown to induce the oxidation of the catalytic cysteine of MKP-1 and PTEN in angiomyolipoma cells (BiovinB-2008-9959). Thus, the low PK$_a$ of the catalytic cysteine of the dual specificity (and other) PTPs make them particularly susceptible to oxidation. This oxidation is reversible and leads to temporary catalytic inactivation of the enzyme.

Interestingly, members of three subfamilies of the dual specificity PTPS, the PTENs and the PRLs, and the CDC14s, seem to be regulated by oxidation of the catalytic cysteine of the phosphatase binding loop in a more complex manner. In both PRL-1 (Sun, Wang et al. 2005) and PRL-3 (Kozlov, Cheng et al. 2004), it has been shown that the catalytic cysteine is oxidized and forms a reversible, intramolecular disulfide bridge with a neighboring, invariant cysteine residue, Cys$^{49}$. Since these residues are conserved
among all PRLs, it is likely that this represents a conserved phenomenon in the PRL family of dual specificity PTPs. This disulfide bridge formation not only catalytically inactivates the protein but causes local conformational changes which disrupt substrate binding (Sun, Wang et al. 2005).

The formation of an oxidation-dependent disulfide bridge involving the catalytic cysteine of the phosphate binding loop and a nearby cysteine has also been observed in two other dual specificity PTPs. The dual specificity PTP, PTEN, undergoes oxidation of its active site cysteine to form a disulfide bond with a neighboring cysteine, Cys\textsuperscript{71} in response to oxidative stress (Lee, Yang et al. 2002). Furthermore, in the CDC14 dual specificity PTP, CDKN3/KAP, formation of an oxidation-dependent disulfide bridge can occur between the catalytic cysteine and a nearby cysteine, Cys\textsuperscript{79} (Song, Hanlon et al. 2001).

It has been suggested that the formation of oxidation-dependent disulfide bridges involving the catalytic cysteine represents a regulatory mechanism designed to either downregulate the activity of the dual specificity phosphatases, or protect them from irreversible oxidation during signaling involving reactive oxygen species, and thus can potentially serve as an important means of post-translational regulation of these enzymes.
1.4.5 Prenylation

One family of dual specificity PTPs, the PRLs, has also been shown to be post-translationally modified by prenylation. Prenylation involves the addition of a lipid moiety to the cysteine terminus of a protein. The prenyl group inserts into the lipid bilayer of membranes, thus anchoring the prenylated protein to the membrane. Aside from Rab proteins (which are Ras family monomeric G-proteins), proteins are prenylated at a consensus CaaX motif, where the C is a cysteine residue, the a’s are aliphatic residues and the X is any amino acid. The identity of the final amino acid in the motif determines whether the CaaX box is farnesylated (15-carbon isoprenoid) or geranylgeranylated (2-carbon isoprenoid) (Terry, Casey et al. 2006).

The PRLs contains a CaaX motif located at the C-terminus of the enzyme in a conserved region consisting of multiple basic residues which undergoes farnesylation (Cates, Michael et al. 1996; Zeng, Si et al. 2000; Bardelli, Saha et al. 2003). Farnesylation of the PRLs on the CaaX cysteine, Cys\textsuperscript{170}, is required for the localization of the PRLs to the cell membrane and the endoplasmic reticulum and, in the absence of farnesylation, all three PRLs re-localize to the nucleus (Zeng, Si et al. 2000). Furthermore, in the case of PRL-1, in dividing cells, localization to the nucleus occurs in a cell cycle-dependent manner, with nuclear re-direction occurring during mitosis. This nuclear re-direction of PRL-1 in dividing cells is farnesylation-independent and results in PRL-1 becoming
associated with γ-tubulin at centrosomes during prometaphase, and microtubules at the base of the spindles near centrosomes during late metaphase and anaphase (Wang, Kirby et al. 2002). This association is of functional consequence as mitotic delay occurs when the catalytic activity of PRL-1 is attenuated, thus indicating that the phosphatase activity of PRL-1 is required for normal mitosis. The farnesylation of PRL-1 is also required for normal mitosis as mitotic defects occur with unfarnesylated, but catalytically competent PRL-1 (Wang, Kirby et al. 2002).

The impact on function of post-translational modification of the PRLs by prenylation appears to be primarily due to influencing their localization and hence the pool of potential substrates with which these dual specificity PTPs can interact. Prenylation may also influence the activity of these dual specificity PTPs at their site of action in a manner that is independent of catalytic competence as demonstrated by the findings noted above with PRL-1 (Wang, Kirby et al. 2002). In addition, prenylation-dependent localization of the PRLs to particular membranes has been postulated to increase their local concentration at these sites, thus potentially promoting the formation of oligomeric forms which may then exhibit altered activities (Sun, Wang et al. 2005). Thus, taken together, the available evidence suggest prenylation impacts the PRL dual specificity phosphatases mainly by influencing localization and interaction and this has important consequences for activity.
1.4.6 Oligomerization

While the previously described methods of post-translational regulation of the dual specificity PTPs all involve covalent modification of the protein itself, one method for control of the activity of selected dual specificity PTPs has emerged which does not. Oligomerization, most notably the formation of dimers and trimers, has been observed for some dual specificity PTPs, and the formation of these quaternary structures has been postulated to be a means of regulating the function of these enzymes.

Perhaps the best example of the regulation of dual specificity PTPs via oligomerization comes from the myotubularin family of dual specificity PTPs. Interestingly, the myotubularin family contains several catalytically inactive members which lack the catalytic cysteine and/or the stabilizing arginine residue(s) of the PTP consensus catalytic motif, and it has been shown that these phosphatase-dead myotubularin family members effect post-translational regulation of the catalytic family members via dimerization. The catalytically inactive MTMR5 dimerizes with the catalytically active MTMR2 to increase its phosphatidylinositol phosphatase activity and direct its subcellular localization (Kim, Vacratsis et al. 2003), while the catalytically inactive MTMR12 dimerizes with the catalytically active MTMR1 to cause relocalization from the plasma membrane to the cytosol (Nandurkar, Layton et al. 2003). In addition, the catalytically inactive MTMR9 dimerizes with the catalytically active MTMR7 to
increase its phosphatidylinositol phosphatase activity (Mochizuki and Majerus 2003) and MTMR9 also dimerizes with MTMR6 to increase its binding to substrate, catalytic activity and stability (Zou, Chang et al. 2009).

Loss or mutation of the catalytically inactive myotubularins has been found to be as capable of causing severe disease phenotypes as the loss or mutation of their catalytically active partners in the heterodimer pair. This is exemplified by the fact that mutation of the gene encoding the catalytically active MTMR2 is associated with Charcot-Marie-Tooth disease type 4B (Bolino, Muglia et al. 2000), while mutation of the gene encoding the catalytically inactive MTMR13 is associated with Charcot-Marie-Tooth neuropathy type 4B2/11p15 in humans (Senderek, Bergmann et al. 2003) and loss of MTMR13 has been shown to lead to the development of Charcot-Marie-Tooth 4B2-like disease in mice (Robinson, Niesman et al. 2008).

Beyond the myotubularins, other dual specificity PTPs have also been revealed to form oligomers that are believed to be biologically relevant. Protein crystallization efforts by two different groups have revealed that PRL-1 forms a trimer in the crystal structure that involves residues that are conserved among the different PRLs (Jeong, Kim et al. 2005; Sun, Wang et al. 2005). Interestingly, in the trimer, the C-terminal polybasic tails of the three PRL-1 molecules are all located on one face of the quaternary structure, forming a basic patch which is oriented towards membranes in cells (Jeong,
Mutations in the polybasic tail of PRL-1 abolishes membrane association (Sun, Wang et al. 2005; Sun, Luo et al. 2007) while deletion of the CaaX motif in PRL-3 abrogates trimer formation (Sun, Luo et al. 2007). In addition, crosslinking experiments have demonstrated that membrane-associated PRL-1 exists as a trimer in cells (Jeong, Kim et al. 2005; Sun, Wang et al. 2005) and cells containing a non-trimerizing mutant of PRL-1 show reduced growth and migration (Sun, Luo et al. 2007). Thus, the trimerization of the PRLs is postulated to have functional consequence in terms of localization to membranes and hence activity, with trimerization and prenylation acting cooperatively to target the PRLs to membranes.

It has also very recently been reported that VH1, the Vaccinia virus ortholog of the human VHR dual specificity PTP, exists as a dimer in the protein crystal (Koksal, Nardozzi et al. 2009). In the dimer, the VH1 active sites occupy the same face of the quaternary structure and are located about 30Å apart. Since VH1 selectively binds the cytoplasmic, DNA-free pool of its substrate STAT1, but not the nucleic, DNA-bound form of STAT1 (Koksal, Nardozzi et al. 2009), it tempting to speculate whether the dimer structure of VH1 confers some selectivity for the DNA-free cytoplasmic form of STAT1.

One of the members of the MAPK phosphatase family of dual specificity PTPs has also shown evidence of oligomer formation in cells. MKP-3 was detected in dimers, trimers and higher oligomeric forms in nine different pancreatic lysates (Mark, Aubin et
al. 2008). There is evidence that this oligomerization probably occurs via interdomain binding and comprises part of a mechanism of negative self-regulation of MKP-3 activity as oligomeric forms of MKP-3 did not exhibit detectable phosphatase activity (Mark, Aubin et al. 2008).

Thus, oligomerization of the dual specificity PTPs is fast emerging as a potential method of post-translational regulation of the dual specificity PTPs which does not involve post-translational covalent modification of the enzymes themselves. Oligomerization of the dual specificity PTPs has been shown to occur in both homo- and hetero- forms, with MKP-3, VH1 and the PRLs forming homo-oligomers and the myotubularins forming hetero-oligomers. In contrast to the receptor PTPs, where oligomerization has been identified as a post-translational method of negatively regulating activity (den Hertog, Ostman et al. 2008), thus far, oligomerization seems to mainly be a positive method of post-translational control for the dual specificity PTPs. In the case of the dual specificity PTPs, oligomerization appears to assist, not only in targeting the enzymes to the appropriate subcellular compartment where the appropriate substrate can be engaged at the appropriate time, but also in enhancing intrinsic phosphatase activity in some cases but notably, in the case of MKP-3, of abrogating phosphatase activity.
In summary, the dual specificity PTPs are post-translationally regulated by a number of covalent modifications. These include phosphorylation, ubiquitination, prenylation, acetylation and oxidation. Additionally, the dual specificity PTPs are post-translationally regulated by one method which does not include physical modification of the protein. Given the recent discoveries of the occurrence of regulatory dimers and trimers in various classes of dual specificity PTPs, it would not be surprising if oligomerization emerges as a major regulator of the activity of the dual specificity PTPs as we learn more about this important class of enzymes.

As we seek to elucidate the function of various dual specificity PTPs and to develop therapeutic interventions to disrupt their function, it will be important to consider the potential means by which these enzymes are post-translationally regulated. On the one hand, these methods of post-translational control may confound our efforts to effectively target particular dual specificity PTPs, or conversely, it may be possible to exploit these post-translational regulatory mechanisms to achieve more effective and/or selective targeting.
1.5 Inhibitors of the dual specificity PTPs

One means of controlling the activity of enzymes is through the use of small molecule chemical inhibitors. These inhibitors, by reversibly or irreversibly binding to the enzyme, may impact activity in several ways including precluding interaction of the enzyme with its cognate substrate, decreasing the intrinsic activity of the enzyme or trapping the enzyme with bound substrate thus preventing the release of product. Thus, identification of effective and selective small molecule inhibitors of specific enzymes affords us a means of regulating the activity of enzymes in the cell.

As our knowledge of the action and functions of many protein tyrosine phosphatases has grown and the involvement of these PTPs in various disease states has become apparent, numerous PTPs have been identified as therapeutic targets and thus have become the subject of inhibitor screens. Conversely, as the gaps and limits in our understanding of the function of many newly discovered PTPs have been revealed, additional PTPs have been subjected to screens in a bid to increase our knowledgebase via chemical genetics approaches, particularly as these approaches also offer the potential for the identification of new drugs. Thus, these two logics for engaging in screens for PTP inhibitors have converged to offer a rapidly growing cache of inhibitors of various protein tyrosine phosphatases.
The traditional forward chemical genetics approach to identifying inhibitors of selected enzymes for potential use as drugs has involved screening a large library of candidate compounds in vitro for inhibition of the purified enzyme, and then testing these compounds in cells and/or whole animals. As our repertoire of structures of proteins has grown, this approach has been modified to structure-based inhibitor identification, wherein a subset of potential inhibitory compounds is chosen based on their potential to interact in an inhibitory manner with specific structural features of the enzyme of interest. The in silico evaluation of the inhibitory potential of particular compounds is usually based on modeling of the fit of the compound into the available protein structure. This approach has achieved most utility in the identification of competitive inhibitors of selected enzymes, since these inhibitors can be modeled into the active site of the protein. However, this approach is also limited by the availability of high quality protein structural information for a sufficient number of enzymes.

Several modifications of the traditional forward chemical genetics method have emerged over time. These modifications have predominantly involved either new ways of defining the pool of compounds to be considered for inhibitor screening or new ways of defining the context in which the target enzyme is assayed. For example, one modification of the traditional chemical genetics method which refines the pool of compounds considered for inhibitor screening and has been used to screen for
inhibitors of the atypical dual specificity PTP, VHR, is BIOS or biology-oriented synthesis (Koch, Wittenberg et al. 2004; Noren-Muller, Reis-Correa et al. 2006). This method relies on the selection of compounds for inhibitor screening based on their similarity to chemical scaffolds found in nature. The presumption is that these natural products have already been validated in nature via evolution and thus they are more likely to be biologically relevant and bioavailable.

Another modification of the chemical genetics method which refines the context in which the enzyme is assayed and has been used to screen for inhibitors of the MKP dual specificity PTPs, has been referred to as chemical complementation (Vogt, Cooley et al. 2003; Vogt, Tamewitz et al. 2005). This method, which has been used for identification of MKP inhibitors, incorporates aspects of reverse chemical genetics by exploiting the in-cell phenomenon associated with decreased activity of the enzyme of interest. In summary, since inhibition of certain MKPs is associated with increased ERK phosphorylation in the nucleus in response to particular chemical stimuli, the screen for inhibitors of the MKPs utilizes cells transfected to overexpress the MKP of interest. The cells are then treated with potential inhibitors in the context of chemical stimulation of ERK phosphorylation. The presumption is that if a bonafide inhibitor of the MKP is present, the quenching of the phospho-ERK signal normally associated with
overexpressed levels of the MKP will be rescued, thus resulting in the detection of increased ERK phosphorylation (Vogt, Cooley et al. 2003).

With the recognition of the large number of dual specificity phosphatases in the human genome (Alonso, Sasin et al. 2004) and the growing appreciation of their importance in regulating normal cell signaling as well as their involvement in disease states, the need for tools to interrogate the biology of these enzymes and the quest for inhibitors to therapeutically target these enzymes, has grown in prominence. Thus, it seems timely to review our current knowledge of the known potent (at least low micromolar range), small molecule, chemical inhibitors of the dual specificity PTPS.

1.5.1 Inhibitors of the MAPK phosphatases

Since the MAPK phosphatases control the duration and intensity of signaling downstream of the MAP kinases, they have been implicated in signaling events which control growth, development, apoptosis and the response to extracellular stress (Jeffrey, Camps et al. 2007). Not surprisingly, derailment of MKP signaling has been observed in several cancers (Jeffrey, Camps et al. 2007). Hence, the MAPK phosphatases have been the subject of several small molecule inhibitor screens.

The first MKP inhibitor, which was identified in 2003, was an inhibitor of MKP-3 (Vogt, Cooley et al. 2003). MKP-3 has been shown to play crucial roles in embryogenesis including cell specification and limb development (Kawakami, Rodriguez-Leon et al. 2007).
2003; Tsang, Maegawa et al. 2004; Smith, Sweetman et al. 2005) and MKP-3 is postulated to act as a tumor suppressor in the development of pancreatic cancer (Furukawa, Fujisaki et al. 2005). The MKP-3 inhibitor identified was a benzophenanthridine compound and inhibited the enzyme in cells with an IC$_{50}$ = 8.0 μM (Vogt, Cooley et al. 2003). While the compound did not significantly inhibit VHR, it was not tested against other dual specificity PTPs, including other MKPs, so its true degree of selectivity remains unclear. More recently, quinine-type compounds originally synthesized as potential Cdc25B inhibitors have demonstrated inhibition of MKP-1 and MKP-3 with IC$_{50}$ values lower than 10 μM in vitro (Cao, Murphy et al. 2009). These compounds were based on the structure of the most potent known inhibitor of Cdc25B, adociaquinone, or on a general naphthoquinone moiety. Of the adociaquinones, only adociaquinone itself inhibited MKP-3 significantly (IC$_{50}$ = 1.53 μM). Of the naphthoquinones, two compounds, compound 15 and compound 20, inhibited MKP-3 significantly (IC$_{50}$ = 6.9 and 1.35 μM respectively). However, these compounds all also significantly inhibited MKP-1 and demonstrated more potent inhibition of Cdc25B, and hence were not truly selective.

The first inhibitor of MKP-1 was identified in 2005 after screening a natural products library using an in cell chemical complementation assay. MKP-1 has been demonstrated to protect cells from apoptosis and is overexpressed in prostate, breast,
pancreatic and gastric cancer. Elevated MKP-1 expression is associated with shorter progression-free survival times in patients with invasive ovarian cancer (Denkert, Schmitt et al. 2002) and reduction of MKP-1 expression in pancreatic cancer cells resulted in decreased tumorigenicity in a nude mouse model (Liao, Guo et al. 2003). Thus, the identification of selective MKP-1 inhibitors is an attractive prospect in terms of cancer treatment.

The natural product sanguinarine inhibited MKP-1 in cells with an IC$_{50}$ of 10 μM and in vitro with an IC$_{50}$ = 17.3 μM (Vogt, Tamewitz et al. 2005). Sanguinarine selectively inhibited MKP-1 compared to MKP-3, Cdc25B, VHR and PTP1B. The closely related compound chelerythrine also inhibited MKP-1 in vitro, with an IC$_{50}$ = 16.2 μM. (Vogt, Tamewitz et al. 2005). Several biological effects have been reported for sanguinarine, but it is unknown whether these effects can be attributed solely to impact on the MKPs (Vogt, Tamewitz et al. 2005). Subsequently, a benzofuran compound, NU-126, was shown to inhibit MKP-1 with an IC$_{50}$ = 28.8 μM in vitro. NU-126 was selective in its inhibition of MKP-1 compared to MKP-3 (Lazo, Nunes et al. 2006). The inhibition of MKP-1 was reversible and not purely competitive, but of mixed character. However, this compound did not exhibit any cellular activity against MKP-1, suggesting that it might not be bioavailable.
More recently a high throughput *in vitro* screen was carried out using 3-O-methylfluorescein phosphate as a substrate in the identification of inhibitors of MKP-1. Screening of an NIH diversity library of 65,000 compounds resulted in the identification of 11 compounds which inhibited MKP-1 with an IC$_{50}$ $<$ 10 μM (Johnston, Foster et al. 2007). Four compounds exhibiting structures based on quinolinedione or naphthoquinone moieties were the most potent inhibitors of MKP-1 with IC$_{50}$ values of 0.1 – 0.2 μM, while other compounds containing furan and quinolinecarboxylic moieties inhibited the enzyme in the range of 1 - 10 μM. Also, use of a fluorescence-based chemical complementation assay demonstrated that a quinine-based compound, NSC-95397, which had previously been evaluated as a Cdc25B inhibitor, produced reactive oxygen species and inhibited MKP-1 and MKP-3. In cells, the IC$_{50}$ for both MKP-1 and MKP-3 was 13 μM, while *in vitro*, the IC$_{50}$ for MKP-1 was 65 μM and that for MKP-3 was 25 μM. In an MKP-1-dependent cell survival model, NSC-95397 caused MKP-1-dependent cell death and thus phenocopied the effect of decreasing cellular MKP-1 levels using RNA interference. (Vogt, McDonald et al. 2008).

As mentioned above, recently some Cdc25 inhibitors based on the adociaquinone structure and a general naphthoquinone moiety have demonstrated inhibition of MKP-1 with IC$_{50}$ values lower than 10 μM *in vitro* (Cao, Murphy et al. 2009). Adociaquinone inhibited MKP-1 with IC$_{50}$ = 1.10 μM, while amongst the
naphthoquinones, compound 11 inhibited with IC$_{50}$ = 8.45 μM, compound 15 inhibited with IC$_{50}$ = 9.37 μM, and compound 20 inhibited with IC$_{50}$ = 0.82 μM. However, these compounds all also demonstrated more potent inhibition of Cdc25B and hence were not selective. To date, the inhibitors of the MKPs that have been discovered have almost all been competitive with respect to substrate and thus target the active site of the enzyme. However, the revelation of the importance of residues in the kinase interaction motif at the N-terminus of the MKPs, for substrate selectivity and activation of the phosphatase activity of the MKPs upon binding of their cognate MAPK substrate (Camps, Nichols et al. 1998; Nichols, Camps et al. 2000; Tanoue, Yamamoto et al. 2002), raises the possibility of development of non-active site-directed inhibitors of the MKPs (Jeffrey, Camps et al. 2007).

1.5.2 Inhibitors of the atypical dual specificity PTPs

Amongst the atypical dual specificity PTPs, VHR has probably been the target of the largest number of inhibitor screens. VHR is constitutively expressed in T cells, and through its inhibition of ERK and JNK phosphorylation/activation moderates T cell receptor-initiated T cell activation (Alonso, Saxena et al. 2001; Alonso, Rahmouni et al. 2003). In addition, cells in which VHR protein levels have been decreased by RNA interference, arrest at the G$_1$-S and G$_2$-M transition of the cell cycle with a concomitant decrease in telomerase activity (Todd, Tanner et al. 1999). Thus, VHR is an attractive
target for inhibitor screening designed to uncover new therapeutics for the treatment of immune disease and cancer.

In 1992, one of the first inhibitors of VHR was identified in a screen of bacterial metabolites. Compound RK-682 (3-hexadecanoyl-5-hydroxymethyltetronic acid), which was isolated from *Streptomyces* (Hamaguchi, Sudo et al. 1995), inhibited VHR *in vitro* with an IC$_{50}$ value of 11.6 μM (Sodeoka, Sampe et al. 2001). Structure activity relationship studies and molecular modeling of the compound into the crystal structure for VHR revealed that the free tetronic acid moiety was important for inhibition, suggesting that it might mimic the phosphate of the substrate (Sodeoka, Sampe et al. 2001). RK-682 showed kinetics consistent with a competitive mode of inhibition (Usui, Kojima et al. 2001), while the second order plots suggested that 2 molecules of the compound might bind a single VHR molecule (Usui, Kojima et al. 2001). An optimized RK-682 dimer inhibited VHR with an IC$_{50}$ of 1.8 μM, showed a mixed competitive/non-competitive inhibition and bound with a 1:1 stoichiometry (Usui, Kojima et al. 2001).

Although the bioavailability of RK-682 was poor, cell-based experiments revealed that treatment with RK-682 increased the level of tyrosine phosphorylation of proteins approximately 90 and 110 kDa in size. RK-682 did not significantly inhibit Cdc25b, thus indicating some degree of selectivity. In addition, RK-689 inhibited cell cycle progression at the G$_1$ phase (Hamaguchi, Sudo et al. 1995). This is particularly interesting since it has
recently been reported that loss of VHR causes cell cycle arrest at $G_1$-$S$ and $G_2$-$M$ (Rahmouni, Cerignoli et al. 2006), and that the *Vaccinia* virus ortholog of VHR (VH1) exists as a dimer in the protein crystal (Koksal, Nardozzi et al. 2009).

Although not as potent as RK-682, benzofurans have also been identified as inhibitors of VHR (Malamas, Sredy et al. 2000). Benzofuranbiphenyls emerged as inhibitors of VHR as part of an *in vitro* screen for inhibitors of PTP1B using a tri-phosphorylated peptide of the insulin receptor kinase regulatory domain. The lead benzofuran compound was shown to inhibit VHR with an $IC_{50} = 15.9 \mu M$ (Malamas, Sredy et al. 2000). A microbial metabolite, 4-isoavenaciolide (4-iA), has also been identified as an inhibitor of VHR (Ueda, Usui et al. 2002). 4-iA inhibited VHR with an $IC_{50}$ value of $1.2 \mu M$, but this inhibition was not selective for VHR as the compound also significantly inhibited laforin, Cdc25B and PTP1B. Inhibition of VHR by 4-iA was time-dependent and irreversible. 4-iA achieved inhibition by binding covalently to the active site cysteine Cys$^{124}$ of VHR under non-reducing conditions, and further studies suggested that the Cys$^{124}$ and nearby Cys$^{171}$ were both modified by bound 4-iA during inhibition.

More recently, virtual screening has led to the identification of more VHR inhibitors. A glucosamine-based compound, glucosamine-aminoethoxytriphenyltin, (GATPT), was identified as a potent inhibitor of VHR using a structure-based virtual
screening and docking strategy, where potential inhibitors were modeled into the active site of VHR in a high resolution crystal structure and then validated for binding to VHR (Shi, Tabassum et al. 2007). This method resulted in the identification of GATPT as a potent inhibitor of VHR, displaying a competitive mode of inhibition with $K_i = 2.92 \mu M$. Modeling of the structure of GATPT into the active site of VHR suggests that inhibition results from hydrogen bonding of the glucosamine ring to the catalytic cysteine Cys$^{124}$ as well as hydrogen bonding of the NH group linking the glucosamine moiety to the triphenyl ring moiety to Asp$^{92}$ which acts as a general acid in the catalytic reaction. The activity of GATPT as an inhibitor of VHR in cells was verified by demonstrating that the compound phenocopied the effects of knockdown of VHR using RNA interference. These effects included stimulation of ERK and JNK phosphorylation as well as arrest of cells in the G$_1$ phase of the cell cycle. Further evidence that the effect of GATPT was targeted towards VHR came from the demonstration that the effects of GATPT were negligible in the context of knockdown of VHR levels by RNA interference.

Six other inhibitors of VHR, comprising 4 structural classes have also been recently identified using a structure-based virtual screening and docking strategy (Park, Jung et al. 2008). The four structural classes that were identified as inhibitory to VHR included a ([2-(2,5-dioxo-imidazolidin-4-ylidenemethyl)-pyrrol-1-yl]-benzoic acid scaffold, and a 1-(3,4-dihydroxyphenyl)-2-(4H-[1,2,4]triazol-3-ylsulfanyl)ethanone
scaffold, with the former displaying greatest potency. The two compounds possessing this scaffold exhibited IC$_{50}$ values of 3.7 μM and 4.7 μM. This potency has been attributed, via evaluation of docking simulations, primarily to hydrogen bonding of the benzoate moiety to the active site cysteine, thus mimicking the phosphate group of the substrate.

The atypical dual specificity PTP, DUSP26 is also an attractive target for inhibitor screening due to its involvement in tumorigenesis and tumor progression (Vasudevan, Skoko et al. 2005; Yu, Imoto et al. 2007; Tanuma, Nomura et al. 2009). The only DUSP26 inhibitor reported to date is NSC-87877 (Song, Park et al. 2009), which had previously been reported as an inhibitor of SHP-1 and SHP-2. NSC-87877 was identified as an inhibitor of DUSP26 in an in vitro screen and demonstrated kinetics consistent with competitive inhibition of DUSP26 with a K$_i$ value of 16 μM. NSC-87877 was fairly selective as, aside from its previously demonstrated inhibition of SHP-1 (IC$_{50}$ = 40 μM) and SHP-2, it did not significantly inhibit any of the other PTPs tested. NSC-87877 inhibited the dephosphorylation of p38 MAPK by DUSP26, and also abrogated the inhibition of kinase activity of p38 MAPK by DUSP26, in a dose-dependent manner in vitro and in cells (Song, Park et al. 2009). Taken together, these data suggest that NSC-87877 is a bonafide inhibitor of DUSP26 which is potent and fairly selective. It will be interesting to determine if inhibitors with better potency and selectivity can be
developed based on the NSC-87877 pharmacophore and how NSC-87877 affects derailed p38 MAPK signaling in anaplastic thyroid cancer cells.

1.5.3 Inhibitors of the PRLs

The PRL family of dual specificity phosphatases is involved in cell growth, migration and invasion (Zeng, Dong et al. 2003). Importantly, PRL-3 is highly expressed in metastatic lesions of colorectal cancer in the liver (Bradbury 2001; Saha, Bardelli et al. 2001) and overexpression of PRL-3 promotes cell transformation and growth (Matter, Estridge et al. 2001). Thus, PRLs are an attractive target for the screening of inhibitors of dual specificity phosphatases with the potential to impact disease.

In 2002, pentamidine was identified as an irreversible inhibitor of the PRLs. Pentamidine was evaluated as a potential protein tyrosine phosphatase inhibitor based on the observed similarity of its effects against intracellular leishmania with those observed with sodium stibogluconate, which had also been shown to be an inhibitor of particular PTPs (Pathak, Dhawan et al. 2002). Pentamidine was demonstrated to be an irreversible inhibitor of all three PRLs (PRL-1, PRL-2 and PRL-3) both in vitro and in cells. Pentamidine was also shown to inhibit the growth of several cancer cell lines which endogenously express PRLs (Pathak, Dhawan et al. 2002).

Four years later, following high throughput screening of a chemical library, it was discovered that the rhodanine moiety shows inhibition of PRL-3. Several
salicylaldehyde and naphthaldene rhodanine derivatives were then synthesized and evaluated for their ability to inhibit PRL-3 (Ahn, Kim et al. 2006). The inhibitory salicylaldehyde rhodanine derivatives exhibited IC$_{50}$ values in the range 0.9 μM to 9.5 μM. Compound 5e and compound 7d were the most potent with IC$_{50}$ value = 0.9 μM. The naphthaldene rhodanine derivatives exhibited IC$_{50}$ values in the range 1.7 μM to 3.1 μM, with a 2-chloro-6-fluorobenzyl substitution being the most potent (Ahn, Kim et al. 2006). The crystal structure of PRL-1 was then analyzed with a view to extrapolating features of the most potent rhodanine inhibitors which might contribute to their ability to inhibit PRL-3. This analysis suggested that the best rhodanine derivative inhibitors might owe their potency to (i) deprotonation of their NH group which would mimic negatively-charged substrate and (ii) the hydrophobicity of substituents which would bind the highly hydrophobic active site (Ahn, Kim et al. 2006). Investigators also tested potent salicylaldehyde rhodanine and naphthaldene rhodanine derivatives in a cell invasion model and showed that both compounds caused a reduction in cell invasion, suggesting that they were bioactive and potentially targeted PRL-3 in cells (Ahn, Kim et al. 2006).

Two biflavonoids isolated from the young branches of *T. cuspidata* have also been reported as inhibitors of PRL-3. Ginkgetin inhibited recombinant PRL-3 *in vitro* with an IC$_{50}$ value of 25.8μM, while sciadopitysin inhibited PRL-3 with an IC$_{50}$ value of 46.2
μM (Choi, Oh et al. 2006). Unfortunately, the selectivity of these compounds was not tested. Most recently, a small molecule, 7-amino-2-phenyl-5H-thieno[3,2-c]pyridine-4-one (a thienopyridone compound) was reported as an \textit{in vitro} inhibitor of all three PRLs (Daouti, Li et al. 2008). Molecules with fused pyridine structure were identified as inhibitors of PRL-1, PRL-2 and PRL-3 following a high throughput screen of the Roche chemical library \textit{in vitro} using a peptide substrate. 7-amino-2-phenyl-5H-thieno[3,2-c]pyridine-4-one was identified as the most potent of these compounds and reversibly inhibited PRL-1 with an IC$_{50}$ value of 0.173 μM, PRL-2 with an IC$_{50}$ value of 0.277 μM and PRL-3 with an IC$_{50}$ value of 0.128 μM. Compared to other non-PRL phosphatases, the inhibition of PRL-3 by the thienopyridone compound was selective.

The effect of 7-amino-2-phenyl-5H-thieno[3,2-c]pyridine-4-one was also tested in cells. The thienopyridone compound was shown to induce the proteolytic cleavage of p130Cas (Daouti, Li et al. 2008) and focal adhesion kinase (FAK). Cleavage of p130Cas and FAK were associated with the induction of apoptosis which involved caspase-8 cleavage and was independent of p53 activity, with induction of apoptosis occurring in cancer cells but not normal cells (Daouti, Li et al. 2008). The thienopyridone also inhibited anchorage-independent growth of human colorectal cancer cell lines with an EC$_{50}$ value of approximately 3.0 μM. Additionally, the thienopyridone inhibited HUVEC
cell migration but not proliferation, an effect which is consistent with inhibition of PRL-3 (Daouti, Li et al. 2008).

The inhibitors of the PRLs identified thus far have mainly been compounds selected from synthesized chemical libraries, and if kinetically characterized, most have been competitive inhibitors. These have mainly targeted PRL-3 and have demonstrated high potency both in vitro and in cells. However it is expected that most of these inhibitors will be active against all three enzymes since there is such high homology among the PRLs in the active site, and this may confound use of these compounds for truly targeted inhibition.

1.5.4 Inhibitors of the PTENs and related phosphatases

Amongst the PTEN dual specificity PTPs, PTEN itself has probably been the target of the largest number of small molecule inhibitor screens. The role of PTEN is now widely recognized as that of a tumor suppressor since the mutation or absence of PTEN in cells is correlated with various cancers. While this suggests that inhibitors of PTEN would not serve a therapeutic role, interestingly PTEN has also been shown to be a negative regulator of insulin signaling in adipocytes (Nakashima, Sharma et al. 2000; Ono, Katagiri et al. 2001). Thus, inhibitors of PTEN will aid not only in further study of PTEN and the dissection of the various mechanisms by which dysregulation of PTEN
contributing to the development of cancer, but also dissection of the mechanisms by which PTEN impacts insulin signaling.

Amongst the most potent and best characterized PTEN chemical inhibitors is a group of compounds with a bisperoxovanadium core structure (Schmid, Byrne et al. 2004). Although the bisperoxovandium compounds were originally characterized as PTP inhibitors which resulted in activation of insulin receptor kinase (Posner, Faure et al. 1994; Cuncic, Desmarais et al. 1999), further evaluation with respect to inhibition of PTEN in particular revealed that the subgroup of bisperoxovanadium compounds possessing polar _N,O_ ligands, dipotassium bisperoxo(5-hydroxypyridine-2-carboxyl)oxovanadate (bpV(HOpic)) and dipotassium bisperoxo(picolinato)oxovanadate (bpV(pic)) were highly potent inhibitors of PTEN. bpV(HOpic) and bpV(pic) inhibited PTEN with low nanomolar IC\textsubscript{50} values of 14 nM and 31 nM respectively (Schmid, Byrne et al. 2004) \textit{in vitro} using recombinant PTEN and synthetic dipalmitoyl-phosphatidylinositol 3,4,5-triphosphate as substrate.

The inhibition of PTEN by the bisperoxovanadium compounds was selective as the inhibition of PTP-β and PTP-1B by these compounds occurred with an IC\textsubscript{50} value that was at least 2 log units higher. The subgroup of bisperoxovanadium compounds possessing neutral _N,N_ ligands also inhibited PTEN with low nanomolar IC\textsubscript{50} values. The compound potassium bisperoxo(bipyridine)oxovanadate (bpV(bipy)) inhibited PTEN with
IC$_{50}$ = 18 nM while the compound potassium bisperoxo(1,10-phenanthroline)oxovanadate (bpV(phen)) inhibited PTEN with IC$_{50}$ = 38 nM. However, the selectivity of these two compounds for inhibition of PTEN was much lower when compared to inhibition of PTP-β and PTP-1B.

In cells expressing PTEN, the bisperoxovanadate compounds increased phosphorylation of Akt in a dose-dependent manner, while in PTEN-null cells the bisperoxovanadate compounds had no effect. This suggests that the compounds inhibited PTEN in cells, leading to a cellular increase in phosphatidylinositol 3,4,5-triphosphate, which subsequently resulted in increased Akt phosphorylation (Schmid, Byrne et al. 2004). Further exploration of vanadate complexes as potential inhibitors of PTEN revealed a 3-hydroxypicolinate vanadium (IV) complex as a potent inhibitor of PTEN (Rosivatz, Matthews et al. 2006). This compound inhibited PTEN with an IC$_{50}$ value of 35 nM in vitro and was selective for inhibition of PTEN as the IC$_{50}$ values for inhibition of other phosphatases including myotubularin, was at least 2 log orders of magnitude higher. In cells, this compound not only induced a dose-dependent increase in Akt phosphorylation and induced translocation of phosphorylated Akt to the plasma membrane and the perinuclear region, but also induced a substantial increase in the cytoplasmic levels of PI(3,4,5)P3 (Rosivatz, Matthews et al. 2006).
More recently, the naphthoquinone derivative compound, shikonin, which is isolated from the dried root of the plant *Lithospermum erythrorhizon* has also been shown to be an inhibitor of PTEN (Nigorikawa, Yoshikawa et al. 2006). *In vitro* shikonin inhibited recombinant PTEN with IC$_{50}$ = 2.7 μM using PI(3,4,5)P3 as substrate (Nigorikawa, Yoshikawa et al. 2006), and in cells shikonin treatment resulted in the accumulation of PI(3,4,5)P3 and activation of Akt (Nigorikawa, Yoshikawa et al. 2006). However, this effect was not selective as shikonin also significantly inhibited recombinant PTP1B with an IC$_{50}$ = 17 μM. This is perhaps not surprising as naphthoquinone derivatives have also been shown to inhibit the MKPs with IC$_{50}$ values lower than 1 μM (Johnston, Foster et al. 2007), thus suggesting that this pharmacophore may not be particularly selective for PTEN.

To date, the bisperoxovanadate compounds are amongst the most potent and selective inhibitors of PTEN. Indeed, as the IC$_{50}$ for their inhibition of PTEN is in the low nanomolar range, these compounds number amongst the most potent inhibitors identified for any dual specificity PTP to date. Their effectiveness in inhibition of PTEN can at least partially be attributed to their mimicry of the phosphate group of the PTEN substrate combined with their bulky structure which allows them to fit into the wide, shallow active site of PTEN while presumably excluding them from the more narrow active sites of other PTPs (Rosivatz, Matthews et al. 2006).
A screen for new phosphatases based on the sequence of the catalytic motif of PTEN resulted in the discovery of the dual specificity phosphatase PTPMT1, Protein Tyrosine Phosphatase Localized to the Mitochondrion 1 (Merlot, Meili et al. 2003). Interestingly, PTPMT1 has been identified in several highly metabolic tissues including the liver and pancreas (Pagliarini, Wiley et al. 2005). In the β-cell of the pancreas, reduction of PTPMT1 protein levels resulted in an increase in cellular ATP levels which correlated with an increase in insulin secretion (Pagliarini, Wiley et al. 2005). As detailed in Chapters 2, 3 and 4 of this thesis, we have recently identified alexidine dihydrochloride, a dibiguanide compound, as a fairly selective, potentially non-active site-directed inhibitor of PTPMT1 with an IC\textsubscript{50} value of 1 μM. This inhibitor is expected to aid in further study of PTPMT1 and exploration of its potential role as a therapeutic target in the treatment of Type II diabetes.

Thus, in summary, the inhibitors of the dual specificity PTPs identified to date have almost all been competitive ones and thus target the active site. Since there is a high degree of homology between the catalytic motifs of the dual specificity PTPS, there is sometimes overlap in targeting of compounds to specific subfamilies of these enzymes. The most selective competitive inhibitors of the dual specificity PTPs have exploited features within or near to the active site, such as hydrophobic crevices which are fairly distinct to a particular subfamily of dual specificity PTP. One other potential
approach to identifying selective inhibitors of particular dual specificity PTPs may be to pursue the identification of non-active site-directed inhibitors. Since there is very low homology amongst the dual specificity PTPs outside of the active site, this approach may potentially yield more selective inhibitors for particular dual specificity PTP subfamilies.
Table 1: Inhibitors of the dual specificity protein tyrosine phosphatases

<table>
<thead>
<tr>
<th>Pharmacophore/Inhibitor Name</th>
<th>Inhibitor Structure</th>
<th>Dual Specificity PTP Subfamily/Protein</th>
<th>IC$_{50}$ (µM)</th>
<th>IC$_{50}$ detail$^1$</th>
<th>Inhibition Model$^2$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzophenanthridine/NSC357756</td>
<td><img src="image1" alt="Structure" /></td>
<td>MAPK phosphatase/ MKP3</td>
<td>8.0</td>
<td>v</td>
<td>C</td>
<td>Vogt, 2003</td>
</tr>
<tr>
<td>Adociaquinone/Adociaquinone B</td>
<td><img src="image2" alt="Structure" /></td>
<td>MAPK phosphatase/ MKP1 MKP3</td>
<td>1.1 1.53</td>
<td>v v</td>
<td>n/a</td>
<td>Cao 2009</td>
</tr>
<tr>
<td>Naphthoquinone/5,8-dihydroxy-naphthoquinone</td>
<td><img src="image3" alt="Structure" /></td>
<td>MAPK phosphatase/ MKP1 MKP3</td>
<td>9.37 6.9</td>
<td>v v</td>
<td>n/a</td>
<td>Cao 2009</td>
</tr>
</tbody>
</table>

$^1$ IC$_{50}$ detail: v: IC$_{50}$ measured in vitro, c: IC$_{50}$ measured in cells

$^2$ Inhibition Model: C: competitive inhibitor, N: non-competitive inhibitor, U: uncompetitive inhibitor, M: mixed model inhibitor
Table 1: Inhibitors of the dual specificity protein tyrosine phosphatases (continued)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Structure</th>
<th>MAPK phosphatase/ MKP1</th>
<th>IC₅₀ (μM)</th>
<th>pIC₅₀</th>
<th>Comment</th>
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<tr>
<td>Naphthoquinone/ 4-ethoxynaphthalene-1,2-dione</td>
<td><img src="image" alt="Structure" /></td>
<td>MAPK phosphatase/ MKP1 MKP3</td>
<td>0.82 1.35</td>
<td>v v</td>
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<tr>
<td>Benzofuran/ NU-126</td>
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<td>MAPK phosphatase/ MKP1</td>
<td>28.8</td>
<td>v</td>
<td>n/a</td>
</tr>
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</table>
Table 1: Inhibitors of the dual specificity protein tyrosine phosphatases (continued)

<table>
<thead>
<tr>
<th>Inhibitor Description</th>
<th>Chemical Structure</th>
<th>Target(s)</th>
<th>IC50 (μM)</th>
<th>Selectivity Factor</th>
<th>Source</th>
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<td>47.6</td>
<td>v</td>
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<tr>
<td>Pyrroldin-naphthyridine/ Pubchem SID 3712249</td>
<td><img src="image" alt="Pyrroldin-naphthyridine" /></td>
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<td>0.136</td>
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<td>v</td>
<td>Johnston 2007</td>
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<td>v</td>
<td>Johnston 2007</td>
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<tr>
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<td><img src="image" alt="Pyrimido-triazinedione" /></td>
<td>MAPK phosphatase/MKP1</td>
<td>0.2</td>
<td>v</td>
<td>Johnston 2007</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>MAPK phosphatase/MKP1</td>
<td>IC50</td>
<td>v</td>
<td>n/a</td>
<td>Source</td>
</tr>
<tr>
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<td>Compound</td>
<td>Structure</td>
<td>MAPK phosphatase/ MKP1</td>
<td>IC50</td>
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</tr>
<tr>
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**Table 1: Inhibitors of the dual specificity protein tyrosine phosphatases (continued)**
<table>
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<tr>
<th>Inhibitor</th>
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<th>IC50</th>
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<td>Inhibitor</td>
<td>Activity</td>
<td>IC₅₀ (μM)</td>
<td>Grade</td>
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<td>Table 1: Inhibitors of the dual specificity protein tyrosine phosphatases (continued)</td>
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<td>Target</td>
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<td>D</td>
<td>C</td>
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<td>Quinolyn-oxosulfonium/NCS87877</td>
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Table 1: Inhibitors of the dual specificity protein tyrosine phosphatases (continued)

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<th>Inhibitor</th>
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<th>PRLs/ PRL</th>
<th>IC50 (μM)</th>
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<td>PRLs/ PRL3</td>
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<td>v</td>
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<tr>
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<td>IC50</td>
<td>v</td>
<td>n/a</td>
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</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
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<td>--------------</td>
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<tr>
<td>Bisperoxovanadium/dipotassium bisperoxo (5-hydroxypyridine-2-carboxyl)oxovanadate</td>
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<td>v</td>
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<td>Bisperoxovanadium/Dipotassium bisperoxo(picolinato)oxovanadate</td>
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<td>Mode</td>
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<td>3-hydroxypicolinate vanadium/</td>
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<td>Dibiguanide/ Alexidine dihydrochloride</td>
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<td>1</td>
<td>v</td>
<td>U</td>
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2 Screening for an inhibitor of the dual specificity PTP, PTPMT1

2.1 Introduction

PTPMT1, Protein Tyrosine Phosphatase Localized to the Mitochondrion 1, was discovered in 2003, following a bioinformatics screen using the sequence of the catalytic motif of PTEN to search for potential dual specificity phosphatases which bound phosphoinositides (Merlot, Meili et al. 2003). The crystal structure of PTEN, published 4 years earlier (Lee, Yang et al. 1999), revealed that the selectivity of PTEN for its substrate phosphatidylinositol (3,4,5) tri-phosphate was partially due to the wide shallow active site of PTEN and the occurrence of two basic lysine residues within the catalytic motif of the phosphate binding loop (HCKAGKGR). These basic residues result in a positively charged active site which facilitates binding of phosphoinositides. It was postulated that other dual specificity phosphatases possessing basic residues in similar positions in the catalytic motif would also potentially utilize phosphoinositides as substrate. Thus, PTPMT1, which possessed the sequence HCKAGRGRS in its catalytic motif, was discovered, and subsequently evaluated as a potential phosphoinositide phosphatase.

Experiments revealed that PTPMT1 was indeed able to utilize various phosphoinositides as substrates and that it exhibited a preference for phosphatidyl inositol 5-phosphate (Merlot, Meili et al. 2003). It was initially thought that PTPMT1 was
not a protein phosphatase as its catalytic activity towards phosphorylated proteins, such as myelin basic protein and casein was extremely low (Pagliarini, Worby et al. 2004). Moreover, the specific activity of PTPMT1 towards PI(5)P was approximately 2 log units higher compared to phosphorylated protein and 1 log unit higher compared to other phosphoinositides (Pagliarini, Worby et al. 2004). In addition, PTPMT1 showed higher activity towards phosphotidylinositol 5-phosphate species possessing longer acyl carbon chains as it demonstrated 1 log unit higher activity towards PI(5)P possessing an 8-carbon acyl chain compared to PI(5)P possessing a 4-carbon acyl chain (Pagliarini, Worby et al. 2004). However, in spite of the similarity of the sequence of the catalytic motif of the active site of PTPMT1 to that of PTEN and the in vitro evidence that PTPMT1 utilizes PI(5)P as a substrate, no demonstration of its utilization of phosphatidylinositol as a substrate in vivo has yet been found. Thus, the possibility that under some circumstances, PTPMT1 might dephosphorylate protein in vivo cannot be ruled out.

Investigation of the role of PTPMT1 in the pancreatic β-cell, revealed that knockdown of protein levels in the cell using RNA interference, resulted in an increase in cellular ATP levels that was accompanied by an increase in insulin secretion (Pagliarini, Wiley et al. 2005). However, there was no evidence of any change in PI(5)P levels in the cell (Pagliarini, Wiley et al. 2005). While pointing to the potential importance of PTPMT1 in the pancreatic β-cell, these experiments also pointed to the dearth of
knowledge regarding the function of PTPMT1 that still existed, and called into question whether PI(5)P served as a substrate of PTPMT1 in the pancreatic β-cell. This suggested that identification of an inhibitor of PTPMT1 might not only aid in further study of the enzyme, but, given the positive effect of reduction of PTPMT1 levels on insulin secretion, might support the role of PTPMT1 as a potential target in the treatment of Type II diabetes. There is good precedence for the use of small molecule inhibitors of phosphatases in the interrogation of the biology of these enzymes and selective inhibitors of phosphatases may well prove valuable in the treatment of diseases impacted by their dysregulation (Lai, Bao et al. 2009). Hence, we elected to perform a screen of diverse chemical structures as an approach towards identifying a selective inhibitor of PTPMT1.
2.2 Methods

2.2.1 PTPMT1 phosphatase assays

Assays were carried out using recombinant, murine, GST/His$_6$-tagged PTPMT1 which was expressed and purified as previously described (Pagliarini, Worby et al. 2004). A three component buffer consisting of 0.1 M sodium acetate, 0.05 M bis Tris, 0.05 M Tris was used in all PTPMT1 phosphatase assays. Assays designed to verify the optimal pH and Mg$^{2+}$ dependency of PTPMT1, utilized the three component assay buffer at a range of pH from 5.5 to 9.0 supplemented with 1 mM Mg$^{2+}$. Product formation was monitored continuously either by fluorescence emission at 520 nm or absorbance at 477 nm, with initial velocity readings being used for the analysis.

2.2.2 Screening of the chemical library

The Prestwick library of small molecules (Prestwick Chemical Inc., Washington, DC) was screened in vitro using a 384-well plate format with a Beckman Biomek FX robot under conditions where the Z-factor, a measure of the quality of a particular high throughput assay (Zhang, Chung et al. 1999), was greater than 0.5. Briefly, the enzyme assay was carried out in a total reaction volume of 40 µl using a three component buffer consisting of 0.1 M sodium acetate, 0.05 M bis Tris, 0.05 M Tris at pH 5.5, 50 µM O-MFP substrate, 50 µM test compound in 5% DMSO and 44 nM PTPMT1. The reaction was initiated by addition of enzyme, allowed to proceed for 40 min at room temperature
then quenched with 40 μl of 0.4 M NaOH. Product formation was determined by reading fluorescence emission at 520 nm.

The ten compounds showing the most potent inhibition of PTPMT1 were then subjected to a second round of screening using both 50 μM and 5 μM compound concentration. In these assays, product formation was monitored by both fluorescence emission at 520 nm and absorbance at 477 nm in order to ensure that decreased fluorescence indicated decreased product formation and hence true inhibition of PTPMT1 rather than quenching of the fluorescence signal.
2.3 Results

2.3.1 Characterization of $K_m$, optimal pH, and $\text{Mg}^{2+}$ dependency of PTPMT1

Our first order of business in developing a protocol for a screen for inhibitors of PTPMT1 was to settle on an appropriate substrate. The reported $K_m$ of PTPMT1 with pNPP, an artificial phosphatase substrate, is about 6 mM (Pagliarini, Worby et al. 2004). Since PTPMT1 possessed such low activity towards pNPP, we explored using another small molecule phosphatase substrate, 3-$O$-methylfluorescein phosphate ($O$-MFP), in our in vitro screen for an inhibitor. Under these conditions, we found that the $K_m$ of PTPMT1 with $O$-MFP was approximately 27.5 μM at pH = 5.5 and 39 μM at pH = 7.0, Figure 2. This was comparable to the reported $K_m$ of 37.5 μM at pH = 5.5 using PI(5)P as a substrate (Pagliarini, Worby et al. 2004).

The previously reported optimal pH for PTPMT1 was 5.5, and the enzyme has been reported to show no $\text{Mg}^{2+}$ dependency (Pagliarini, Worby et al. 2004). Since an acidic pH optimum would be unusual for a mitochondrion-localized enzyme, we decided to verify the pH at which optimal activity for PTPMT1 was obtained using the substrate $O$-MFP, and also reassess its $\text{Mg}^{2+}$ dependence. Using $O$-MFP as a substrate, the optimal pH for activity of PTPMT1, appeared to be 7.0, with this pH representing less than a log unit increase in activity compared to activity at pH = 5.5. There was no significant difference in the activity of PTPMT1 in the presence of 1 mM $\text{Mg}^{2+}$. Figure 2.
Figure 2: $K_m$ and optimum pH for PTPMT1 phosphatase activity

(A) $K_m$ at pH = 5.5 using absorbance-based assay. $K_m = 27.5 \pm 2.8$ μM. (B) $K_m$ at pH = 7.0 using fluorescence-based assay. $K_m = 38.9 \pm 10.8$ μM. (C) pH and Mg$^{2+}$ dependence of phosphatase activity. Optimum pH = 7.0. PTPMT1 activity is not Mg$^{2+}$-dependent. All assays used O-MFP as substrate.
2.3.2 Identification of lead inhibitors of PTPMT1

We chose O-MFP as the enzyme substrate in the screen for an inhibitor because PTPMT1 showed good activity using O-MFP as a substrate, and it was amenable to both fluorescence- and absorbance-based experiments, thus facilitating development of a high-throughput assay. There was also good precedence for its use as a small molecule substrate in various protein tyrosine phosphatase assays (Gottlin, Xu et al. 1996; Johnston, Foster et al. 2007; Song, Park et al. 2009). We screened the Prestwick Chemical Library®, a commercially available library of approximately 1000 small molecules with previously characterized pharmacokinetic properties, since this would increase the likelihood of identifying an inhibitor with good bioavailability. In our initial screen, approximately 8% of the compounds reduced enzyme activity by more than 50% at 50 µM concentration. The Z-factors ranged from 0.57 to 0.85 indicating good quality of the data (Zhang, Chung et al. 1999). Subsequent rescreening of the most potent inhibitors revealed that less than 1% of the library compounds inhibited PTPMT1 by more than 50% at 5 µM concentration in both fluorescence-and absorbance-based assays.

The 10 most potent inhibitors identified in the screen were anthralin, ursolic acid, lynestrenol, oxytetracycline dehydrate, clofazimine, Chicago Sky Blue, gramicidin, chrysene 1, 4-quinone, gossypol and alexidine dihydrochloride. Figure 3. Amongst these
10 compounds, one was a polypeptide (gramicidin), 8 compounds contained saturated or unsaturated 6-carbon ring moieties (anthralin, ursolic acid, lynestrenol, oxytetracycline dehydrate, clofazimine, Chicago Sky Blue, chrysene 1, 4-quinone and gossypol), 4 compounds were bidentate (anthralin, Chicago Sky Blue, gossypol and alexidine dihydrochloride) and a single compound was aliphatic (alexidine dihydrochloride). Figure 4. We chose the 3 most potent inhibitors for follow up studies.

The three most potent inhibitors of PTPMT1, gossypol, Chicago Sky Blue and alexidine dihydrochloride, were all non-peptidic and demonstrated similar activity in both fluorescence- and absorbance-based assays. All 3 compounds exhibited IC$_{50}$ values in the low micromolar or high nanomolar range, with gossypol exhibiting IC$_{50}$ value $110 \pm 11$ nM, Chicago Sky Blue exhibiting IC$_{50}$ = 331 ± 64 nM and alexidine dihydrochloride exhibiting IC$_{50}$ = $1.08 \pm 0.08 \mu$M. Table 2, Figure 5. Interestingly, these three most potent inhibitors of PTPMT1 were all bi-dentate compounds. Figure 5. In addition, two of the three most potent compounds, gossypol and alexidine dihydrochloride, exhibited Hill coefficients close to 2. Table 2.
Figure 3: Comparison of the activity of the 10 most potent inhibitors of PTPMT1
Activity of the 10 compounds showing greatest inhibition of PTPMT1 at 5 μM concentration in the fluorescence-based assay using O-MFP as the substrate
Figure 4: Structures of the 10 most potent inhibitors of PTPMT1
Figure 5: Comparison of IC₅₀ curves and structures of the 3 lead inhibitors of PTPMT1

(A) Inhibition of PTPMT1 by gossypol, Chicago Sky Blue and alexidine dihydrochloride. Assays were carried out using O-MFP as the substrate (n=3). Data represent the mean ± SEM. (B) Structures of the 3 lead inhibitors of PTPMT1. All 3 leads are bidentate.
<table>
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<tr>
<th>Compound</th>
<th>$\text{IC}_{50}$ (μM)</th>
<th>Hill coefficient</th>
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<td>gossypol</td>
<td>$0.111 \pm 0.011$</td>
<td>$2.15 \pm 0.36$</td>
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<tr>
<td>Chicago Sky Blue</td>
<td>$0.331 \pm 0.064$</td>
<td>$1.5 \pm 0.3$</td>
</tr>
<tr>
<td>alexidine dihydrochloride</td>
<td>$1.08 \pm 0.08$</td>
<td>$2.16 \pm 0.31$</td>
</tr>
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2.4 Discussion

In the screen for an inhibitor of PTPMT1, a convenient substrate, amenable to high throughput screening was needed and thus the artificial phosphatase $O$-MFP was used. The $K_m$ of PTPMT1 using $O$-MFP as substrate was in close agreement with the $K_m$ obtained using an identified substrate of PTPMT1, PI(5)P. This suggested that $O$-MFP would be a suitable substrate for the in vitro screen for an inhibitor. However, the initial screen was carried out at pH 5.5 when pH = 7.0 was later found to be the optimal pH for PTPMT1 using $O$-MFP as a substrate, with pH 7.0 giving 7-fold higher activity compared to pH 5.5. The $K_m$ obtained using $O$-MFP as the substrate at pH = 5.5 was 27.5 μM, while at pH = 7.0 the $K_m$ obtained was 38.9 μM. Thus, although the screen for the inhibitor was carried out at a sub-optimal pH, the close agreement between the $K_m$ values at pH = 5.5, compared to pH = 7.0, suggests that that the main disadvantage of the lower pH was loss of signal-to-noise resolution. Moreover, the Z-factor scores for the screen were all above 0.5, indicating good quality of the data in the high throughput assay.

The 10 lead compounds which emerged from the initial inhibitor screen showed similar potency in both fluorescence- and absorbance-based assays, allowing us to verify that the effectiveness of inhibition of PTPMT1 by the compounds was not due to simple quenching of the fluorescence signal of the product. Examination of the 10 compounds revealed several interesting points. For example, 4 of the 10 compounds exhibited
bidentate structure, causing us to ponder whether duplication of moieties which bound
the protein aided in targeting of PTPMT1. In addition, 2 of the 3 lead compounds
exhibited Hill coefficients near 2, also suggesting a multivalent mode of binding.

In choosing a single compound for extensive study from amongst our 3 lead
compounds, I looked to reports in the scientific literature for information regarding the
3 leads. Initial investigation of the literature revealed that gossypol, the most potent of
the lead compounds, was reported to be highly toxic to cells as it inhibits Bcl-X(L) and
Bcl-2, thus inducing apoptosis (Zhang, Liu et al. 2003; Oliver, Miranda et al. 2005; Lei,
Chen et al. 2006). In one study, gossypol was reported to inhibit Bcl-X(L) \textit{in vitro} with IC$_{50}$
= 500 nM (Kitada, Leone et al. 2003). In addition, gossypol inhibits the activity of the
serine/threonine phosphatase, calcineurin, (Baumgrass, Weiwad et al. 2001; Carruthers,
Dowd et al. 2007). Thus, I was concerned not only that the inhibition of PTPMT1 by
gossypol might not be selective, but that the induction of apoptosis by gossypol might
preclude cellular studies of its inhibition of PTPMT1. Investigation of the literature
revealed that Chicago Sky Blue inhibited the serine/threonine phosphatase,
Ca$^{2+}$/calmodulin-dependent protein kinase phosphatase (CaMKP), with an IC$_{50}$ = 4.1 μM
(Sueyoshi, Takao et al. 2007). Although this value was a log unit higher than its inhibition
of PTPMT1, I was somewhat concerned that Chicago Sky Blue might be a general
phosphatase inhibitor, rather than selective for the dual specificity phosphatases.
The di-biguanide compound, alexidine dihydrochloride emerged as a leading candidate for further study as an inhibitor of PTPMT1. Firstly, alexidine dihydrochloride had not been previously reported to inhibit any other phosphatase. In addition, alexidine dihydrochloride had high potency, displaying an IC$_{50}$ value of approximately 1 μM. Alexidine dihydrochloride also possessed a unique structure amongst the 10 lead compounds; its lipophilic dicationic structure suggested that it might efficiently target the mitochondria (Ross, Da Ros et al. 2006) where PTPMT1 was known to be localized in cells. Thus, alexidine dihydrochloride emerged from the initial screen as our lead inhibitor of PTPMT1.
3 Kinetic characterization of alexidine dihydrochloride, an inhibitor of PTPMT1

3.1 Introduction

Having chosen alexidine dihydrochloride as the lead inhibitor of PTPMT1, our next order of business was to perform follow up experiments to determine the selectivity of the inhibition of the compound for PTPMT1, the mode of inhibition of the compound and the identity of the major pharmacophore responsible for inhibition. As mentioned previously, in general, the dual specificity PTPs all share a common sequence HCX₅RS in the catalytic motif of the phosphate-binding loop. As a result of this homology in the active site, as well as the wide, shallow shape of the active site of dual specificity PTPs and other factors, it is often difficult to identify potent inhibitors which show a high degree of selectivity for particular subfamilies of this class of enzyme (Bakan, Lazo et al. 2008). Indeed, given the fact that PTPMT1 was initially identified based on the similarity of the sequence of its catalytic motif to that of PTEN, it might be expected that identifying an active site-directed, selective inhibitor would be difficult.

To date, most of the inhibitors of the dual specificity phosphatases which have been identified have been competitive ones (see Chapter 1) and thus are active site-directed. Selectivity of inhibition of these compounds for particular subfamilies has been conferred by exploitation of unique binding interactions within the active site of the enzyme, and as such these have been tremendously aided, and proved most successful,
when a high resolution structure of the enzyme (preferably bound to substrate) has been available (Bakan, Lazo et al. 2008). This has allowed modeling of the fit of potential inhibitors of the dual specificity PTPs into the active site of the enzyme and thus the selection of compounds containing moieties most likely to have favorable binding interactions. One potential, alternative means of discovering selective inhibitors may be to identify non-active site-directed ones. Since the dual specificity PTPs often share very little sequence homology outside of the active site, non-active site-directed inhibitors may exploit these differences and thus achieve a fair degree of selectivity. Once a potent and selective inhibitor has been identified, it can then be further studied and the essential pharmacophores isolated. These pharmacophores can then typically be used as scaffolds in structure activity relationship studies in an effort to refine the basic inhibitor structure to produce more potent and effective inhibitors.
3.2 Methods

3.2.1 Kinetic experiments

Kinetic studies were carried out using an independent source of alexidine dihydrochloride (Toronto Research Chemicals). Chlorhexidine dihydrochloride, phenformin and metformin were purchased from Sigma-Aldrich. Half alexidine (N-(2-ethylhexyl)-N’-propylimidodicarbonimidic diamide) was synthesized by the Duke Center for Chemical Biology, with the purity of the final product being determined by mass spectrometry and NMR. IC$_{50}$ determinations for inhibition of PTPMT1 were carried out using 44 nM of recombinant enzyme (prepared in the laboratory of Jack Dixon at University of California, San Diego using a previously described protocol (Pagliarini, Worby et al. 2004)) in a total reaction volume of 100 μl in a three-component buffer consisting of 0.1 M sodium acetate, 0.05 M bis Tris and 0.05 M Tris. Since pH 7.0 had been determined to support optimal PTPMT1 phosphatase activity, all assays involving PTPMT1 were carried out at pH 7.0.

VHR phosphatase assays were carried out using 21.8 nM recombinant enzyme (provided by the laboratory of Jack Dixon at University of California, San Diego and prepared as previously described (Denu, Zhou et al. 1995)), in the three component acetate/Tris/Bis Tris buffer at pH 6.5, which was determined to support optimal VHR phosphatase activity (data not shown). λ protein phosphatase and T-cell PTP assays
were carried out in buffers provided by the supplier, using 44.8 nM and 7.2 nM recombinant enzyme (purchased from New England Biolabs) respectively.

All assays using PTPMT1, VHR, λ protein phosphatase and T-cell PTP were carried out using 50 μM O-MFP (Sigma-Aldrich) as substrate, and product formation was monitored continuously by fluorescence at 520 nm with initial velocity readings being used for the analysis. Kinetic characterization of the inhibition of PTPMT1 by alexidine dihydrochloride was carried out using 88 nM enzyme, in order to optimize signal-to-noise ratio, in a total reaction volume of 100 μl at pH 7.0 in the three-component acetate/Tris/bis Tris buffer described above.

PTEN assays were carried out as described previously (Maehama, Taylor et al. 2000) using 0.4 μM recombinant enzyme (prepared in the laboratory of Jack Dixon at University of California, San Diego using an established protocol (Maehama and Dixon 1998)), and DiC16-PI 3,4,5P$_3$ substrate (Echelon Biosciences Inc., Salt Lake City UT).

3.2.2 Kinetic modeling and analysis

Kinetic analysis and modeling were carried out using GraphPad Prism 5; IC$_{50}$ values were fit with variable slope and inhibition was modeled using the following equations:
Competitive inhibition

$$K_{m\text{Apparent}} = Km*(1+[I]/K_i), \quad Y = V_{\text{max}}*X/(K_{m\text{Apparent}}+X)$$

Non-competitive inhibition

$$V_{\text{max\text{Apparent}}} = V_{\text{max}}/(1+I/K_i), \quad Y = V_{\text{max\text{Apparent}}}*X/(K_m+X)$$

Uncompetitive inhibition

$$V_{\text{max\text{Apparent}}} = V_{\text{max}}/(1+I/\alpha K_i), \quad K_{m\text{Apparent}} = K_m/(1+I/\alpha K_i)$$

$$Y = V_{\text{max\text{Apparent}}}*X/(K_{m\text{Apparent}}+X)$$

Mixed model inhibition

$$V_{\text{max\text{Apparent}}} = V_{\text{max}}/(1+I/(\alpha*K_i)), \quad K_{m\text{Apparent}} = K_m*(1+I/K_i)/(1+I/(\alpha*K_i))$$

$$Y = V_{\text{max\text{Apparent}}}*X/(K_{m\text{Apparent}}+X)$$

$$\alpha = 1$$ indicates non-competitive inhibition,

$$\alpha >> 1$$ indicates predominantly competitive inhibition, and

$$0 < \alpha <<1$$ indicates predominantly uncompetitive inhibition
3.3 Results

3.3.1 Selectivity of inhibition of PTPMT1 by alexidine dihydrochloride

Initial kinetic analysis of the capacity of alexidine dihydrochloride to inhibit PTPMT1 using O-MFP as a substrate, gave an IC\textsubscript{50} value of 1.08 ± 0.08 µM. In order to validate these results, we also evaluated the ability of alexidine dihydrochloride to inhibit PTPMT1 using phosphatidylinositol 5-phosphate (PI5P), a potential biological substrate of the enzyme. Encouragingly, the IC\textsubscript{50} obtained using O-MFP as a substrate was in very close agreement with the IC\textsubscript{50} obtained using PI5P (unpublished observations of Ji Zhang and Jack Dixon - University of California, San Diego). This supports the assignment of alexidine dihydrochloride as a bonafide inhibitor of PTPMT1, as well as the applicability of O-MFP as an appropriate substrate for these inhibition studies.

Having established alexidine dihydrochloride as an inhibitor of PTPMT1, we then investigated whether the inhibition was selective. Alexidine dihydrochloride proved to be a fairly selective inhibitor of PTPMT1 as, in assays using O-MFP as a substrate, it did not significantly inhibit a variety of other phosphatases. These phosphatases included the serine/threonine protein phosphatase, lambda protein phosphatase; the classical, tyrosine-specific PTP, T-cell protein tyrosine phosphatase; the dual-specificity PTP, VH1-related phosphatase and the phosphoinositol PTP, PTEN. Figure 6. The latter was particularly noteworthy since the sequence of the catalytic motif of the active site of
PTEN formed the basis for the bioinformatics screen which led to the discovery of PTPMT1, and the two enzymes differ in this sequence by just two amino acids (Merlot, Meili et al. 2003).
Figure 6: Selective inhibition of PTPMT1 by the dibiguanide alexidine dihydrochloride

(A) Structure of alexidine dihydrochloride (B) Inhibition of various phosphatases by alexidine dihydrochloride. PTPMT1, VHR phosphatase, λ-PPase and T-Cell PTPase assays were carried out using O-MFP as the substrate (n=3) while PTEN assays were carried out using lipid substrate (n=2). Data are presented as the means ± SEM of independent experiments (C) Comparison of the sequences of the catalytic motif of the protein tyrosine phosphatases assayed. Boxed residues are those conserved within the catalytic motif. Bold represents basic residues, bold underlined represent hydroxyl, amine and basic residues excluding Q, bold lower case represents small, hydrophobic and aromatic excluding Y.
3.3.2 Kinetic characterization of the inhibition of PTPMT1 by alexidine dihydrochloride

Following the preliminary studies which established the degree of potency and selectivity of alexidine dihydrochloride as an inhibitor of PTPMT1, we next sought to identify the kinetic model of this inhibition. Incubation of PTPMT1 with alexidine dihydrochloride resulted in reductions in both $V_{\text{max}}$ and $K_m$ with increasing inhibitor concentration. Table 3. This suggested that alexidine dihydrochloride did not inhibit PTPMT1 in a competitive nor a non-competitive manner. In addition, fitting of the enzyme’s activity in the presence of varying concentrations of the inhibitor to models for competitive, non-competitive, uncompetitive and mixed inhibition revealed that the data fit the uncompetitive and mixed inhibition models best, giving $R^2$ values of 0.9244 and 0.9275 respectively. Table 4.

We also compared the fit of the data to the models of competitive, non-competitive and uncompetitive inhibition using Akaike's Information Criterion. This comparison revealed that the model for uncompetitive inhibition fit the data best with a 99.99% probability that it was correct compared to the competitive and non-competitive models. In addition, the model for mixed inhibition, which is a general one incorporating competitive, non-competitive and uncompetitive inhibition, gave a value of $\alpha = 0.097 \pm 0.071$ indicating significant uncompetitive character (GraphPad Prism 5).
Figure 7: Characterization of inhibition of PTPMT1 by alexidine dihydrochloride

(A) PTPMT1 activity in the presence of varying concentrations of alexidine dihydrochloride using O-MFP as substrate. Alexidine dihydrochloride reduced both V<sub>max</sub> and K<sub>m</sub>. Data represent means ± SEM of 3 independent experiments.

(B) Lineweaver – Burk plot of the data presented in (A)
Table 3: Impact of alexidine dihydrochloride on the kinetics of PTPMT1

<table>
<thead>
<tr>
<th>[alexidine] (μM)</th>
<th>Apparent $V_{\text{max}}$ (Δ Fluor min$^{-1}$)</th>
<th>Apparent $K_m$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5087</td>
<td>79</td>
</tr>
<tr>
<td>2</td>
<td>1979</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>904</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>587</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>478</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 4: Comparison of the fit of the major kinetic models to inhibition of PTPMT1 by alexidine dihydrochloride

<table>
<thead>
<tr>
<th>Model of Inhibition</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Competitive</td>
<td>0.8525</td>
</tr>
<tr>
<td>Non-competitive</td>
<td>0.9094</td>
</tr>
<tr>
<td>Uncompetitive</td>
<td>0.9244</td>
</tr>
<tr>
<td>Mixed</td>
<td>0.9275</td>
</tr>
</tbody>
</table>

3.3.3 Importance of the di-biguanide pharmacophore of alexidine dihydrochloride

The effectiveness of the inhibition of PTPMT1, demonstrated by alexidine dihydrochloride, led us to ponder which structural features of the compound might contribute to this inhibition. The striking duplication inherent in the structure of alexidine dihydrochloride prompted us to investigate whether this duplication contributed to the ability of the compound to inhibit PTPMT1 and whether other biguanide and di-biguanide compounds also significantly inhibited PTPMT1. Interestingly, we found that the di-biguanide compound chlorhexidine dihydrochloride significantly inhibited PTPMT1, albeit at a log-fold higher concentration compared to
alexidine dihydrochloride. Chlorhexidine dihydrochloride inhibited PTPMT1 with an IC$_{50}$ of 19.7 ± 3.3 µM with the substrate O-MFP. In contrast, a mono-biguanide compound comprising half of the alexidine dihydrochloride structure only inhibited PTPMT1 very weakly with IC$_{50}$ = 207.1 ± 43.1 µM. Table 5, Figure 8. This represented a potency of inhibition two log orders of magnitude lower than the inhibition demonstrated by the full-length alexidine dihydrochloride molecule. In addition, two other mono-biguanide compounds, the Type II diabetes drugs, metformin and phenformin, did not significantly inhibit PTPMT1. Thus, the dibiguanide pharmacophore conferred a significantly higher level of potency of inhibition of PTPMT1 compared to the monobiguanide structure.

Interestingly, the Hill coefficient for the inhibition of PTPMT1 by alexidine dihydrochloride was 2.16 ± 0.3, while that for inhibition by the half alexidine molecule was 0.7 ± 0.1. This suggests that the dibiguanide structure of alexidine dihydrochloride is also responsible for the Hill coefficient near 2 which is observed upon inhibition of PTPMT1 by alexidine dihydrochloride.
Figure 8: Importance of the dibiguanide pharmacophore in inhibition of PTPMT1

(A) Inhibition of PTPMT1 by various biguanide and di-biguanide compounds. Data represent the mean ± SEM of 3 independent experiments. (B) Structures of dibiguanide and biguanide compounds tested for inhibition of PTPMT1.
Table 5: Comparison of the inhibition of PTPMT1 by various biguanide compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>alexidine dihydrochloride</td>
<td>1.08 ± 0.8</td>
<td>2.16 ± 0.3</td>
</tr>
<tr>
<td>chlorhexidine dihydrochloride</td>
<td>19.7 ± 3.3</td>
<td>1.28 ± 0.3</td>
</tr>
<tr>
<td>half alexidine</td>
<td>207.1 ± 43.1</td>
<td>0.70 ± 0.1</td>
</tr>
<tr>
<td>metformin</td>
<td>&gt; 1000</td>
<td>n/a</td>
</tr>
<tr>
<td>phenformin</td>
<td>&gt; 1000</td>
<td>n/a</td>
</tr>
</tbody>
</table>
3.4 Discussion

In our screen to identify a pharmacological inhibitor of PTPMT1, alexidine dihydrochloride emerged as an effective and fairly selective inhibitor of the enzyme. Subsequent studies to characterize the inhibition of PTPMT1 by alexidine dihydrochloride revealed several important points. Firstly, alexidine dihydrochloride did not inhibit the dual specificity phosphatase PTEN which utilizes 3-phosphoinositides as substrates. Since the sequence of the catalytic P-loop of PTEN formed the basis of the screen which led to the discovery of PTPMT1 (Merlot, Meili et al. 2003), the two enzymes share very close homology in the catalytic motif and in fact PTPMT1 has also demonstrated dephosphorylation of phosphoinositides in vitro. Thus, the lack of inhibition of PTEN by alexidine dihydrochloride suggests that the compound most likely exploits features that are unique to PTPMT1 compared to other dual specificity protein tyrosine phosphatases. It also implies that the compound might not bind the catalytic motif of the enzyme or at minimum does not exploit features within the catalytic motif which enable PTPMT1’s phosphatidylinositol phosphatase activity in vitro. Further evidence in support of this hypothesis comes from the finding that inhibition of PTPMT1 by alexidine dihydrochloride did not seem to be competitive in nature.

Our modeling of the inhibition of PTPMT1 by alexidine dihydrochloride revealed that the inhibition was predominantly uncompetitive in nature, implying that alexidine
dihydrochloride likely targets the enzyme-substrate complex and may not bind within the active site of the enzyme. Furthermore, in the study characterizing the IC$_{50}$ for inhibition of PTPMT1 by alexidine, we noted a Hill coefficient of 2, suggesting co-operativity of binding of the inhibitor to the enzyme. This co-operativity might be achieved where binding of the inhibitor one site facilitates binding to a second site on the same molecule (Krstic, Krinulovic et al. 2004) or where the enzyme functions as dimer and binding of one enzyme molecule facilitates binding of the second. Considering these observations together with alexidine dihydrochloride’s striking bidentate structure, we have speculated whether the compound might work by binding two similar sites on a single enzyme molecule or single sites on each of two molecules of an enzyme dimer which are available in close proximity following substrate binding. However, in the absence of a structure for PTPMT1 this is would be very difficult to determine.

Following up on the idea that alexidine dihydrochloride might bind PTPMT1 in a distinctive manner due to its bidentate structure, we investigated whether the di-biguanide structure of the compound played an important part in its ability to inhibit the enzyme. Encouragingly, previous reports have demonstrated that multi-dentate compounds which bind two sites of the enzyme at the same time are more potent and selective protein tyrosine phosphatase inhibitors (Yu, Sun et al. 2007; Zhang and Zhang
2007; Fang, Zhang et al. 2008). We found that although the half alexidine molecule inhibited PTPMT1, it was with an IC_{50} 200 times greater than the full length molecule and a Hill coefficient which approached 1. This indicates significantly lower potency and suggests a lack of co-operativity of binding. Based on our modeling of alexidine dihydrochloride’s inhibition of PTPMT1 as predominantly uncompetitive in nature, we suspect that the compound binds to the enzyme at two locations which are both outside of the active site. We have further speculated that these two locations may not reside on a single enzyme molecule but may comprise similar sites on an enzyme dimer.

The existence of a PTPMT1 dimer would not be implausible as, as detailed in Chapter 1 of this dissertation, several dual specificity protein tyrosine phosphatases, including the myotubularins, VH1 and MKP-3 have been recently revealed to exist as dimers, and in the case of PRL-1, as a trimer. This suggests that multidentate compounds might be more effective at inhibiting such enzymes. Indeed, as detailed in Chapter 1 and Table 1 of this dissertation, several inhibitors of dual specificity phosphatases, including the PRL inhibitor pentamidine; the MKP inhibitors NSC357756, PubchemSID 3712249 and sanguinarine; the VHR inhibitor, the RK-682 dimer and the DUSP26 inhibitor, NCS878777, have exhibited somewhat bidentate structure, although the link between the multidentate structure of these inhibitors and the existence of
their target dual specificity protein tyrosine phosphatases as dimers or trimers has not
generally been remarked upon.

In summary, our experiments strongly suggest that dibiguanide compounds are
more potent inhibitors of PTPMT1 compared to (mono-)biguanide compounds. We
further postulate that this is most likely a result of some co-operativity of binding of the
dibiguanide to PTPMT1 compared to the mono-biguanide which probably also confers
some degree of selectivity.
4 Evaluation of the impact of alexidine dihydrochloride on PTPMT1 in cells

4.1 Introduction

PTPMT1 enjoys the distinction of being amongst the first phosphatases found to primarily localize to mitochondria, where it resides on the inner membrane facing the mitochondrial matrix (Pagliarini, Wiley et al. 2005). PTPMT1 has been identified in the mitochondria of several highly metabolic tissues including the liver and pancreas. In the \( \beta \)-cell of the pancreas; the sole insulin producing cell in the body, knockdown of PTPMT1 protein levels using RNA interference resulted in changes in the phosphoprotein profile as well as an increase in cellular ATP levels. Importantly, the increase in cellular ATP levels in the pancreatic \( \beta \)-cell correlated with an increase in insulin secretion (Pagliarini, Wiley et al. 2005).

While the localization PTPMT1 to the mitochondrion and its impact on insulin secretion pointed to a potential role in \( \beta \)-cell metabolism, further interrogation of the biology of PTPMT1 was somewhat limited by the paucity of tools available to target the enzyme, particularly during short term studies. Indeed, even the endogenous substrates of PTPMT1 are still being investigated since, in spite of the similarity of its catalytic motif to that of PTEN and its ability to utilize phospholipid substrates \textit{in vitro} (Pagliarini, Worby et al. 2004), evidence of its action on phosphoinositides \textit{in vivo} remains to be demonstrated. Thus, in order to facilitate further study of PTPMT1, and its role in \( \beta \)-cell
metabolism in particular, we pursued a search for inhibitors of the enzyme, and subsequently identified alexidine dihydrochloride as an inhibitor of PTPMT1 in vitro.

Since reduction of PTPMT1 protein levels in the pancreatic β-cell, using RNA interference, results in increased insulin secretion and changes in the mitochondrial phosphoprotein profile (Pagliarini, Wiley et al. 2005), it is expected that an effective, bioavailable, chemical inhibitor of PTPMT1 would mimic these effects. For such a chemical inhibitor of PTPMT1 to be useful, it would have to be able to access the enzyme in its sub-cellular location on the inner mitochondrial membrane and show selectivity of inhibition of PTPMT1 in cells with minimal off-target effects. Thus, in order to complete our studies of alexidine dihydrochloride as an inhibitor of PTPMT1, it was necessary to evaluate its efficacy in cells.

We also felt that our in vitro kinetic studies of the inhibition of PTPMT1 by alexidine dihydrochloride had provided some clues regarding a possible aspect of the biology of PTPMT1. The bidentate structure of alexidine dihydrochloride, combined with the Hill coefficient of 2 which accompanied the IC$_{50}$ curve describing the inhibition of PTPMT1 by alexidine dihydrochloride, as well as the high potency of inhibition of PTPMT1 by the full length alexidine dihydrochloride molecule compared to the half alexidine molecule, all suggested that alexidine dihydrochloride might be inhibiting a dimeric form of PTPMT1 in vitro. Since several dual specificity protein tyrosine
phosphatases have recently been demonstrated to exist as dimers, we believed that investigation into the possible existence of dimers of PTPMT1 in cells might be warranted.
4.2 Methods

4.2.1 Tissue culture, insulin secretion and cytotoxicity assays

INS-1 cells were cultured in RPMI 1640 medium containing 8 mM glucose, 2 mM glutamine, 10 mM HEPES and 1 mM sodium pyruvate. Islets were harvested from Wistar rats weighing approximately 250 g under a protocol approved by the Duke University Animal Care and Use Committee. Rat islets were isolated by collagenase digestion of the pancreas followed by separation on a density gradient as previously described (Milburn, Hirose et al. 1995) and maintained in RPMI 1640 with 8 mM glucose supplemented with 10 % fetal bovine serum, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 20 units/ml penicillin, 20 µg/ml streptomycin and 0.05 µg/ml amphotericin B).

Islets were used in static glucose-stimulated insulin secretion assays as previously described (Joseph, Jensen et al. 2006), with some modifications. In summary, equal numbers of islets of similar size were plated in 12-well tissue culture plates in a modified Krebs-Ringer phosphate buffer (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.16 mM MgSO$_4$, 20 mM HEPES pH 7.2, 25 mM NaHCO$_3$, 0.25 M CaCl$_2$, 0.2 % bovine serum albumin) containing 2.8 mM glucose, washed for a 1 hr at 37°C in fresh buffer containing 2.8 mM glucose, then successively incubated for 1 hr intervals at 37°C in 500 µl of buffer per well in 24-well plates, first in buffer containing 2.8 mM glucose, then in buffer containing 2.8 mM glucose and drug, and finally in buffer containing 16.7 mM glucose and drug. Secreted insulin was assessed from assay buffer following islet
incubations, while islet insulin content was assessed from islet lysates. The amount of insulin present was determined via radioimmunoassay (DPC/Siemens Healthcare Diagnostics). For dose response experiments using alexidine dihydrochloride, insulin secretion assays were carried out on the day following islet isolation using 30 islets in triplicate for each drug concentration. For assays requiring the use of recombinant adenovirus, pools of approximately 100 rat islets were infected with \(2.3 \times 10^8\) infectious units of adenovirus on the day of islet isolation, adenovirus was removed about 15 hr later and insulin secretion assays were performed 72 hr later using 20 islets in triplicate for each condition.

Cell viability/drug cytotoxicity was determined using a cell membrane integrity-based cytotoxicity assay; the Toxilight BioAssay (Lonza), which utilizes leakage of the cytoplasmic protein, adenylate kinase across damaged cell membranes into surrounding medium as a measure of reduced cell membrane integrity and thus increased cytotoxicity. In summary, leakage of adenylate kinase from islets into surrounding medium was assessed during insulin secretion assays following the manufacturer’s instructions. Islets treated with 0.2% Triton X-100 instead of alexidine dihydrochloride during the insulin secretion assay, to induce cell lysis, served as the 100% cytotoxicity reference.
Recombinant adenovirus containing control shRNA or shRNA targeted against PTPMT1 was obtained from the laboratory of Jack Dixon at University of California, San Diego (Pagliarini, Wiley et al. 2005). Recombinant adenovirus for the overexpression of wildtype PTPMT1 was produced by subcloning murine, c-terminal v5-His-tagged, wildtype PTPMT1 from a pcDNA3.1D vector (Pagliarini, Wiley et al. 2005), obtained from the laboratory of Jack Dixon at University of California, San Diego, into the pAdTrack-CMV vector. The resulting plasmid was allowed to undergo homologous recombination with the AdEasy-1 plasmid in AdEasier-1, BJ5183-AD-1 competent cells (Stratagene) to produce the recombinant adenoviral plasmid.

4.2.2 Analysis of mitochondrial protein phosphorylation

Approximately $2.5 \times 10^6$ INS-1 cells were plated in 10 cm plates with 3 plates being used for each of the 3 conditions tested. Following 24 hr incubation, cells were infected with $2.3 \times 10^8$ infectious units of adenovirus coding for control shRNA or PTPMT1-targeted shRNA, or were left uninfected. After an additional 48 hr the cells were washed in phosphate-buffered saline without magnesium and calcium, then incubated in the modified Krebs-Ringer buffer utilized in insulin secretion assays (see above) containing 2.8 mM glucose for 1 hr, following which, the buffer was replaced; uninfected cells received buffer containing 4 μM alexidine dihydrochloride. After an additional 2 hr incubation, the cells were washed with phosphate-buffered saline and
mitochondria-enriched cell lysate was isolated as previously described (Ronnebaum, Ilkayeva et al. 2006). In summary, the cells were harvested by scraping in phosphate-buffered saline followed by centrifugation at 500 x g, the pellet was resuspended in ice-cold buffer containing 220 mM mannitol, 70 mM sucrose, 5 mM HEPES, homogenized in a 2 ml Dounce homogenizer and the homogenate centrifuged at 500 x g. The supernatant was then centrifuged at 16,000 x g to obtain the mitochondria-enriched pellet. The mitochondria-enriched pellet was resuspended in a modified NP-40 cell lysis buffer (50 mM Tris pH 7.7, 150 mM NaCl, 1% igepal, 10% glycerol, 2 mM vanidate, 10 mM NaF, 2.5 mM MgCl2 with protease inhibitors) and 100 μg of protein was resolved on a 4 – 20% tris-glycine gel (Invitrogen) and transferred to nitrocellulose for immunoblot analysis.

4.2.3 Protein crosslinking
Approximately 2.5 x 10^6 INS-1 cells were plated in 10 cm plates. Following 72 hr incubation, cells were infected with 2.3 x 10^8 infectious units of adenovirus coding for wildtype PTPMT1 (containing a v5 epitope at the c-terminus) or left uninfected. 10 hr later, cells were washed in phosphate-buffered saline without magnesium and calcium, then incubated for 2 hr in the modified Krebs-Ringer buffer utilized in insulin secretion assays (see above) containing 2.8 mM glucose. Mitochondria were then isolated as described above. Following isolation, mitochondria were suspended in 20 mM HEPES pH
= 7.2. For each crosslinking sample, 25 μg of mitochondria were lysed in a solution containing 1% igepal and 10 mM HEPES. The lysate was supplemented with 3.75 mM BS³ (Bis[sulfosuccinimidyl] suberate) crosslinking reagent (Pierce/Thermo Scientific) and the crosslinking reaction allowed to proceed at room temperature. At various time points the crosslinking reaction was terminated by the addition of 20 mM Tris. Mitochondrial protein was then resolved on a 14% tris-glycine gel (Invitrogen) and transferred to nitrocellulose for immunoblot analysis.

4.2.4 Western blotting

Immunoblotting was carried out, following transfer of proteins resolved on a tris-glycine gel (Invitrogen) to nitrocellulose membranes. Immunoblotting utilized the Odyssey system by LI-COR Biosciences following the manufacturer’s protocol, using near infrared fluorescence detection. Membranes were blocked in a 50% LI-COR Odyssey Blocking Buffer/Tris-buffered saline solution, while primary antibodies were diluted in a 50% LI-COR Odyssey Blocking Buffer solution supplemented with 0.1% Tween-20 and infrared dye-labeled secondary antibodies (Invitrogen) were diluted in a 50% LI-COR Odyssey Blocking Buffer solution supplemented with 0.1% Tween and 0.01% sodium dodecyl sulfate. Infrared signal at 680 nm or 800 nm was detected using the LI-COR Odyssey scanner. Images of western blots were produced and relative amounts of protein on the nitrocellulose membrane were quantified using the LI-COR Odyssey
software version 2.1. In experiments where the blocking peptide to a particular antibody was used, a 2-fold excess mass of the blocking peptide (compared to the mass of antibody used for a particular western blot) was preincubated with the primary antibody of interest in a 50% LI-COR Odyssey Blocking Buffer/50% Tris-buffered saline solution supplemented with 0.1% Tween-20, at room temperature for 2 hr with gentle shaking. This solution was then used in place of the primary antibody in the western blotting protocol described above.

PTPMT1 rabbit polyclonal antibody was provided by the laboratory of Jack Dixon at University of California, San Diego. PTPMT1 goat polyclonal antibody, PTPMT1 goat polyclonal antibody blocking peptide and α-tubulin mouse monoclonal antibody were purchased from Santa Cruz Biotechnology. Phosphothreonine rabbit polyclonal antibody was purchased from Cell Signaling Technology. The v5 epitope tag monoclonal antibody was obtained from Sigma-Aldrich.

4.2.5 Statistical Analysis

Graphing and statistical analysis were carried out using GraphPad Prism 5. Data are reported as mean ± SEM. Statistical significance was assessed using ANOVA and appropriate post hoc tests.
4.3 Results

4.3.1 Alexidine dihydrochloride stimulates insulin secretion in a dose-dependent manner

Having determined that alexidine dihydrochloride was an effective inhibitor of PTPMT1 \textit{in vitro}, we were keen to explore whether the compound would inhibit PTPMT1 in cells. Evaluation of the efficacy of alexidine dihydrochloride as an inhibitor of PTPMT1 in β-cells is somewhat confounded by our ignorance of the identity of the true endogenous substrate of PTPMT1 in the pancreatic β-cell. Although decreased expression of PTPMT1 in the pancreatic β-cell has been associated with increased insulin secretion, no changes in the level of PI(5)P, the postulated substrate of PTPMT1, were detected (Pagliarini, Wiley et al. 2005). Thus, our studies of the effect of alexidine dihydrochloride in cells necessarily utilized the more distal readouts of insulin secretion and global changes in the mitochondrial phosphoprotein profile rather than the more proximal one of changes in the phosphorylation state of a known substrate.

PTPMT1 is resident on the matrix-facing side of the inner mitochondrial membrane, so we hoped alexidine dihydrochloride would successfully target the enzyme, as molecules with lipophilic dicationic structure have previously been shown to target the mitochondria; their hydrophobicity allowing them to cross the membranes while their positive charge encourages accumulation in the matrix (Ross, Da Ros et al. 2006; Murphy and Smith 2007). As the role of PTPMT1 had previously been studied in
the pancreatic β-cell, we employed this cell type as our model and utilized changes in insulin secretion as our main readout for changes in enzyme activity. Since lipophilic dications have also been reported to show cellular toxicity at high concentrations (Severina, Vyssokikh et al. 2007), and most previous studies of alexidine dihydrochloride have focused on its antimicrobial or cytotoxic effects (Gilbert and Moore 2005; Yip, Ito et al. 2006), we first needed to ensure that our assays were performed at concentrations which did not impact cell viability. In order to make certain that any apparent increases in insulin secretion observed were not simply the result of decreased cell viability resulting in cell membrane damage and thus leakage of cellular insulin into surrounding medium, we employed a membrane integrity-based cytotoxicity assay. The cytotoxicity assay indicated that alexidine dihydrochloride did not significantly affect membrane permeability and hence cell viability at concentrations less than or equal to 4 µM. Figure 9.

Knockdown of endogenous PTPMT1 levels in a pancreatic β-cell line has been shown to result in increased insulin secretion (Pagliarini, Wiley et al. 2005), hence we investigated whether treatment of β-cells with alexidine dihydrochloride would phenocopy this result. Rather than a cell line, we elected to use primary rat islets in these experiments as these represented a more physiologically relevant model. To this end, we treated isolated rat islets with alexidine dihydrochloride at both basal (2.8 mM)
and stimulatory (16.7 mM) glucose concentrations and monitored the accompanying changes in insulin secretion. At concentrations of up to and including 4 μM, alexidine dihydrochloride induced a dose-dependent increase in insulin secretion from isolated rat islets, at both basal and stimulatory glucose concentrations. At both basal and stimulatory glucose concentrations, this effect was found to be statistically significant using ANOVA. In addition, a Dunnett post hoc analysis indicated that the effect of 4 μM alexidine dihydrochloride on insulin secretion was statistically significant at both basal (p < 0.05), and stimulatory (p < 0.001) glucose concentrations compared to untreated controls.
Figure 9: Alexidine dihydrochloride stimulates dose-dependent insulin secretion

Isolated rat islets were treated with various concentrations of alexidine dihydrochloride in the presence of 2.8 mM glucose for 1 hr and subsequently treated with the same concentration of alexidine dihydrochloride in the presence of 16.7 mM glucose for 1 hr. Alexidine dihydrochloride induced a dose-dependent increase in insulin secretion which was significant at both basal (2.8 mM) and stimulatory (16.7 mM) glucose concentrations. At both glucose concentrations, the increased insulin secretion stimulated by 4 μm alexidine dihydrochloride was significant compared to untreated controls. Data represent the means ± SEM of 6 independent experiments and are displayed as a bar graph plotted on the y-axis on the left. Data were analyzed by ANOVA with post hoc Dunnett test for significance.

The effect of various concentrations of alexidine dihydrochloride on islet cell viability was assessed during the insulin secretion assay utilizing a membrane integrity-based cytotoxicity assay. Alexidine dihydrochloride was not significantly cytotoxic relative to 0.2% Triton X-100 (100% cytotoxicity reference) applied to the cells for the same period of time to induce cell lysis. Data represent the means ± SEM of 3 independent experiments and are displayed as a line graph plotted on the y-axis on the right.
4.3.2 Alexidine dihydrochloride targets PTPMT1 in cells

Having obtained evidence that alexidine dihydrochloride phenocopied the effect of knockdown of PTPMT1 in cells by inducing increased insulin secretion, we next sought to determine whether the compound targeted PTPMT1 in cells. To this end, we investigated whether the cellular effects of alexidine dihydrochloride were influenced by perturbations of the endogenous level of PTPMT1. We rationalized that if alexidine dihydrochloride caused increased insulin secretion via inhibition of PTPMT1 in the β-cell, then reduction of the cellular protein levels of PTPMT1 should reduce the alexidine dihydrochloride-stimulated increase in insulin secretion.

In isolated rat islets, partial knockdown of PTPMT1, typically to approximately 45 - 60% of the endogenous level, resulted in a significant increase in insulin secretion. In addition, in islets in which the level of expression of PTPMT1 was unperturbed, treatment with 4 μM alexidine dihydrochloride also resulted in a significant increase in insulin secretion. However, in islets in which the level of expression of PTPMT1 was reduced by RNA interference, the alexidine dihydrochloride-stimulated increase in insulin secretion was smaller in magnitude. Statistical analysis using ANOVA and the Bonferroni test post hoc, showed that while the effect of alexidine dihydrochloride was significant in islets in which the level of expression of PTPMT1 was unperturbed, the effect of the drug was not significant in islets in which the level of expression of PTPMT1 was reduced by RNA interference.
had been significantly reduced. This suggests that the alexidine dihydrochloride-stimulated increase in insulin secretion in pancreatic β-cells may be dependent on the presence of a substantial level of PTPMT1, and thus may be selective for PTPMT1.
Figure 10: Alexidine dihydrochloride-stimulated insulin secretion is influenced by the cellular protein levels of PTPMT1

(A) Immunoblot showing level of reduction of PTPMT1 protein levels in islets (typically 45 – 60%) of the endogenous level when quantified relative to VDAC. (B) In rat islets in which the expression of PTPMT1 was reduced, there was a significant increase in insulin secretion. In addition, in islets in which the expression of PTPMT1 was unperturbed, 4.0 μM alexidine dihydrochloride induced a significant increase in insulin secretion. However, when cellular expression of PTPMT1 was reduced, alexidine dihydrochloride no longer induced a significant increase in insulin secretion. Data represent the mean ± SEM of 6 independent experiments.
4.3.3 Alexidine dihydrochloride impacts the β-cell mitochondrial phosphoprotein profile

In order to obtain additional evidence that alexidine dihydrochloride selectively targeted PTPMT1 in cells, we attempted to determine whether the cellular effects of the compound resulted from a mechanism of action equivalent to that of knockdown of cellular protein levels of PTPMT1 using RNA interference. Since RNA interference-induced reduction of cellular PTPMT1 levels impacted the phosphoprotein profile of the mitochondria (Pagliarini, Wiley et al. 2005), we investigated whether treatment of β-cells with alexidine dihydrochloride resulted in similar changes. To facilitate the collection of sufficient pancreatic β-cell mitochondria for the analysis of phosphorylation of constituent proteins, we decided to use a pancreatic β-cell line for our studies.

In our experiments, treatment of INS-1 cells with 4 μM alexidine dihydrochloride resulted in recognizable changes in the threonine phosphorylation of at least 3 mitochondrial proteins in INS-1 cells. Alexidine dihydrochloride induced an increase in the phosphorylation of an 80 kDa protein and a decrease in the phosphorylation of a 90 kDa as well as a 45 kDa protein. Figure 11. However, the extent of the changes in threonine phosphorylation of these proteins varied somewhat from experiment to experiment.

Encouragingly, our observed alexidine dihydrochloride-stimulated decrease in threonine phosphorylation of the 45 kDa mitochondrial protein, recapitulated the
reported decrease in threonine phosphorylation of a 45 kDa mitochondrial protein following RNA interference-induced reduction of the expression of PTPMT1 (Pagliarini, Wiley et al. 2005). However, in our experiments, this effect was not statistically significant with treatment of cells with 4 μM alexidine dihydrochloride, nor following RNA interference-induced knockdown of the expression of PTPMT1. This may be a result of the level of knockdown of expression of PTPMT1 that we achieved in our experiments, which was typically about 40% of the endogenous level. Figure 11.

Although alexidine dihydrochloride induced a significant increase in the level of threonine phosphorylation of an 80 kDa mitochondrial protein, the increase in phosphorylation of the 80 kDa mitochondrial protein induced by knockdown of PTPMT1 expression by RNA interference was not statistically significant. Figure 11. This was likely due to the high degree of variability in the effect of knockdown of PTPMT1 protein levels on the threonine phosphorylation of this 80 kDa protein.

Alexidine dihydrochloride also induced a significant decrease in the phosphorylation of a 90 kDa mitochondrial protein. However, knockdown of PTPMT1 expression by RNA interference did not induce a significant decrease in the phosphorylation of this 90 kDa mitochondrial protein. Figure 11. This is partially a result of some variability in the effect of knockdown of PTPMT1 protein levels on the threonine phosphorylation of this 90 kDa protein.
Figure 11: Alexidine dihydrochloride impacts mitochondrial protein phosphorylation

(A) Representative western blot comparing alexidine-induced changes, to PTPMT1 shRNA-induced changes, in threonine phosphorylation of proteins in the mitochondria-enriched fraction of INS-1 cell lysate. (B) Quantification of phosphorylation changes in 90 kDa, 80 kDa and 45 kDa mitochondrial proteins. Amounts of phosphorylated protein were quantified relative to VDAC then normalized relative to phosphorylated protein in ctrl shRNA treated cells. Data represent mean ± SEM of 4 experiments
4.3.4 Potential dimers of PTPMT1 can be crosslinked in mitochondria

Following our studies to determine the efficacy of inhibition of PTPMT1 by alexidine dihydrochloride in cells, we decided to undertake some initial studies of the biology of PTPMT1. In our in vitro kinetic studies of the inhibition of PTPMT1 by alexidine dihydrochloride we made a couple of observations which suggested that alexidine dihydrochloride might be binding a dimeric form of PTPMT1. Firstly, the bidentate structure of alexidine dihydrochloride suggested that the molecule might bind two similar or identical sites on the enzyme. One explanation for such binding would be the existence of an enzyme dimer with alexidine dihydrochloride binding the same site on each of the two enzyme molecules comprising the dimer. Secondly, the IC$_{50}$ curve for the inhibition of PTPMT1 by alexidine dihydrochloride yielded a Hill coefficient of 2, which suggests cooperativity of binding, while the IC$_{50}$ curve for the significantly less potent half alexidine molecule yielded a Hill coefficient closer to 1. One possible explanation for this would also be the existence of a PTPMT1 dimer.

In order to investigate the possible existence of dimers of PTPMT1, we decided to undertake in cell crosslinking studies. However, we first needed to ensure that we possessed an antibody capable of efficiently recognizing PTPMT1 in mitochondrial lysates. To this end we characterized the ability of the PTPMT1 goat polyclonal antibody from Santa Cruz Biotechnology to bind to PTPMT1 in INS-1 mitochondrial lysates. This
antibody was reportedly produced using a c-terminal PTPMT1 peptide so we hoped that it would potentially be able to recognize PTPMT1 bound in multimers in the mitochondrial lysates. The PTPMT1 antibody from Santa Cruz Biotechnology bound to 3 lower molecular weight proteins at 25, 20 and 18 kDa, and an additional higher molecular weight protein at approximately 45 kDa, whose expression was downregulated by PTPMT1-targeted shRNA. In addition, the blocking peptide for the PTPMT1 antibody rendered the 25 kDa protein and the 45 kDa protein undetectable with the PTPMT1 goat polyclonal antibody from Santa Cruz Biotechnology. Taken together, this suggested that the 25, 20 and 18 kDa proteins which bound the PTPMT1 antibody might represent the 3 potential isoforms of PTPMT1 (NCBI accession.version #: EDL79493.1, EDL79494.1, EDL79495.1) predicted to exist in the rat genome by AIR; Annotation Integrated Resource (Florea, Di Francesco et al. 2005). The identity of the 45 kDa protein is currently unknown.

Having verified that the antibody from Santa Cruz Biotechnology was capable of recognizing PTPMT1 in mitochondrial lysates, we then undertook studies to crosslink endogenous PTPMT1 in the mitochondria of INS-1 cells and carried out western blots using mitochondrial lysates and the PTPMT1 polyclonal antibody from Santa Cruz Biotechnology. As a second approach to identifying potential dimers of PTPMT1 in cells, we overexpressed v5-tagged PTPMT1 in INS-1 cells and carried out western blots using
mitochondrial lysates and a v5-epitope monoclonal antibody. Our crosslinking experiments revealed that major protein bands potentially representing endogenous PTPMT1 could be found at 20 and 25 kDa in the uncrosslinked sample, with additional protein bands appearing at approximately 50, 100 and 200 kDa in BS\textsuperscript{3} crosslinked mitochondrial lysates. In addition, major protein bands representing the exogenous v5-tagged PTPMT1 could be found at 25 and approximately 30 kDa in the uncrosslinked sample, with an additional band appearing at approximately 50 kDa in the BS\textsuperscript{3} crosslinked mitochondrial lysates. Taken together, the appearance of protein complexes, containing PTPMT1 (as evidenced by their recognition by the PTPMT1 and v5 antibodies) around twice (~50 kDa) and four times (~100 kDa) the predicted molecular weight of a single PTPMT1 molecule (~25 kDa), in the crosslinked mitochondrial lysates, strongly suggests that PTPMT1 may exist as dimers in the mitochondrion.
**Figure 12: Crosslinking of potential dimers of PTPMT1 in β-cell mitochondria**

(A) Western blots demonstrating the ability of the PTPMT1 goat polyclonal antibody from Santa Cruz Biotechnology to bind PTPMT1. Proteins of interest recognized by the antibody are downregulated by PTPMT1-targeted shRNA or rendered undetectable by blocking peptide. (B) Crosslinking of INS-1 mitochondrial lysates using 3.75 mM BS$_3$ causes a reduction in the amount of 25 kDa PTPMT1 monomer and the appearance of higher molecular weight complexes of PTPMT1 at approximately 2 and 4 times the molecular weight of the monomer. Arrows indicate major proteins of interest.
4.4 Discussion

Analysis of the impact of alexidine dihydrochloride on insulin secretion from pancreatic β-cells verifies that the compound is able to successfully target PTPMT1 in cells. It also confirms the positive effect of inhibition of PTPMT1 on insulin secretion (Pagliarini, Wiley et al. 2005) and strengthens the case for potential targeting of PTPMT1 in the treatment of Type II diabetes.

Current Type II diabetes drugs which target the β-cell, act to either inhibit the KATP channel (e.g. sulfonylureas and meglitinides), or enhance GLP-1 signaling (e.g. GLP-1 analogs and DPP4 inhibitors). However, one exciting new class of drugs in development augments glucokinase activity. Glucokinase activators have the advantage of acting at both the β-cell and the liver, thus increasing insulin secretion and, at the same time, decreasing hepatic glucose output, to mimic combination therapy for Type II diabetes (Grimsby, Sarabu et al. 2003; Grimsby, Berthel et al. 2008). Interestingly, PTPMT1 is expressed in the liver and muscle as well as the pancreatic β-cell. Although reduction of the activity of PTPMT1 has been shown to increase cellular ATP levels in the β-cell, its effect in the liver and skeletal muscle has not yet been explored. Thus, the effect of reduced PTPMT1 activity in these tissues would need to be examined before a drug which inhibits PTPMT1 could truly be considered as a potential therapeutic for Type II diabetes. The use of alexidine dihydrochloride may facilitate such studies.
Since protein phosphorylation is a dynamic process, the use of a selective small molecule inhibitor is more likely to mimic endogenous modulation of enzyme activity compared to knockdown of protein expression using RNA interference. Thus, as demonstrated in our mitochondrial phosphoprotein profile experiments, acute inhibition of PTPMT1 in cells by alexidine dihydrochloride may facilitate detection of mitochondrial proteins whose phosphorylation is impacted by reduced PTPMT1 activity.

We believe that the use of alexidine dihydrochloride to study changes in protein phosphorylation in mitochondria in which PTPMT1 has been inhibited would aid in the discovery of other proteins in the signaling pathways in which PTPMT1 is involved. Identification of the substrate of PTPMT1 may be advanced by the fact that alexidine dihydrochloride seems to be a predominantly uncompetitive inhibitor of PTPMT1. Since alexidine dihydrochloride likely binds to the enzyme-substrate complex, it may assist in trapping endogenous substrate in the active site of PTPMT1 in inhibitor-treated cells and perhaps aid in the identification of its physiological substrate. Thus, alexidine dihydrochloride may prove useful in further study of PTPMT1 including elucidation of its cellular substrate and its true role in the cell. In addition, alexidine dihydrochloride may also provide a scaffold for the development of other PTPMT1 inhibitors and thus potentially serve as a model for a new class of drugs for the treatment of Type II diabetes.
Our evidence for the possible existence of dimers of PTPMT1 in cells, based on our in cell crosslinking studies, supports our in vitro studies which suggested that the inhibitor alexidine dihydrochloride might be binding an enzyme dimer. This not only aids in the validation of our in vitro kinetic studies, but implies that bidentate molecules would number among the most effective inhibitors of PTPMT1. The latter theory is also supported by the fact that the three most potent inhibitors identified in our screen for inhibitors of PTPMT1 were all bidentate compounds (see Chapter 2 of this dissertation).

Of course, the potential existence of dimers of PTPMT1 also raises questions regarding the possible purpose of the dimerization of PTPMT1 and whether there exist circumstances under which the formation of PTPMT1 dimers (or higher multimeric forms) is favored over the monomeric form of the enzyme. In addition, since the existence of at least 3 isoforms (NCBI accession.version #: EDL79493.1, EDL79494.1, EDL79495.1) of PTPMT1 has been predicted (Florea, Di Francesco et al. 2005), the possible existence of multimeric forms of PTPMT1 in the cell also raises questions regarding the composition of these multimeric forms and it will be interesting to determine whether these potential multimers of PTPMT1 in the cell are comprised of identical isoforms (homo-multimers) or different isoforms (hetero-multimers).
5 Concluding Remarks

Alexidine dihydrochloride: a pharmacological tool to study PTPMT1

Over the last decade, the immense volume of data that has become available as the result of genome sequencing efforts has led to the identification of many new protein phosphatases (Alonso, Sasin et al. 2004). Within the protein phosphatase family in particular, the number of known protein tyrosine phosphatases has grown exponentially, and with these new discoveries has come an increased recognition of their importance in cellular signaling. Investigation of the interactions, cellular substrates and modes of action of some of these enzymes has helped us better appreciate their cellular roles and how derailment of their function may contribute to disease (Hendriks, Elson et al. 2008; Pulido and Hooft van Huijsduijnen 2008; Vang, Miletic et al. 2008; Yi and Lindner 2008). However, while these advancements have been significant, it is also clear that the rate of elucidation of the cellular function of protein tyrosine phosphatases has somewhat lagged behind their rate of discovery (Alonso, Sasin et al. 2004). In the quest to gain insight into the cellular roles of protein tyrosine phosphatases, small molecule chemical inhibitors have proven valuable in selectively interdicting signaling in pathways in which these enzymes are involved, and there is much hope that they will soon be applied in disease treatment (Jeffrey, Camps et al. 2007; Jiang and Zhang 2008).
A screen for new phosphatases based on the sequence of the catalytic motif of PTEN resulted in the discovery of the dual specificity phosphatase PTPMT1, Protein Tyrosine Phosphatase Localized to Mitochondrion 1 (Merlot, Meili et al. 2003). Interestingly PTPMT1 is one of the first phosphatases found to primarily localize to mitochondria. Within the mitochondrion PTPMT1 is localized in the mitochondrial matrix, tightly associated with the inner mitochondrial (Pagliarini, Wiley et al. 2005). PTPMT1 has been identified in several highly metabolic tissues including the liver and pancreas (Pagliarini, Wiley et al. 2005). Initial studies of the role of PTPMT1 in the β-cell of the pancreas revealed that knockdown of PTPMT1 levels resulted in changes in the mitochondrial phosphoprotein profile as well as an increase in cellular ATP levels which correlated with an increase in insulin secretion (Pagliarini, Wiley et al. 2005).

The close association of PTPMT1 with the inner mitochondrial membrane and its impact on insulin secretion suggests that PTPMT1 may play a potential role in β-cell metabolism. However, further studies of the biology of PTPMT1 have been somewhat limited by a need for new tools to target the enzyme, particularly during short term studies. Indeed, even the endogenous substrates of PTPMT1 are still being investigated since, in spite of the similarity of its catalytic motif to that of PTEN, and its ability to utilize phospholipid substrates in vitro (Pagliarini, Worby et al. 2004), evidence of its action on phosphoinositides in vivo remains to be demonstrated. Hence, in order to
facilitate further study of PTPMT1, and its role in β-cell metabolism in particular, we
decided to pursue a search for inhibitors of the enzyme. Small molecule inhibitors of
phosphatases have proved useful in the study of the biology of these protein tyrosine
phosphatases and selective inhibitors of these enzymes may prove valuable in the
treatment of diseases impacted by their dysregulation. Since the absence of a crystal
structure for PTPMT1 limited the applicability of rational drug design, we adopted an
unbiased screen of diverse chemical structures as the best approach towards identifying
a selective inhibitor of PTPMT1.

As detailed in Chapter 2 of this dissertation, screening of a commercially-
available, small molecule chemical inhibitor library, yielded alexidine dihydrochloride, a
dibiguanide compound, as a potent inhibitor of PTPMT1. As discussed in Chapter 3,
alexidine dihydrochloride was a fairly selective inhibitor of PTPMT1, displayed rather
interesting kinetics and yielded the dibiguanide structure as an essential
pharmacophore for inhibition of PTPMT1. Our modeling of the inhibition of PTPMT1 by
alexidine dihydrochloride revealed that the inhibition was predominantly
uncompetitive, implying that alexidine dihydrochloride likely targets the enzyme-
substrate complex and may not bind within the active site of the enzyme. Furthermore,
the Hill coefficient of 2 which was observed in the inhibition of PTPMT1 by alexidine
dihydrochloride, suggested cooperativity of binding of the inhibitor to the enzyme and
led to speculation that PTPMT1 might exist as a dimer. As revealed in the cell-based studies described in Chapter 4 of this dissertation, alexidine dihydrochloride phenocopied the effect of RNA interference-induced knockdown of PTPMT1 protein levels in the pancreatic β-cell, and induced insulin secretion in a dose-dependent and potentially PTPMT1-dependent manner. Alexidine dihydrochloride also mimicked the changes in phosphorylation of some mitochondrial proteins observed upon knockdown of cellular levels of PTPMT1 using RNA interference.

Taken together, the studies detailed in Chapters 2, 3 and 4 of this dissertation support the ability of alexidine dihydrochloride to effectively inhibit PTPMT1 both in vitro and in cells. Although the dibiguanide structure has been identified as a good pharmacophore for this inhibition, more extensive structure activity relationship studies are needed to optimize potency and minimize toxicity of this compound. As alexidine dihydrochloride is possibly not an active site-directed inhibitor, we suspect that this may contribute to the selectivity of its inhibition. Significantly, the sequence of the catalytic motif of the active site of the dual specificity phosphatases is highly conserved, and as a result, in the past it has proven somewhat difficult to identify truly selective inhibitors of the dual specificity phosphatases in the absence of a high resolution structure of the enzyme to aid in modeling. However, based on the high degree of selectivity for PTPMT1 exhibited by alexidine dihydrochloride, we posit that the pursuit of non-active
site directed inhibitors, for example, compounds such as alexidine dihydrochloride which display uncompetitive inhibition of a particular dual specificity phosphatase, might be a viable strategy in identifying selective inhibitors of this class of enzymes in the absence of structural data. Active screening for uncompetitive inhibitors of particular enzymes would theoretically require few changes in the current approach to high throughput screening for inhibitors and thus could be quickly and efficiently implemented (Copeland 2005).

Our studies of the kinetics of the inhibition of PTPMT1 by alexidine dihydrochloride hinted at the possibility that PTPMT1 may exhibit dimerization, a phenomenon which has been shown to post-translationally regulate the activity of various dual specificity PTPs. The possible existence of regulatory dimers of PTPMT1 is certainly worth investigating and our initial crosslinking studies strongly suggest that PTPMT1 may indeed exist as a dimers in β-cell mitochondria. Thus, in this respect, the kinetic studies of the inhibition of PTPMT1 by alexidine dihydrochloride validate the utility of this inhibitor as a tool to probe the biology of PTPMT1. Alexidine dihydrochloride has also shown applicability as a tool to study PTPMT1 in our evaluation of the impact of inhibition of PTPMT1 on the phosphorylation of mitochondrial proteins. The ability to acutely inhibit PTPMT1 that is conferred by alexidine dihydrochloride, combined with its binding of the enzyme-substrate complex and hence potential
substrate trapping abilities, may support the identification of phosphorylated mitochondrial proteins which may serve as potential substrates of PTPMT1.

Finally, our studies of the inhibition of PTPMT1 by alexidine dihydrochloride in cells supports the notion that PTPMT1 could serve as a pharmacological target in the treatment of Type II diabetes. Treatment of β-cells with alexidine dihydrochloride demonstrates the proof of principle that inhibition of PTPMT1 by a small molecule, chemical inhibitor leads to increased insulin secretion. Currently we believe that the mechanism of this increased insulin secretion is probably related to an increase in metabolism following inhibition of PTPMT1 in the β-cell. In addition, since PTPMT1 is also expressed in the liver and muscle, it would be necessary to investigate the effect of inhibition of PTPMT1 in these tissues, before a full consideration of the true potential of PTPMT1 as a therapeutic target in the treatment of Type II diabetes could be completed.
Appendix

Fatty acid-stimulated G-protein signaling in the pancreatic β-cell

Abstract

In studying insulin secretion from the pancreatic β-cell, fatty acid-stimulated G-protein signaling has also been examined in this dissertation. Acute exposure to free fatty acids has been shown to potentiate insulin secretion from the pancreatic β-cell at high glucose concentration via both changes in metabolism and stimulation of the G-protein-coupled free fatty acid receptor. While the majority of evidence suggests that the free fatty acid receptor GPR40/FFA1 is coupled to the Gq family of heterotrimeric G-proteins in the β-cell, no extensive study of its potential coupling to other G-proteins and its downstream effectors has yet been undertaken. In this work, the use of overexpression of regulator of G-protein signaling molecules to selectively interdict signaling downstream of particular heterotrimeric G-proteins has potentially revealed functional coupling of the free fatty acid receptor to the G12 family. This suggests that both Gq and G12-induced free fatty acid signaling may need to be considered in the development of therapeutics which target GPR40/FFA1 for the treatment of Type II diabetes.
**Introduction**

**Mechanism of glucose-stimulated insulin secretion**

Glucose-stimulated insulin secretion comprises one of the body’s most important mechanisms of glucose homeostasis, and functionally links a rise in plasma glucose, the body’s primary metabolic fuel, to a rise in plasma insulin levels. A rise in plasma glucose levels leads to increased uptake of glucose into the β-cell. Subsequent metabolism of the glucose in the β-cell leads to the production of ATP and hence an increase in the ATP to ADP ratio in the cell. The rise in ATP levels causes closure of ATP-sensitive K$^+$ channels in the β-cell membrane leading to membrane depolarization. This then leads to activation and opening of voltage-dependent L-type Ca$^{2+}$ channels in the cell membrane and the influx of Ca$^{2+}$ into the cell. The resulting rise in intracellular calcium is associated with transport of insulin secretory vesicles to the cell membrane and insulin release by exocytosis. Figure 13.

Examination of insulin release in response to a rise in blood glucose using a hyperglycemic clamp reveals two phases of insulin secretion. The first or early phase of insulin secretion occurs within the first 10 minutes after a rise in plasma glucose levels and is seen as a sharp but brief (about 2 minutes) rise in plasma insulin levels. This is followed by a second or late phase of insulin secretion which lasts over an hour after the first phase ends and is characterized by a less intense but longer-sustained rise in plasma insulin levels. The early phase of insulin secretion is believed to be due to rapid
exocytosis from plasma membrane-localized secretory granules located in close proximity to the L-type Ca^{2+} channels. This proximal pool of granules is often referred to as the readily-releasable pool as it is able to respond quickly to the glucose-induced rise in intracellular calcium levels. The late phase of insulin secretion is believed to result from exocytosis from more distally located cytosolic secretory granules which experience a slightly delayed, less intense rise in calcium concentration (Ohara-Imaizumi, Nishiwaki et al. 2004; Rutter 2004). In Type II diabetics, the first phase of insulin secretion is lost and the second phase of insulin secretion is greatly reduced (Eriksson, Franssila-Kallunki et al. 1989; Ohara-Imaizumi, Nishiwaki et al. 2004).
Figure 13: Model of glucose-stimulated insulin secretion
GPR40 is a free fatty acid-stimulated G-protein-coupled receptor

The discovery of a free fatty acid-stimulated G-protein-coupled receptor, GPR40, which is expressed in the β-cell suggested that some of the observed effects of fatty acids on insulin secretion in the β-cell may be mediated by engagement of heterotrimeric G-proteins. G-protein-coupled receptors (GPCRs) are seven transmembrane domain receptors which bind ligand on the extracellular face of the plasma membrane and associate with guanine nucleotide-binding proteins or G-proteins on the intracellular face, hence facilitating the transmission of signals across the membrane. G-proteins are heterotrimeric subunit proteins consisting of Gα, Gβ and Gγ subunits. The Gα subunit binds guanine nucleotides; in its inactive state it binds GDP but when activated, exchanges GDP for GTP. The tight association of the Gα subunit with the Gβ and Gγ subunits inhibits GDP release, thus maintaining the G-protein in an inactive state. According to conventional theory, upon binding and stimulation by an agonist ligand, the GPCR undergoes a conformational change causing it to stimulate release of GDP by Gα at the cytoplasmic face of the membrane. Gα then exchanges GDP for GTP and is activated. The heterotrimeric G-protein then separates into two signaling units; the GTP-bound Gα subunit and the tightly-coupled Gβγ complex, both of which may then separately initiate signaling cascades within the cell.
The Gα subunit possesses intrinsic GTPase activity. Hence Gα-stimulated signaling is terminated when GTP is hydrolyzed back to GDP and Gα re-associates with the Gβγ subunits and the receptor. Termination of Gα-stimulated signaling may be hastened by the action of regulators of G-protein signaling (RGS) proteins which act as GTPase-accelerating proteins (GAPs) for particular Gα subunits. Hence RGS proteins terminate Gα-stimulated signaling in the cell, and overexpression of RGS proteins in cells has been employed as a means to attenuate G-protein signaling.

Heterotrimeric G-proteins are divided into 4 families Gs, Gi, Gq and G12 based on sequence homology of the Gα subunits as well as the nature of the signals they initiate within the cell when activated. Traditionally, one could preliminarily identify the Gα family activated by a particular receptor by determination of the intracellular signal initiated (e.g. rise in intracellular cAMP for Gαs or Ca^{2+} levels for Gαq) and assessment of the sensitivity of that signal to the bacterial toxin pertussis toxin, which selectively inactivates Gαi proteins. However, with the increasing number of functions ascribed to specific Gα proteins and to the Gβγ complex, as well as the increasing evidence of crossover in signaling (Gu, Muller et al. 2002; Gavard and Gutkind 2008; Jiang, Collins et al. 2008) it is not possible to definitively ascribe a specific G-protein class to a given GPCR using only this type of data, particularly if signaling is pertussis toxin-insensitive. In 2003, when three labs independently reported that the orphan G-protein-coupled...
receptor, GPR40 was stimulated by free fatty acids; they also reported that stimulation of GPR40 induced a rise in intracellular Ca\(^{2+}\) levels (Briscoe, Tadayyon et al. 2003; Itoh, Kawamata et al. 2003; Kotarsky, Nilsson et al. 2003), activation of ERK (Itoh, Kawamata et al. 2003; Kotarsky, Nilsson et al. 2003) and was pertussis toxin-insensitive (Itoh, Kawamata et al. 2003; Kotarsky, Nilsson et al. 2003). This suggested that the receptor was predominantly coupled to the Gq family of G-proteins. Later, the report that a selective small molecule inhibitor of Gαq, YM-254890, diminished the intracellular rise in calcium levels induced by palmitate in the β-cell, confirmed the link between GPR40 and Gq (Shapiro, Shachar et al. 2005).

**Signaling downstream of GPR40 leading to insulin secretion**

Using a functional readout of increases in intracellular calcium in cells overexpressing GPR40, the receptor was found to be stimulated by a wide range of physiologically-relevant medium and long chain free fatty acids including saturated fatty acids 12 to 16 carbons long and unsaturated fatty acids 18 and 20 carbons long (Itoh, Kawamata et al. 2003). Amongst saturated fatty acids, those 15 or 16 carbons long were most potent (Briscoe, Tadayyon et al. 2003). GPR40 has now been shown to mediate some of the effects of free fatty acid on insulin secretion from the β-cell (Briscoe, Tadayyon et al. 2003; Fujiwara, Maekawa et al. 2005; Shapiro, Shachar et al. 2005; Steneberg, Rubins et al. 2005).
Fatty acids; the body’s secondary metabolic fuel, can act as secretagogues for the β-cell. Fatty acids potentiate glucose-stimulated insulin secretion, with this effect being more striking at high or stimulatory glucose concentrations (>10 mM), rather than low or basal glucose concentrations (<3 mM). However, while acute exposure of β-cells to free fatty acids in the presence of high glucose concentrations induces insulin secretion, chronic exposure diminishes insulin secretion. This suggests that fatty acids usually function as positive regulators in insulin homeostasis. The free fatty acid-induced rise in insulin secretion is at least partially a result of stimulation of GPR40 since siRNA-mediated downregulation of GPR40 levels reduced insulin secretion in response to fatty acid (Itoh, Kawamata et al. 2003). siRNA targeted against GPR40 also abolished intracellular calcium rises resulting from acute treatment of β-cells with fatty acid (Fujiwara, Maekawa et al. 2005; Shapiro, Shachar et al. 2005).

Although both high glucose and high fatty acid induce a rise in intracellular calcium, these effects seem to be distinct from each other as siRNA-mediated downregulation of GPR40 in single β-cells abrogates the calcium rise induced by acute treatment with the fatty acid oleate but has little effect on the calcium rise induced by high glucose concentrations (Fujiwara, Maekawa et al. 2005). GPR40-induced calcium rises in β-cells appear to require extracellular calcium since calcium-free medium eliminates the observed fatty acid-stimulated calcium rise (Fujiwara, Maekawa et al. 2005).
Significantly, nifedipine, a voltage-dependent L-type calcium channel blocker has been shown to reduce fatty acid-potentiated insulin secretion from β-cells (Itoh, Kawamata et al. 2003). This implies that, although the rise in intracellular calcium and subsequent insulin secretion induced by GPR40 stimulation is distinct from that induced by high glucose concentration, the two may be physiologically linked since the fatty acid-induced rise in intracellular calcium appears to be at least partially mediated by influx of calcium through voltage-dependent L-type calcium channels in the plasma membrane (Fujiwara, Maekawa et al. 2005). The GPR40-induced rise in intracellular calcium levels likely also involves efflux from intracellular calcium stores like the endoplasmic reticulum as cyclopiazonic acid, an inhibitor of sarcoplasmic reticulum calcium ATPases, which causes depletion of intracellular calcium stores, diminished the palmitate-induced calcium rise in β-cells (Shapiro, Shachar et al. 2005).

Signaling downstream of fatty acid-induced activation of heterotrimeric G-proteins is at least partly a function of activation of phospholipase C, since use of U73122, a pharmacological inhibitor of phospholipase C, reduced the intracellular calcium rises induced by fatty acid (Fujiwara, Maekawa et al. 2005; Shapiro, Shachar et al. 2005). Since Gαq activation is known to stimulate phospholipase C this also argues for the coupling of GPR40 to Gq heterotrimeric G-protein family members.
Furthermore, this supports the possible contribution of efflux from intracellular calcium stores to the rise in intracellular calcium observed following treatment of β-cells with fatty acid, since phospholipase C activation typically leads to production of inositol-1,4,5-triphosphate which in turn can mediate release of calcium from ER stores.

In addition to an induced rise in intracellular calcium levels and activation of phospholipase C, activation of ERK1/2 in β-cells has been observed downstream of fatty acid-induced activation of heterotrimeric G-proteins (Itoh, Kawamata et al. 2003). This activation of ERK1/2 may be significant to fatty acid-induced G-protein signaling in β-cells since the Gq family of heterotrimeric G-proteins has been shown to often activate ERK1/2 (Osmond, Sheehan et al. 2005). Beyond supporting the hypothesis that GPR40 is coupled to Gq, activation of ERK1/2 downstream of GPR40 may also support a role for ERK1/2 in fatty acid-potentiated insulin secretion. However, in one study, inhibition of ERK1/2 using the pharmacological inhibitor PD098059 apparently did not diminish oleate or linoleate-potentiated insulin secretion from MIN6 cells (Itoh, Kawamata et al. 2003). In another study a cytoplasmic pool of ERK1/2 which was activated during glucose-stimulated insulin secretion in β-cells was found to bind to and phosphorylate an insulin secretory vesicle membrane-associated protein, synapsin I, and use of PD098059 or siRNA targeted against ERK1/2 diminished glucose-stimulated insulin secretion (Longuet, Broca et al. 2005). This latter report, combined with the observed
activation of ERK in β-cells in response to fatty acid, suggests that activation of ERK1/2 by fatty acid may possibly play a role in fatty acid-potentiated glucose-stimulated insulin secretion.

The 6 years since the discovery of GPR40 have witnessed an eruption of research on the receptor as a potential pharmacological target in the treatment of Type II diabetes (Stoddart, Smith et al. 2008). However, while much research has focused on the impact of perturbations of the level of expression or activation of the GPR40 receptor on insulin secretion, limited attention has been paid to elucidation of the effectors and signaling events downstream of the receptor that impact this insulin secretion. Since the potential contribution of GPR40 activation to the detrimental effects of β-cell exposure to chronic, elevated, free fatty acid levels, and thus its true potential utility as a pharmacological target is still being debated, a more detailed understanding of the signaling events downstream of receptor stimulation may prove useful in resolving this issue.

It is now well documented that GPCRs that couple to the Gq family of heterotrimeric G-proteins in many cases also couple to the G12 family (Gu, Muller et al. 2002; McLaughlin, Shen et al. 2005). Hence, engagement of a single receptor has the potential to activate two disparate signaling pathways in the same cell, although these pathways may then converge at particular downstream effectors. In some cases in
which such cross-talk between the Gq and G12 families have been demonstrated, the
receptor may be activated by different ligands which then trigger preferential
stimulation of different heterotrimeric G-proteins (McLaughlin, Shen et al. 2005). Since
it has been demonstrated that GPR40 is activated by a range of physiologically-relevant
saturated fatty acids within the C12 to C16 range as well as unsaturated fatty acids in
the C18 to C20 range (Itoh, Kawamata et al. 2003), and furthermore that these fatty
acids stimulate GPR40 to different extents, it will be important to ascertain whether
such crosstalk occurs with GPR40 activation and whether disparate signaling occurs
downstream of the heterotrimeric G-proteins coupled to GPR40.
Methods

Analysis of the cellular phosphoprotein profile

For adenoviral-mediated changes in protein expression, 1.6 x 10^6 INS-1 cells were plated in each well of a 6-well plate and cultured in RPMI 1640 growth medium containing 8 mM glucose, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 10% fetal bovine serum for 24 hr, then cells were infected with 1.95 x 10^{10} infectious units of adenovirus per well. Adenovirus for overexpression of RGS2, p115RGS and RGS10 (Kelly, Moeller et al. 2006) or expression of mutationally activated forms of Gαq, Gα13 (Kelly, Moeller et al. 2006) were used. After 4 hr, growth medium was replaced with serum-free, RPMI 1640 starvation medium containing 0.5 mM glucose, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 0.1% fatty acid free, bovine serum albumin. For siRNA-mediated changes in protein expression, INS-1 832/13 cells were nucleofected (using the Amaxa system) with 0.2 nM siRNA, then cultured in RPMI 1640 growth medium. After 24 hr, growth medium was replaced with serum-free, RPMI 1640 starvation medium. After 18 hr of culture in serum-free, RPMI 1640 starvation medium, adenovirally-infected or siRNA-nucleofected cells were treated with drug (0.4 mM palmitate or 30 μM carbachol) for 20 min, washed with phosphate-buffered saline, then lysed using a modified NP-40 cell lysis buffer (50 mM Tris pH 7.7, 150 mM NaCl, 1% igepal, 10% glycerol, 2 mM vanadate, 10 mM NaF, 2.5 mM MgCl2 with protease inhibitors).
Proteins from lysate were resolved on a 12% polyacrylamide gel using sodium dodecyl sulfate polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes for immunoblotting. Membranes were blocked in a 5% bovine serum albumin/Tris buffered saline solution supplemented with 0.1% Tween-20. Primary and secondary antibodies were diluted in the same 5% bovine serum albumin blocking solution described above. Secondary antibodies were horseradish peroxidase-conjugated (Pierce Biotechnology) and were detected by chemiluminescence reaction following incubation with substrate (Amersham Pharmacia) and documented by exposure to X-ray film (Kodak BioMax). Phospho-Akt, phospho-ERK1/2, phospho-S6 ribosomal antibodies were purchased from Cell Signaling Technology. Phospho JNK antibody was purchased from Santa Cruz Biotechnology. Gq antibody was obtained from Santa Cruz Biotechnology.

**Intracellular calcium flux assays**

INS-1 832/13 cells were plated on glass slides and cultured in RPMI 1640 medium containing 8 mM glucose, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 10% fetal bovine serum for 24 hr. Cells were then loaded with fura-2-acetoxymethyl dye by incubation for 30 min in the dark in a solution of 4 μM fura-2-acetoxymethyl in Hanks’ balanced salt solution supplemented with bovine serum albumin. Cells were then transferred to a modified Krebs-Ringer phosphate buffer (see method for insulin
secretion assays) containing 3 mM glucose and incubated in the dark for a further for 30 min. Cells were then perifused with modified Krebs-Ringer buffer containing 10 mM glucose supplemented with 0.4 mM palmitate at a rate of approximately 1 ml/min. Changes in intracellular calcium concentration were monitored by recording absorbance at 340 nm and 380 nm with emission at 510 nm, with the 340/380 nm ratio being reported.

**Insulin secretion assays**

INS-1 832/3 cells were cultured in RPMI 1640 medium containing 8 mM glucose, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 10% fetal bovine serum. INS-1 832/3 cells were used in static glucose-stimulated insulin secretion assays as previously described (Joseph, Jensen et al. 2006), with some modifications. In summary, 0.8 x 10⁶ cells were plated in 12-well tissue culture plates, cultured for 24 hr, then infected with 6.5 x 10⁹ infectious units of adenovirus per well. Medium was changed after 24 hr and insulin secretion assay was performed approximately 12 hr later. Cells were rinsed with a modified Krebs-Ringer phosphate buffer (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES pH 7.2, 25 mM NaHCO₃, 0.25 M CaCl₂, 0.2% bovine serum albumin) containing 2.8 mM glucose, washed for a 1 hr at 37°C in fresh buffer containing 2.8 mM glucose, then successively incubated for 1 hr intervals at 37°C in 1 ml of buffer per well, first in buffer containing 2.8 mM glucose, then in buffer containing 2.8 mM glucose.
containing 2.8 mM glucose and 0.4 mM sodium palmitate (Sigma-Aldrich), and finally in buffer containing 16.7 mM glucose and 0.4 mM sodium palmitate. Secreted insulin was assessed from assay buffer via radioimmunoassay (DPC/Siemens Healthcare Diagnostics).
**Results**

**Palmitate-induced signaling in the β-cell is transduced by Gaq**

In order to study palmitate-induced G-protein signaling in the β-cell, we utilized INS-1 and high insulin-secreting INS-1-derived β-cell lines and examined the impact of acute treatment with palmitate on potential downstream effectors by means of western blot using phospho-specific antibodies. Acute treatment of INS-1 cells with palmitate resulted in a striking increase in phosphorylation/activation of ERK1/2 and JNK1/2 with a concomitant decrease in phosphorylation/activation of Akt. Significantly, in these same cells, adenoviral-mediated overexpression of RGS2 to diminish Gq signaling markedly reduced activation of ERK1/2 and JNK1/2 following acute treatment with palmitate, and increased activation of Akt. Overexpression of p115RGS, the G12-specific GTPase-accelerating protein, did not have a significant effect on the observed changes in phosphorylation of Akt, ERK1/2 and JNK1/2 upon acute stimulation with palmitate. Figure 14.

Interestingly, expression of mutationally-activated Gaq (and to a very minor extent Gα13), recapitulated the effect of acute treatment of INS-1 cells with the fatty acid palmitate and resulted in increased phosphorylation/activation of both ERK1/2 and JNK1/2 and decreased phosphorylation/activation of Akt. Figure 14. A similar effect was seen when mutationally-activated Gaq (Gaql) and Gα13 (Gα13QL) were expressed in the
MIN6 and RIN5mF β-cell lines (data not shown). The distinct attenuation of at least three of the palmitate-induced signaling events, by the Gq selective downregulator of signaling, RGS2, combined with the recapitulation of palmitate-induced signaling by constitutively active Gαq in β-cells, corroborates previous evidence suggesting the coupling of Gαq to GPR40 and induction of the activation of Gαq in response to stimulation of the receptor by free fatty acid. Taken together, these data strongly support the hypothesis that the free fatty acid receptor GPR40, is coupled to the Gq family of heterotrimeric G-proteins in the β-cell.

In the related, high insulin-secreting INS-1-derived β-cell line cell, INS-1 832/13, siRNA-mediated downregulation of Gαq expression combined with acute treatment with palmitate results in decreased activation of ERK1/2 and JNK1/2. Figure 14. This partially reflects the results seen with RGS2-induced attenuation of Gq signaling upon acute treatment of INS-1 cells with palmitate. A similar reduction in the phosphorylation of ERK1/2 and JNK1/2, following siRNA mediated downregulation of Gq expression, was seen in the positive control experiment where cells were treated with carbachol, a known stimulator of Gq signaling.

In particular, the effect of acute treatment of β-cells with palmitate on ERK1/2 phosphorylation/activation is noteworthy, because activation of a cytoplasmic pool of ERK1/2 which binds to and activates an insulin vesicle membrane-localized protein
synapsin I, has been implicated in the mechanism of glucose-stimulated insulin secretion, and pharmacological or siRNA-mediated inhibition of ERK1/2 results in reduced insulin secretion (Longuet, Broca et al. 2005). Thus, the observed palmitate-induced activation of ERK1/2 may possibly be related to the mechanism of palmitate-potentiated glucose-stimulated insulin secretion.
Figure 14: Gαq transduces palmitate-induced signaling in the pancreatic β-cell

(A) Western blot showing changes in phosphorylation of Akt, ERK1/2 and JNK1/2 in INS-1 cells in response to acute stimulation with palmitate following overexpression of various RGS proteins or expression of mutationally activated G-proteins. The selective downregulator of Gq signaling, RGS2, abrogates palmitate induced cell signaling, while mutationally-activated Gαq, GαqQL recapitulates it. (B) Western blot showing phosphorylation of Akt, ERK1/2, JNK1/2 and S6 ribosomal protein in INS-1 832/13 cells in response to stimulation with palmitate or carbachol following si-RNA mediated knockdown of Gq expression. Reduced Gq expression results in decreased phosphorylation of both ERK1/2 and JNK1/2
**RGS2 diminishes palmitate-induced intracellular Ca\(^{2+}\) increases**

Acute stimulation of β-cells with palmitate has previously been demonstrated to induce an increase in cellular calcium levels, and this increase in intracellular calcium is associated with stimulation of the G-protein coupled receptor, GPR40. Thus, we pondered whether attenuation of Gq signaling by overexpression of the selective downregulator of Gq signaling, RGS2, in the pancreatic β-cell, would abrogate this palmitate-induced intracellular Ca\(^{2+}\) rise.

In INS-1 832/13 cells, overexpression of RGS2 markedly decreased the rise in intracellular Ca\(^{2+}\) levels induced by acute treatment with palmitate in the presence of 10 mM glucose, compared to cells in which the level of expression of RGS2 was unperturbed. Figure 15. This strongly supports the claim that GPR40 is coupled to the Gq family of heterotrimeric G-proteins in the pancreatic β-cell, and that activation of G\(\alpha_q\) by fatty acid engagement of the receptor contributes to the rise in intracellular calcium levels induced by acute stimulation with palmitate.

It will be interesting to determine whether expression of the mutationally activated form of G\(\alpha_q\) (G\(\alpha_q\)QL) recapitulates the increase in intracellular calcium levels induced by treatment of INS-1 832/13 cells with palmitate and to also determine if overexpression of the selective downregulator G12 signaling, p115RGS, has any effect on the palmitate-induced rise in intracellular calcium levels.
Figure 15: RGS2 attenuates the palmitate-induced rise in intracellular Ca\(^{2+}\)

In control cells (GFP), treatment with 0.4 mM palmitate in the presence of 10 mM glucose induces a rise in intracellular calcium levels. In cells in which RGS2 has been overexpressed (RGS2) there is a marked decrease in the rise in intracellular calcium levels induced by palmitate. Changes in intracellular calcium concentration were monitored by monitoring changes in the 340 nm/380 nm absorbance ratio of the intracellular calcium-binding dye fura-2.
RGS2 and p115RGS diminish palmitate-potentiated insulin secretion

Since signaling downstream of activation of the free fatty acid receptor GPR40 culminates in insulin secretion, we decided to examine the effect of selectively decreasing signaling downstream of Gq and G12 on this terminal event. To this end we examined the impact of the selective reduction of Gq and G12 signaling on palmitate-stimulated insulin secretion in INS-1 832/3 cells.

In agreement with published reports, palmitate potentiated glucose-stimulated insulin secretion, particularly at high (16.7 mM) glucose concentrations. Significantly, overexpression of the selective downregulator of Gq signaling, RGS2, diminished the palmitate-induced increase in insulin secretion at high glucose levels. Intriguingly, overexpression of the selective downregulator of G12 signaling, p115RGS, also decreased the palmitate-induced potentiation of insulin secretion, albeit to a slightly lesser extent compared to overexpression of RGS2. Figure 16. Taken together, these data support a role for Gq activation, and potentially G12 activation, in transducing palmitate-potentiated insulin secretion, and imply that both the Gq and the G12 family of heterotrimeric G-proteins may perhaps be coupled to GPR40 in the pancreatic β-cell. However, more extensive investigation will be necessary to confirm this.
Figure 16: RGS2 and p115RGS diminish palmitate-stimulated insulin secretion

(A) Overexpression of either the selective downregulator of Gq signaling, RGS2 or the selective downregulator of G12 signaling, p115RGS decreases palmitate-potentiated, glucose-stimulated insulin secretion in the pancreatic INS-1 832/3 cells. Data represent the mean ± SEM of 2 experiments, each with 3 replicates. (B) Western blot confirming adenovirally-induced overexpression of RGS2 and p115RGS
Discussion

The very first reports that the G-protein-coupled receptor, GPR40, was stimulated by medium- and long chain- saturated and unsaturated free fatty acids were accompanied by the postulation that the receptor was coupled to the Gq family of heterotrimeric G-proteins. While many studies since the original articles have supported this claim, few have set out to deliberately test it. Based on our studies in the pancreatic β-cell following acute treatment with the free fatty acid palmitate, we now believe that the receptor GPR40 may not be only coupled to the Gq family of heterotrimeric G-proteins, but may possibly also be coupled to the G12 family.

Following the initial evidence from our studies that there might be signaling crosstalk involving both Gq and G12 heterotrimeric G-proteins downstream of GPR40 following acute stimulation of with palmitate in the pancreatic β-cell, we have speculated that signaling may diverge downstream of the heterotrimeric G-proteins and that it may occur preferentially via a particular G-protein family under different circumstances of receptor stimulation. For example, stimulation of the GPR40 receptor by different fatty acids may preferentially activate the Gq or G12 family and this may lead to different signaling events which nevertheless converge to support insulin secretion in the short term.
In the experiments we have conducted to date, the similarity in the effect of selectively downregulating Gq versus G12 signaling in the β-cell using overexpression of RGS2 versus overexpression of p115RGS seems greater at the level of insulin secretion than at the level of activation of the MAP kinases ERK1/2 and JNK1/2. This implies that changes in activation of ERK1/2 and JNK1/2 might not be the major route by which G12 signaling impacts fatty acid-potentiated insulin secretion downstream of palmitate-induced activation of GPR40. Hence, more extensive study of the signaling events initiated by fatty acid engagement of GPR40 is needed, particularly with reference to those events which may occur downstream of Gq, and potentially G12 activation.
Concluding Remarks

Fatty acid-stimulated insulin secretion: a Gq and G12 affair

Free fatty acids are important secretagogues in the stimulation of insulin secretion from the pancreatic β-cell. However, numerous studies have shown that short term elevation of free fatty acid levels is associated with increased insulin secretion, while long term elevation of free fatty acid levels is associated with decreased insulin secretion and β-cell death. Thus, acute elevation of free fatty acid levels induces positive effects while chronic elevation has negative effects on the pancreatic β-cell.

The increase in insulin secretion induced by acute exposure of the β-cell to free fatty acid can be attributed to both changes in metabolism and induction of G-protein signaling via the free fatty acid, G-protein-coupled receptor GPR40. The free fatty acid receptor, GPR40, is widely believed to be coupled to the Gq family of heterotrimeric G-proteins but, as detailed in this Appendix, experiments in our laboratory suggest that the receptor may possibly also be coupled to the G12 family of heterotrimeric G-proteins. Such crosstalk between G-protein families downstream of a particular receptor is now more widely being recognized as an important aspect of G-protein-coupled receptor signaling which may have significant consequences.

Activation of different families of G-proteins downstream of stimulation of a particular receptor has been shown to sometimes be dependent on the particular ligand
which engages the receptor. This variation in stimulation of different G-protein families downstream of a particular receptor can have important functional consequence due to divergence of signaling at particular parts of the pathway, although signaling may then converge to produce a particular effect. Since we have discovered that the free fatty acid palmitate potentially induces activation of the G12 family of G-proteins in addition to the Gq family, and the GPR40 receptor has been shown to be activated by a range of medium- and long chain- saturated and unsaturated fatty acids, it would be interesting to determine whether the potential activation of the G12 family by fatty acid is dependent on the subset of fatty acids which engages the receptor. Indeed there are some hints that this may be the case since oleate has been shown to increase activation of ERK1/2 in the absence of JNK1/2 activation, downstream of GPR40 stimulation, albeit in mammary epithelial cells (Yonezawa, Haga et al. 2008).

The investigation of potential G12-stimulated signaling downstream of GPR40 is still in its infancy, thus the identity of the various effectors in signaling downstream of G12 activation remain to be established. Indeed, even the determination of whether both Gα₁₂ and Gα₁₃ potentially contribute equally to signaling downstream of GPR40 engagement or whether the receptor couples preferentially to one of the Gα subunits over the other remains to be investigated. Hence, the questions of whether fatty acid-induced activation of ERK1/2 is stimulated by both Gα₁₂ and Gα₁₃, and whether
activation of either or both of these $\Gamma \alpha$ subunits contribute to the observed increases in intracellular $\text{Ca}^{2+}$ levels which have been linked to fatty acid-potentiated insulin secretion remain to be determined. Thus, the investigation of potential G12-stimulated signaling downstream of activation of GPR40 in the pancreatic β-cell, promises to be an interesting and extensive venture.
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