# Pharmacological Targeting of the Mitochondrial Phosphatase PTPMT1[S]

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## ABSTRACT

The dual-specificity protein tyrosine phosphatases (PTPs) play integral roles in the regulation of cell signaling. There is a need for new tools to study these phosphatases, and the identification of inhibitors potentially affords not only new means for their study, but also possible therapeutics for the treatment of diseases caused by their dysregulation. However, the identification of selective inhibitors of the protein phosphatases has proven somewhat difficult. PTP localized to mitochondrion 1 (PTPMT1) is a recently discovered dual-specificity phosphatase that has been implicated in the regulation of insulin secretion. Screening of a commercially available small-molecule library yielded alexidine dihydrochloride, a dibiguanide compound, as an effective and selective inhibitor of PTPMT1 with an in vitro concentration that inhibits response by 50% of 1.08 $\mu$M. A related dibiguanide ana-log, chlorhexidine dihydrochloride, also significantly inhibited PTPMT1, albeit with lower potency, while a monobiguanide ana-log showed very weak inhibition. Treatment of isolated rat pancreatic islets with alexidine dihydrochloride resulted in a dose-dependent increase in insulin secretion, whereas treatment of a pancreatic $\beta$-cell line with the drug affected the phosphorylation of mitochondrial proteins in a manner similar to genetic inhibition of PTPMT1. Furthermore, knockdown of PTPMT1 in rat islets rendered them insensitive to alexidine dihydrochloride treatment, providing evidence for mechanism-based activity of the inhibitor. Taken together, these studies establish alexidine dihydrochloride as an effective inhibitor of PTPMT1, both in vitro and in cells, and support the notion that PTPMT1 could serve as a pharmacological target in the treatment of type II diabetes.

Phosphorylation of proteins is one of the most important means of regulating signaling events required for basic cellular function. Phosphorylation is reversible and often induces a conformational change that affects the enzymatic activity or scaffolding function of the protein. This in turn affects the propagation of signals in the cell, thus leading to either enhancement or suppression of cellular processes. Changes in protein phosphorylation are controlled by a wide array of protein kinases and phosphatases. Among the protein phosphatases, protein tyrosine phosphatases (PTPs), comprise the largest family. Although these enzymes exhibit widely diverse sequences and structures, they all contain the C(X)5R amino acid sequence in their catalytic cleft (Guan and Dixon, 1990). The invariant cysteine residue in this motif is responsible for the catalytic activity of the enzyme, and substitution of the cysteine for a serine residue abrogates activity (Streuli et al., 1989; Guan and Dixon, 1990; Guan et al., 1991). Within the PTP family, the dual-specificity phosphatases are unique in their ability to catalyze the dephosphorylation of phosphoserine and phosphothreonine residues in addition to phosphotyrosine residues (Guan et al., 1991; Charles et al., 1992; Alessi et al., 1993; Patterson et al., 2009). Notably, the tumor suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome 10), a nontypical member of the dual-specificity PTP family, catalyzes the dephosphorylation of phosphatidylinositides (Myers et al., 1997; Maehama and Dixon, 1998).

A screen for new dual-specificity phosphatases based on the sequence of the catalytic motif of PTEN resulted in the discovery of PTP localized to mitochondrion 1 (PTPMT1)

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**ABBREVIATIONS:** PTP, protein tyrosine phosphatase; PTPMT1, PTP localized to mitochondrion 1; VHR, *Vaccinia virus* VH1-related phosphatase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; VDAC, voltage-dependent anion channel protein; O-MFP, 3-O-methylfluorescein phosphate cyclohexyl ammonium salt; PI5P, phosphatidylinositol 5-phosphate; PBS, phosphate-buffered saline; ANOVA, analysis of variance; IC$_{50}$, concentration that inhibits response by 50%; shRNA, short hairpin RNA.

(Pagliarini et al., 2004). PTPMT1 enjoys the distinction of being among the first protein phosphatases found to localize primarily to mitochondria, where it resides on the inner membrane facing the mitochondrial matrix (Pagliarini et al., 2005). Interestingly, PTPMT1 has been identified in pancreatic islets (Pagliarini et al., 2005). In the β-cell, the sole insulin-producing cell in the body, knockdown of expression of PTPMT1 resulted in a dramatic increase of cellular ATP levels and insulin secretion (Pagliarini et al., 2005), suggesting that PTPMT1 may be a potential target in the β-cell for the treatment of type II diabetes.

Although the localization of PTPMT1 to the mitochondria and its impact on insulin secretion pointed to a potential role in β-cell metabolism, further interrogation of the biology was somewhat limited by the paucity of tools available to target the enzyme, particularly during short-term studies. Indeed, even the endogenous substrate of PTPMT1 in the β-cell is still being investigated because, in spite of the homology of its catalytic motif to that of PTEN and its ability to use phospholipid substrates in vitro (Pagliarini et al., 2004), such activity has not yet been shown in cells (Pagliarini et al., 2005). Thus, to facilitate further study of PTPMT1 and its role in β-cell metabolism in particular, we undertook a search for inhibitors of the enzyme. 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 affected by their dysregulation (Lai et al., 2009).

Because 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 toward identifying an inhibitor of the enzyme. Screening of a commercially available small-molecule library yielded alexidine dihydrochloride, a dibiguanide compound, as an effective inhibitor of PTPMT1. Kinetic studies suggested that alexidine dihydrochloride bound cooperatively and inhibited PTPMT1 in a predominantly uncompetitive manner. In isolated rat pancreatic islets, alexidine dihydrochloride induced insulin secretion in a dose-dependent manner, whereas in a pancreatic β-cell line it affected the mitochondrial phosphoprotein profile, thus phenocopying the effect of knockdown of cellular expression of PTPMT1. Taken together, these studies not only demonstrate the ability of alexidine dihydrochloride to inhibit PTPMT1 and induce increased insulin secretion, thus supporting the notion that PTPMT1 could serve as a pharmacological target in the treatment of type II diabetes, but they also support the use of alexidine dihydrochloride as a tool to facilitate further study of PTPMT1.

## Materials and Methods

**Materials.** Recombinant VHR (*Vaccinia virus* VH1-related phosphatase), PTEN, and PTPMT1 were prepared as described previously (Denu et al., 1995; Maehama and Dixon, 1998; Pagliarini et al., 2004). T-cell PTP and λ protein phosphatase and accompanying buffers were purchased from New England Biolabs (Ipswich, MA). Alexidine dihydrochloride was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), and chlorhexidine dihydrochloride, phenformin, metformin, and 3-*O*-methylfluorescein phosphate cyclohexyl ammonium salt were purchased from Sigma-Aldrich (St. Louis, MO). *N*-(2-ethylhexyl)-*N'*-proplyimidodicarbonimidic diamide (half alexidine) was synthesized by the Duke Center for Chemical Biology (Durham, NC) with purity of the final product (approximately 95%) being determined by mass spectrometry and NMR elemental analysis. PTPMT1-targeted shRNA adenovirus was prepared as described previously (Pagliarini et al., 2005). PTPMT1 rabbit polyclonal antibody was prepared as described previously (Pagliarini et al., 2005), voltage-dependent anion channel protein (VDAC) monoclonal antibody was purchased from Calbiochem (San Diego, CA), and phospho-threonine rabbit polyclonal antibody was purchased from Cell Signaling Technology Inc. (Danvers, MA). The $^{125}$I-insulin radioimmunoassay kit was obtained from DPC/Siemens Healthcare Diagnostics (Deerfield, IL). INS-1 cells were obtained from the laboratory of Christopher Newgard at Duke University (Durham, NC).

**Screening for Inhibitors of PTPMT1.** The Prestwick library of approximately 1000 small molecules dissolved in Me$_2$SO (Prestwick Chemical Inc., Washington, DC) was screened by using the substrate *O*-MFP in an in vitro assay (Gottlin et al., 1996) in a 384-well plate format with a Beckman Biomek FX robot (Beckman Coulter, Brea, CA) under conditions where the Z′ score for the assay (Zhang et al., 1999) was more than 0.5. In brief, the assay was carried out in a total reaction volume of 40 μl in a solution 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, 5% Me$_2$SO, and 44 nM PTPMT1. The reaction was initiated by addition of enzyme, allowed to proceed for 40 min at room temperature, and then quenched with 40 μl of 0.4 M NaOH. Product formation was determined by reading fluorescence emission at 520 nm. The 10 compounds showing the most potent inhibition were then subjected to a second round of screening using compound concentrations of both 50 and 5 μM. In these assays, product formation was monitored by both fluorescence emission at 520 nm and absorbance at 477 nm to ensure that decreased fluorescence indicated decreased product formation and true inhibition, rather than quenching of the fluorescence signal.

**Kinetic Studies.** After the initial screen, it was determined that the optimal pH for PTPMT1 phosphatase activity with the substrate *O*-MFP was 7.0. Hence all kinetic experiments were carried out at this pH. Concentration that inhibits response by 50% (IC$_{50}$) determinations for inhibition of PTPMT1 were carried out by using 44 nM enzyme in a total reaction volume of 100 μl at pH 7.0 in the buffer system described above. VHR phosphatase assays were carried out by using 21.8 nM enzyme in the same buffer system, except that the pH was adjusted to 6.5, and λ protein phosphatase and T-cell PTP assays were carried out in buffers provided by the supplier using 44.8 and 7.2 nM enzyme, respectively. All assays were carried out with 50 μM *O*-MFP as the substrate at the optimal pH for each enzyme, and product formation was monitored continuously by fluorescence with initial velocity readings used for the analysis. Kinetic characterization of the inhibition of PTPMT1 by alexidine dihydrochloride was carried out with 88 nM enzyme to optimize signal and in a total reaction volume of 100 μl at pH 7.0. PTEN assays were carried out as described previously (Maehama et al., 2000) by using 0.4 μM enzyme and di-C16-PI 3,4,5P$_3$ substrate (Echelon Biosciences Inc., Salt Lake City UT). Assays using phosphtidylinositol 5-phosphate (PI5P) as substrate were carried out as described previously (Pagliarini et al., 2004).

Kinetic analysis and modeling were performed by using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). IC$_{50}$ values were fit with variable slope, and inhibition was modeled by using the following equations:

Competitive inhibition: $K_{mApparent} = K_m{}^*(1 + [I]/K_i)$;

$$Y = V_{max}{}^*X/(K_{mApparent} + X) \quad (1)$$

Noncompetitive inhibition: $V_{maxApparent} = V_{max}/(1 + [I]/K_i)$;

$$Y = V_{maxApparent}{}^*X/(K_m + X) \quad (2)$$

Uncompetitive inhibition: $V_{maxApparent} = V_{max}/(1 + [I]/(a{}^*K_i))$;

$K_{mApparent} = K_m/(1 + [I]/(a{}^*K_i)); Y = V_{maxApparent}{}^*X/(K_{mApparent} + X) \quad (3)$

Mixed model inhibition: $V_{\text{maxApparent}} = V_{\text{max}}/(1 + [\text{I}]/(a^*K_i));$

$$K_{\text{mApparent}} = K_m^*(1 + [\text{I}]/K_i)/(1 + [\text{I}]/(a^*K_i));$$

$$Y = V_{\text{maxApparent}}^*X/(K_{\text{mApparent}} + X) \quad (4)$$

**Insulin Secretion Assays and Cytotoxicity Assays.** Islets were harvested from Wistar rats weighing 250 to 300 g under a protocol approved by the Duke University Animal Care and Use Committee. Islets were isolated by collagenase digestion of the pancreas followed by separation on a density gradient as described previously (Milburn et al., 1995), and maintained in RPMI medium 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 then used in glucose-stimulated insulin secretion assays as described previously (Joseph 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 $KH_2PO_4$, 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 and washed for 1 h at 37°C in fresh buffer containing 2.8 mM glucose. Islets were then successively incubated for 1-h 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 after islet incubations, and islet insulin content was assessed from islet lysate, via radioimmunoassay. For alexidine dihydrochloride dose-response experiments, insulin secretion assays were carried out the day after islet isolation by 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 approximately 15 h later, and insulin secretion assays were performed 72 h later by using 20 islets in triplicate for each condition.

Drug cytotoxicity was assessed by using a cell membrane integrity-based cytotoxicity assay, the Toxilight BioAssay (Lonza, Basel Switzerland), according to the manufacturer's instructions. The assay uses leakage of the cytoplasmic protein adenylate kinase across damaged cell membranes into surrounding medium as a measure of reduced cell membrane integrity and hence increased drug cytotoxicity. This cytotoxicity assay was used with the insulin secretion assays described above to assess alexidine dihydrochloride-induced cytotoxicity during the time course of the insulin secretion assay. Alexidine dihydrochloride-induced increase in the release of adenylate kinase was measured relative to levels of adenylate kinase released in the absence of the drug. Islets treated with 0.2% Triton X-100 to induce complete cell lysis served as the 100% cytotoxicity reference.

**Analysis of Mitochondrial Protein Phosphorylation.** INS-1 cells were cultured in RPMI 1640 medium supplemented with 8 mM glucose, 2 mM glutamine, 10 mM HEPES, and 1 mM sodium pyruvate. For the assay, approximately $2.5 \times 10^6$ INS-1 cells were plated in 10-cm plates. After 24-h 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 h, the cells were washed in phosphate-buffered saline (PBS) without magnesium and calcium, and then incubated in insulin assay buffer (see above) containing 2.8 mM glucose for 1 h. The buffer was then replaced, with uninfected cells receiving buffer supplemented with 4 μM alexidine dihydrochloride. After 2-h incubation, the cells were washed with PBS, and a mitochondria-enriched cell lysate was prepared as described previously (Ronnebaum et al., 2006). In brief, the cells were harvested by scraping in PBS followed by centrifugation at 500*g*, the pellet was resuspended in ice-cold

buffer containing 220 mM mannitol, 70 mM sucrose, 5 mM HEPES and homogenized in a 2-ml Dounce homogenizer, and the homogenate was centrifuged at 500*g*. The supernatant was then centrifuged at 16,000*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 $MgCl_2$ with protease inhibitors], and 100 μg of protein was resolved on a 4 to 20% Tris-glycine gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose for immunoblot analysis using antiphosphothreonine antisera.

**Immunoblot Analysis.** Immunoblot analysis was carried out after transfer of proteins resolved on a Tris-glycine gel to nitrocellulose membranes. Analysis used the Odyssey system (LI-COR Biosciences, Lincoln, NE) following the manufacturer's protocol using near-infrared fluorescence detection. Membranes were treated with LI-COR Odyssey Blocking Buffer/Tris-buffered saline (1:1, v/v) solution, with primary antibodies being diluted in this solution supplemented with 0.1% Tween 20, and infrared dye-labeled secondary antibodies (Invitrogen) being diluted in this solution supplemented with 0.1% Tween and 0.01% sodium dodecyl sulfate. Infrared signals at 680 or 800 nm were detected by using the LI-COR Odyssey scanner. Images of the immunoblots were produced, and relative amounts of signal on the nitrocellulose membrane were quantified by using LI-COR Odyssey software version 2.1.

**Statistical Analysis.** Graphing and statistical analysis were carried out with GraphPad Prism 5. Data are reported as mean ± S.E.M. Statistical significance was assessed using ANOVA and appropriate post hoc tests.

## Results

**Identification of Alexidine Dihydrochloride as a Selective Inhibitor of PTPMT1.** To search for an inhibitor of PTPMT1, the first order of business was to develop a suitable assay. Because the endogenous substrates of PTPMT1 are still being investigated, we elected to use a synthetic small-molecule phosphatase substrate in our screens. The compound 3-*O*-methyl fluorescein phosphate (*O*-MFP) was chosen as there is ample precedence for its use as a small-molecule substrate for dual-specificity protein tyrosine phosphatases (Gottlin et al., 1996; Johnston et al., 2007; Song et al., 2009), and because it was amenable to both fluorescence- and absorbance-based readouts, thus facilitating development of a high-throughput assay. In addition, in preliminary determinations the $K_m$ obtained for PTPMT1 with *O*-MFP was 39 μM, which is in close agreement with the $K_m$ of 37.5 μM reported for PTPMT1 with its previously identified substrate phosphtidylinositol 5-phosphate (Pagliarini et al., 2004).

Using the *O*-MFP assay, we screened the Prestwick Chemical Library, a commercially available library of approximately 1000 small molecules with previously characterized pharmacokinetic properties; the use of this library was felt to increase the likelihood of identifying an inhibitor with good bioavailability. In the initial screen (with Z′ scores ranging from 0.57 to 0.85) of the library, approximately 8% of the compounds reduced enzyme activity by more than 50% at 50 μM concentration. Subsequent secondary assays of the compounds showing greatest inhibition revealed that fewer than 1% of the library compounds inhibited PTPMT1 by more than 50% at 5 μM concentration in both the fluorescence- and absorbance-based formats. The dibiguanide compound alexidine dihydrochloride was chosen for further study, as it was among those compounds demonstrating greatest inhibition of the enzyme and showed similar inhibition in both the fluo-

rescence- and absorbance-based assays (reducing phosphatase activity to 20% in the former assay and 13% in the latter assay), thus indicating that its effectiveness was not caused by simple quenching of the fluorescence signal of the product.

Initial kinetic analysis of the capacity of alexidine dihydrochloride to inhibit PTPMT1 demonstrated an IC$_{50}$ of 1.08 ± 0.08 μM with an accompanying Hill coefficient of 2.16 ± 0.31 (Fig. 1). To validate these results, we also evaluated the ability of alexidine dihydrochloride to inhibit PTPMT1 by using phosphatidylinositol 5-phosphate (PI5P), a potential biological substrate of the enzyme. Encouragingly, the IC$_{50}$ obtained using O-MFP as a substrate was in very close agreement with the IC$_{50}$ obtained using PI5P, 1.09 μM ± 0.27 (Supplemental Fig. 1). This supported the assignment of alexidine dihydrochloride as a bona fide inhibitor of PTPMT1 and the applicability of O-MFP as an appropriate substrate for these enzymatic studies.

Having established alexidine dihydrochloride as an inhibitor of PTPMT1, we then investigated whether the inhibition was selective toward this protein phosphatase. Hence, the capacity of the compound to inhibit a variety of related enzymes, including a protein serine/threonine phosphatase, λ protein phosphatase; the classical, tyrosine-specific PTP, T-cell protein tyrosine phosphatase; and the dual-specificity PTP, VH1-related phosphatase and PTEN, under optimal buffer and pH conditions for each enzyme, was assessed. Importantly, none of these protein phosphatases was appreciably inhibited by alexidine dihydrochloride (Fig. 1). The result with PTEN was particularly noteworthy because the

sequence of the catalytic motif of the active site of PTEN formed the basis for the bioinformatics screen that led to the discovery of PTPMT1, and the two enzymes differ in this sequence by just two amino acids (Pagliarini et al., 2004).

**The Dibiguanide Structure of Alexidine Dihydrochloride is Important for Its Inhibition of PTPMT1.** Having established alexidine dihydrochloride as a selective inhibitor of PTPMT1, we were keen to determine the structural features of the compound that contribute to this inhibition. The striking duplication in the structure of alexidine dihydrochloride prompted us to investigate whether this binate aspect contributed to the ability of the compound to inhibit PTPMT1, and whether other biguanide and dibiguanide compounds also inhibited PTPMT1. Interestingly, we found that the dibiguanide compound chlorhexidine dihydrochloride significantly inhibited PTPMT1, albeit with a log-fold higher IC$_{50}$ compared with alexidine dihydrochloride; the IC$_{50}$ determined for the chlorhexidine analog was 19.7 ± 3.3 μM (Fig. 2). In contrast, a monobiguanide compound comprising half of the alexidine dihydrochloride structure inhibited PTPMT1 very weakly with an IC$_{50}$ of 207 ± 43 μM and an accompanying Hill coefficient close to 1 (Fig. 2). In addition, two other monobiguanide compounds, the type II diabetes drugs metformin and phenformin, did not significantly inhibit PTPMT1 (Fig. 2). Thus, the dibiguanide structure conferred a significantly higher level of potency of inhi-
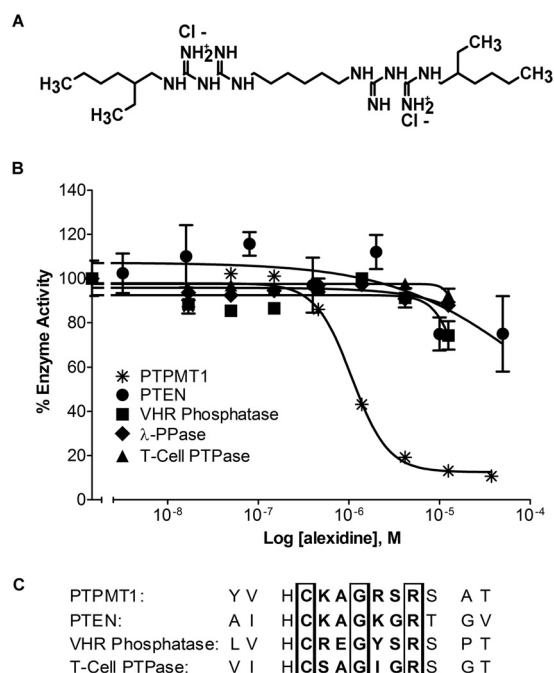


**Fig. 1.** Alexidine dihydrochloride is a selective inhibitor of PTPMT1. A, structure of alexidine dihydrochloride. B, inhibition of selected phosphatases by alexidine dihydrochloride. PTPMT1, VHR phosphatase, λ-Ppase, and T-cell PTPase assays were carried out with O-MFP as the substrate (n = 3), and PTEN assays were carried out with lipid substrate (n = 2). All assays were carried out by using optimum buffer and pH for each enzyme. The IC$_{50}$ with PTPMT1 was 1.08 μM ± 0.08 with a Hill coefficient of 2.16 ± 0.31. Data are presented as the mean ± S.E.M. 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.
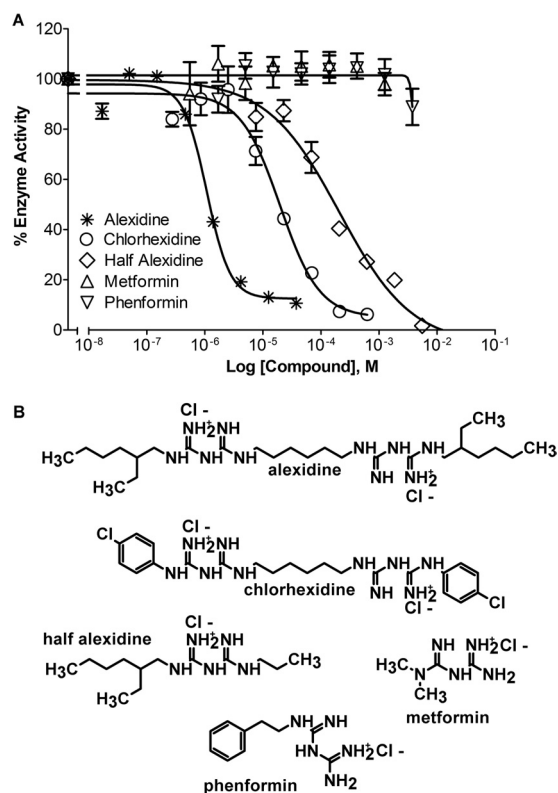


**Fig. 2.** The dibiguanide structure of alexidine dihydrochloride is important for its inhibition of PTPMT1. A, inhibition of PTPMT1 by various biguanide and dibiguanide compounds using O-MFP as the substrate. Inhibition by alexidine dihydrochloride yielded IC$_{50}$ of 1.08 ± 0.08 with Hill coefficient of 2.16 ± 0.31, whereas inhibition by chlorhexidine dihydrochloride yielded IC$_{50}$ of 19.7 ± 3.3 with Hill coefficient of 1.3 ± 0.3, and inhibition by the half alexidine molecule yielded IC$_{50}$ of 207.1 ± 43.1 with Hill coefficient of 0.7 ± 0.1. Data represent the mean ± S.E.M. of three independent experiments. B, structures of dibiguanide and biguanide compounds tested for inhibition of PTPMT1.

bition of PTPMT1 compared with the monobiguanide structure, and the dibiguanide structure appeared to be important for the cooperativity observed in the Hill slope with alexidine dihydrochloride.

**Inhibition of PTPMT1 by Alexidine Dihydrochloride Is Predominantly Uncompetitive.** After our preliminary structure activity relationship studies that established the importance of the dibiguanide structure to the potency of alexidine dihydrochloride as a PTPMT1 inhibitor, we sought to characterize this inhibition in greater detail. Incubation of PTPMT1 with alexidine dihydrochloride resulted in reductions in both $V_{max}$ and $K_m$ with increasing inhibitor concentration (Fig. 3), suggesting that alexidine did not inhibit PTPMT1 in purely competitive or noncompetitive manners. In addition, fitting of the enzyme activity in the presence of varying concentrations of the inhibitor to models for competitive, noncompetitive, uncompetitive, and mixed inhibition revealed that the data fit the uncompetitive and mixed inhibition models best (Fig. 3; Table 1). Using GraphPad Prism 5.0 to fit the data to a model for mixed inhibition, which is a general one incorporating competitive, noncompetitive and uncompetitive inhibition, gave a value of $\alpha = 0.097 \pm 0.071$, suggesting significant uncompetitive character.

**Alexidine Dihydrochloride Stimulates Insulin Secretion from Isolated Rat Pancreatic Islets and Affects**

TABLE 1
Comparison of the fit of the data for inhibition of PTPMT1 by alexidine dihydrochloride to various kinetic models

| Model of Inhibition | $R^2$ Value |
|---|---|
| Competitive | 0.8525 |
| Noncompetitive | 0.9094 |
| Uncompetitive | 0.9244 |
| Mixed | 0.9275 |

**Protein Phosphorylation in a Pancreatic β-Cell Line in a Fashion Similar to Genetic Inhibition of PTPMT1.** Having determined that alexidine dihydrochloride was an effective chemical inhibitor of PTPMT1 in vitro, we were keen to explore whether the compound would inhibit PTPMT1 in cells. We were hopeful of this in part due to PTPMT1 being resident on the matrix-facing side of the inner mitochondrial membrane, and lipophilic dications (a structural class of which alexidine dihydrochloride is a member) 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 et al., 2006; Murphy and Smith, 2007). As the role of PTPMT1 had previously been studied in the pancreatic β-cell, and genetic perturbation had been shown to affect insulin secretion, we used this system as our model. Because lipophilic dications have also been reported to show cellular toxicity at high concentrations (Severina et al., 2007) and most previous studies of alexidine dihydrochloride have focused on its antimicrobial and cytotoxic effects (Gilbert and Moore, 2005; Yip et al., 2006), we first ensured that the assays were performed at concentrations that did not affect cell viability. Through use of a cell membrane integrity-based cytotoxicity assay, we found that alexidine dihydrochloride did not significantly affect membrane permeability and viability of the cells of pancreatic islets at concentrations up to 4 μM, hence this was the maximum concentration used in the assays.

As noted above, genetic inhibition of PTPMT1 using shRNA to knock down endogenous protein levels in the pancreatic β-cell line had previously been shown to result in increased insulin secretion (Pagliarini et al., 2005). Hence, we investigated whether treatment of β-cells with alexidine dihydrochloride would phenocopy this result. We elected to use primary rat islets in these experiments as a more physiological model. To this end, isolated rat islets were treated with alexidine dihydrochloride at both basal (2.8 mM) and stimulatory (16.7 mM) glucose concentrations, and the accompanying changes in insulin secretion and cytotoxicity were monitored. Importantly, alexidine dihydrochloride showed little cytotoxicity (less than 4%) and induced a dose-dependent increase in insulin secretion from the islets at both basal and stimulatory glucose concentrations (Fig. 4). This effect on insulin secretion was found to be statistically significant at both glucose concentrations using ANOVA, and 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 with untreated controls.

Having obtained evidence in pancreatic rat islets that treatment with alexidine dihydrochloride phenocopied the reported effect of PTPMT1 knockdown on insulin secretion from β-cells, we next set out to determine whether the drug
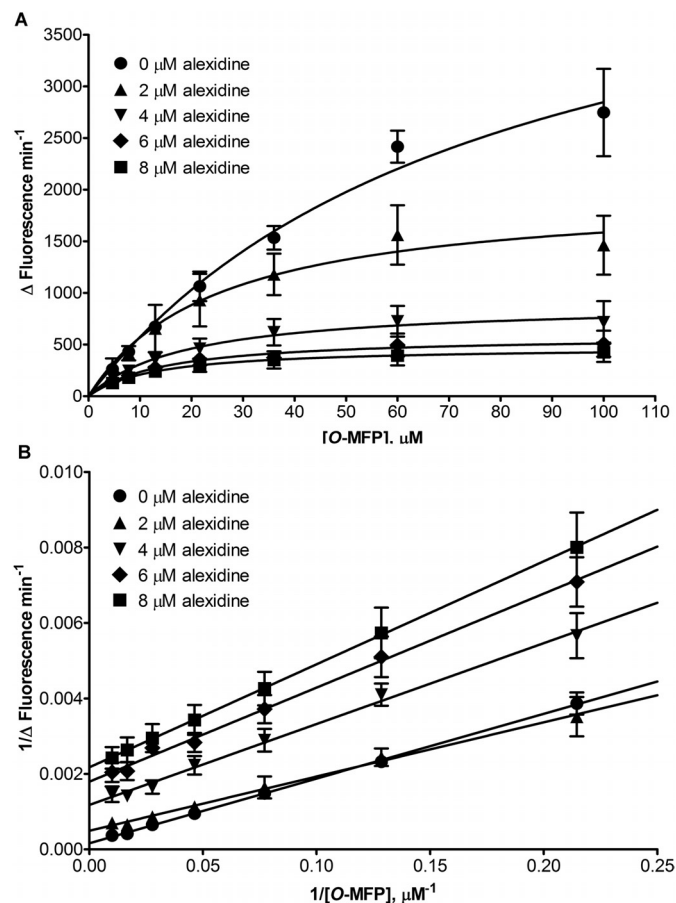


**Fig. 3.** Alexidine dihydrochloride is a predominantly uncompetitive inhibitor of PTPMT1. A, PTPMT1 phosphatase activity in the presence of varying concentrations of alexidine dihydrochloride using $O$-MFP as substrate. Alexidine dihydrochloride reduced both $V_{max}$ and $K_m$. Data represent mean ± S.E.M. of three independent experiments. B, Lineweaver–Burk plot of the data presented in A.
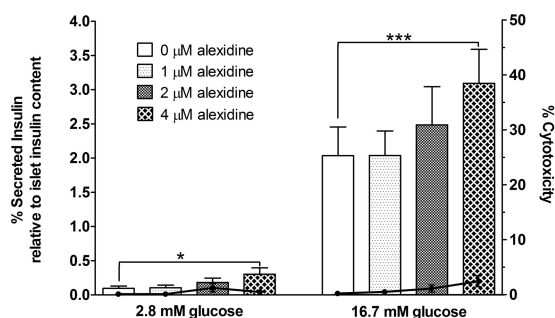
**Fig. 4.** Alexidine dihydrochloride induces a dose-dependent increase in insulin secretion from rat islets. Rat islets were treated with the indicated concentrations of alexidine dihydrochloride, first in the presence of buffer containing basal (2.8 mM) glucose concentration and subsequently in the presence of buffer containing stimulatory (16.7 mM) glucose concentration. The alexidine dihydrochloride-induced dose-dependent increase in insulin secretion (bar graph plotted on the left $y$-axis), was significant at both basal and stimulatory glucose concentrations. At both basal and stimulatory glucose concentrations the effect of 4 $\mu$M alexidine dihydrochloride was significant compared with untreated controls. Data represent the mean $\pm$ S.E.M. of six independent experiments. Data were analyzed by ANOVA with post hoc Dunnett test for significance. A cell membrane integrity-based cytotoxicity assay, using adenylate kinase release from cells as a measure of increased cytotoxicity, was used alongside the insulin secretion assay. The alexidine dihydrochloride-induced cytotoxicity (line graph plotted on the right $y$-axis) did not exceed 4% and was not significant. Data represent the mean $\pm$ S.E.M. of four independent experiments.

could also affect the phosphoprotein profile of the mitochondria in a manner similar to that observed with PTPMT1 knockdown (Pagliarini et al., 2005). To facilitate the collection of sufficient pancreatic β-cell mitochondria for the analysis of phosphorylation of constituent protein, we decided to use a pancreatic β-cell line. Treatment of INS-1 cells with 4 $\mu$M alexidine dihydrochloride resulted in observable changes in the threonine phosphorylation of several mitochondrial proteins. These included changes in phosphorylation of a 90-, 80-, 65-, 55-, 45-, and 39-kDa protein (Fig. 5). Although the increased phosphorylation of the 65- and 39-kDa protein, which was observed in cells treated with alexidine dihydrochloride, was not observed in cells treated with the PTPMT1-targeted shRNA (Fig. 5), the increased phosphorylation of the 80-kDa protein and decreased phosphorylation of the 90-, 55-, and 45-kDa proteins, which was observed upon treatment of cells with alexidine dihydrochloride, was also observed, albeit to greater or lesser extents, in cells treated with the PTPMT1-targeted shRNA (Fig. 5). Importantly, when quantified relative to the protein VDAC and normalized using the cells treated with control shRNA, the effect of alexidine dihydrochloride on the phosphorylation of the 90-, 80-, and 45-kDa proteins was found to be statistically significant (Fig. 5B); a decrease in threonine phosphorylation of a 45-kDa mitochondrial protein has previously been reported to occur upon reduction of expression of PTPMT1 by RNA interference (Pagliarini et al., 2005).

**Evidence that the Impact of Alexidine Dihydrochloride on Insulin Secretion Is Caused by an Impact on PTPMT1.** Having obtained evidence in rat pancreatic islets that alexidine dihydrochloride phenocopied the reported effect of knockdown of PTPMT1 expression on insulin secretion in β-cells, we next sought to confirm that this activity of the compound was caused by its ability to inhibit PTPMT1. To this end, we investigated whether the cellular effects of alexidine dihydrochloride were influenced by perturbations of the
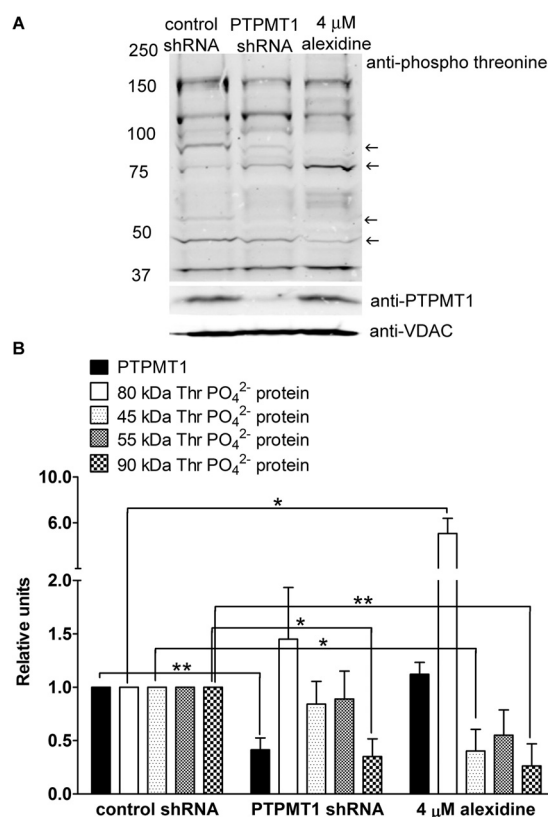


**Fig. 5.** Alexidine dihydrochloride treatment phenocopies the effect of knockdown of PTPMT1 on threonine phosphorylation of mitochondrial proteins. A, representative immunoblot showing the impact of alexidine dihydrochloride on the threonine phosphorylation of mitochondrial proteins. INS-1 cells were treated with PTPMT1-targeted shRNA (48 h) or alexidine dihydrochloride (2 h). Threonine phosphorylation of proteins in the mitochondria-enriched fraction of INS-1 cell lysate was then assessed by immunoblot using a phosphothreonine antibody. Both alexidine dihydrochloride and knockdown of PTPMT1 increased threonine phosphorylation of an 80-kDa protein and decreased threonine phosphorylation of a 90-, 55-, and 45-kDa protein (indicated by arrows). B, quantification of relative amounts of PTPMT1 and threonine-phosphorylated 90-, 80-, 55-, and 45-kDa proteins in the mitochondrial enriched fraction of INS-1 cell lysate after shRNA-mediated knockdown of PTPMT1 protein levels or treatment of cells with alexidine dihydrochloride. Protein was quantified from immunoblots like the representative one shown in A. The amount of each protein of interest was quantified relative to the amount of VDAC protein in the mitochondria-enriched cell lysate and normalized to the amount of that particular protein in the lysate from cells treated with the control shRNA. Data represent mean values $\pm$ S.E.M. from four independent experiments. Data were analyzed by ANOVA with post hoc Dunnett test for significance.

endogenous level of PTPMT1. In isolated rat islets, partial knockdown of PTPMT1 expression, typically to approximately 45 to 60% of the endogenous level, resulted in a statistically significant increase in insulin secretion (Fig. 6). In control islets in which PTPMT1 expression was not knocked down, treatment with 4 $\mu$M alexidine dihydrochloride also resulted in a statistically significant increase in insulin secretion. However, in islets in which the level of expression of PTPMT1 was reduced through use of shRNA, the alexidine dihydrochloride-stimulated increase in insulin secretion was blunted (Fig. 6). Statistical analysis using ANOVA and the Bonferroni test post hoc showed that, although the effect of alexidine dihydrochloride was significant in control islets, the effect of the drug was not significant in islets in which the level of expression of PTPMT1 had been reduced. This suggests that the alexidine dihydrochloride-
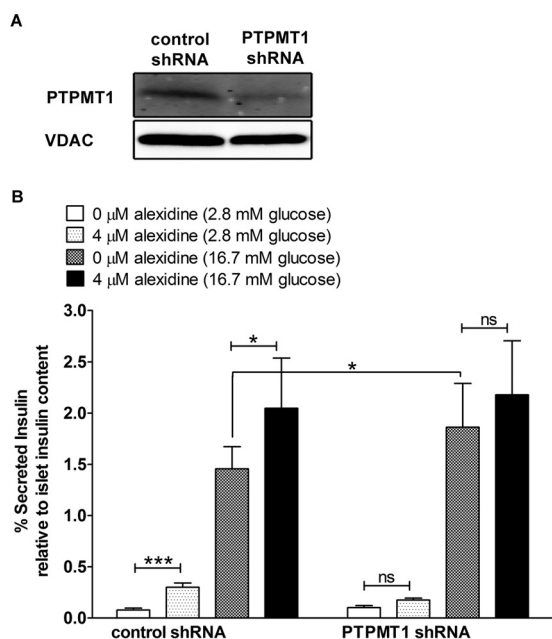
A



B



**Fig. 6.** Stimulation of insulin secretion by alexidine dihydrochloride depends on the presence of PTPMT1. A, representative immunoblot analysis of the level of knockdown of PTPMT1 achieved with PTPMT1-targeted shRNA in islets after 72 h. Typical knockdown of PTPMT1 was 45 to 60% of the endogenous protein level. B, impact of alexidine dihydrochloride on insulin secretion from islets in which PTPMT1 expression is reduced. Rat islets were treated with PTPMT1-targeted shRNA or a control shRNA (72 h). Islets were then treated with 4 μM alexidine dihydrochloride for 1 h, first in the presence of basal (2.8 mM) glucose concentration, then in the presence of stimulatory (16.7 mM) glucose concentration. In rat islets in which the level of PTPMT1 expression was unperturbed, the effect of alexidine dihydrochloride on insulin secretion was significant at both basal and stimulatory glucose concentrations; however, when the level of PTPMT1 expression was reduced, the effect of alexidine dihydrochloride was no longer significant. Data represent the mean ± S.E.M. of six independent experiments and were analyzed using ANOVA with the Bonferroni test applied post hoc.

induced stimulation of increased insulin secretion by β-cells of pancreatic islets depends on the presence of a substantial level of cellular expression of PTPMT1 and provides further evidence for mechanism-based activity of the compound in cells.

## Discussion

Over the last decade, genome sequencing and functional genomics efforts have led to the identification of many new protein phosphatases (Alonso et al., 2004). With these new discoveries has come an increasing recognition of the importance of protein phosphatases 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 et al., 2008; Pulido and Hooft van Huijsduijnen, 2008; Vang et al., 2008; Yi and Lindner, 2008). However, although these advancements have been significant, it is also clear that the rate of elucidation of the cellular function of new protein tyrosine phosphatases has somewhat lagged behind their rate of discovery (Alonso et al., 2004). In the quest to gain insight into the cellular role 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 et al., 2007; Jiang and Zhang, 2008).

PTPMT1 was discovered just 5 years ago and subsequently identified as a new member of the dual-specificity subfamily of protein tyrosine phosphatases (Pagliarini et al., 2004), and data on its substrates and cellular roles are still sparse. Although its expression in several highly metabolic tissues, combined with its localization to mitochondria, suggest that it may play an important role in cell metabolism (Pagliarini et al., 2005), the precise nature of this role remains obscure. Thus, there is a clear need for new tools to aid in the study of PTPMT1, and the identification of a selective chemical inhibitor of the enzyme would provide such a tool. In our screen to identify a pharmacological inhibitor of PTPMT1, alexidine dihydrochloride emerged as an effective and selective inhibitor of the enzyme.

Our subsequent studies to characterize the inhibition of PTPMT1 by alexidine dihydochloride revealed several important points. First, the compound did not significantly inhibit other phosphatases tested, including the dual-specificity phosphatase PTEN. Thus, the lack of inhibition of other phosphatases by alexidine dihydrochloride suggests that the compound most likely exploits features of the dual-specificity phosphatase that are unique to PTPMT1. It also implies that alexidine dihydrochloride might not bind the catalytic motif of PTPMT1 or at minimum does not exploit features within the catalytic motif that enable the phosphatidylinositol phosphatase activity of PTEN and PTPMT1. Further evidence in support of this hypothesis comes from the finding that inhibition of PTPMT1 by alexidine dihydrochloride did not appear to be competitive in nature.

Kinetic 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. Furthermore, in our study characterizing the IC$_{50}$ for inhibition of PTPMT1 by alexidine dihydrochloride, we noted a Hill coefficient of 2, suggesting cooperativity of binding of the inhibitor to the enzyme. This cooperativity might be achieved where binding of the inhibitor to one site on the enzyme facilitates binding to a second site on the same molecule (Krstic et al., 2004) or where the enzyme functions as dimer and binding of one enzyme molecule by the inhibitor facilitates binding of the second enzyme molecule. Considering these observations together with the striking binate structure of alexidine dihydrochloride, and the requirement for the binate structure for effective inhibition of the enzyme, we speculate that the compound works by binding sites on each of two molecules of an enzyme dimer, which are only available in close proximity after substrate binding. However, in the absence of a structure for PTPMT1, this would be difficult to determine. There is precedence for a functional requirement for dimer formation of phosphatases, with the best studied being the myotubularins (Berger et al., 2003). Moreover, the existence of functional dimers of several dual-specificity phosphatases has recently been reported (Mark et al., 2008; Koksal et al., 2009).

The identification of alexidine dihydrochloride as an inhibitor in vitro led us to investigate the cellular activity of the compound. After determining that cell viability was not significantly affected by low concentrations of the drug, we

proceeded to examine its effects on cell function at these concentrations. Investigation of the impact of alexidine dihydrochloride on insulin secretion from isolated rat islets yielded data consistent with successful inhibition of PTPMT1 by this compound. In particular, the attenuation of the alexidine-stimulated increase in insulin secretion in pancreatic islets in which PTPMT1 had been genetically inhibited strongly suggests targeting of PTPMT1 by alexidine dihydrochloride. In addition, the observation that alexidine dihydrochloride affects insulin secretion from the β-cells of isolated rat islets not only confirms the positive effect of inhibition of PTPMT1 on insulin secretion (Pagliarini et al., 2005), but strengthens the case for potential targeting of PTPMT1 in the treatment of type II diabetes. Because PTPMT1 is expressed in the liver and the pancreatic β-cell, it will be interesting to determine whether a compound that effectively inhibits PTPMT1 could be used to combat hyperglycemia in type II diabetics.

Investigation of the effect of alexidine dihydrochloride on mitochondrial protein threonine phosphorylation in a pancreatic β-cell line also yielded data consistent with effective inhibition of PTPMT1 by the drug, with four different mitochondrial proteins showing changes in phosphorylation upon treatment with alexidine dihydrochloride which were mimicked by treatment with PTPMT1-targeted shRNA. However, there were increases in phosphorylation of two proteins (39 and 65 kDa) that were observed in cells treated with the drug, but were not observed in cells treated with PTPMT1-targeted shRNA. The increase in phosphorylation of these proteins observed with alexidine dihydrochloride treatment, but not with genetic inhibition of PTPMT1, may reflect differences in the effect of short-term (drug-induced) versus long-term (shRNA-induced) inhibition of PTPMT1 on the phosphorylation of these particular proteins. However, off-target effects of the drug cannot be excluded at his point.

Because 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 with knockdown of expression using RNA interference. Thus, as demonstrated by the data in Fig. 5, acute inhibition of PTPMT1 in cells by alexidine dihydrochloride may facilitate detection of mitochondrial proteins whose phosphorylation is affected by reduced PTPMT1 activity. Such an application would aid in the discovery of other proteins in the signaling pathways in which PTPMT1 is involved and identification of its physiological substrate and elucidation of its functions in the pancreatic β-cell. Identification of substrates of PTPMT1 may be further advanced by the fact that alexidine dihydrochloride seems to be a predominantly uncompetitive inhibitor of PTPMT1. Because the compound binds to the enzyme–substrate complex, it may assist in trapping endogenous substrate in the active site of the enzyme in inhibitor-treated cells. Furthermore, if PTPMT1 does indeed exist as a dimer, the compound may also prove useful in efforts to study the enzyme's structure using X-ray crystallography by stabilizing the dimer (Tabernero et al., 1999).

Interestingly, previous studies of alexidine dihydrochloride have predominantly characterized it as an antimicrobial agent, with its antibacterial activity thought to be linked to nonspecific disruption of bacterial membrane fluidity (Gilbert and Moore, 2005). However, one study has linked the antifungal activity of alexidine dihydrochloride to possible inhibition of secreted and cytosolic fungal phospholipases (Ganendren et al., 2004). In this latter study, along with the observed inhibition of the phospholipase B of *Cryptococcus neoformans*, some inhibition of purified, recombinant porcine phospholipase A2 was also observed with 2.5 μM alexidine dihydrochloride. Although tests for inhibition of this phospholipase in cells were not carried out, this raises the possibility that some cross-reactivity of the compound with phospholipases in mammalian cells could exist. In terms of toxicity to mammalian cells, one study (Yip et al., 2006) has suggested that alexidine dihydrochloride at low micromolar concentrations may be cytotoxic to some cancer cells but less toxic to untransformed cells. At the concentrations used in our experiments with primary pancreatic islet cells, we did not observe significant cytotoxicity while still observing a significant and PTPMT1-selective effect of alexidine on insulin secretion.

In summary, we have identified an effective small-molecule inhibitor of the mitochondrial phosphatase PTPMT1 and obtained convincing evidence that this inhibitor selectively targets the enzyme both in vitro and in cells. Identification of alexidine dihydrochloride has assisted in interrogation of the biology of PTPMT1 in the pancreatic β-cell and it may not only prove useful in further study of the biology of this important enzyme, but it also provides a scaffold for the development of more potent, less toxic, and more selective PTPMT1 inhibitors.

### References

Alessi DR, Smythe C, and Keyse SM (1993) The human CL100 gene encodes a Tyr/Thr-protein phosphatase, which potently and specifically inactivates MAP kinase and suppresses its activation by oncogenic ras in *Xenopus* oocyte extracts. *Oncogene* **8:**2015–2020.

Alonso A, Sasin J, Bottini N, Friedberg I, Osterman A, Godzik A, Hunter T, Dixon J, and Mustelin T (2004) Protein tyrosine phosphatases in the human genome. *Cell* **117:**699–711.

Berger P, Schaffitzel C, Berger I, Ban N, and Suter U (2003) Membrane association of myotubularin-related protein 2 is mediated by a pleckstrin homology-GRAM domain and a coiled-coil dimerization module. *Proc Natl Acad Sci USA* **100:**12177–12182.

Charles CH, Abler AS, and Lau LF (1992) cDNA sequence of a growth factor-inducible immediate early gene and characterization of its encoded protein. *Oncogene* **7:**187–190.

Denu JM, Zhou G, Wu L, Zhao R, Yuvaniyama J, Saper MA, and Dixon JE (1995) The purification and characterization of a human dual-specific protein tyrosine phosphatase. *J Biol Chem* **270:**3796–3803.

Ganendren R, Widmer F, Singhal V, Wilson C, Sorrell T, and Wright L (2004) In vitro antifungal activities of inhibitors of phospholipases from the fungal pathogen *Cryptococcus neoformans*. *Antimicrob Agents Chemother* **48:**1561–1569.

Gilbert P and Moore LE (2005) Cationic antiseptics: diversity of action under a common epithet. *J Appl Microbiol* **99:**703–715.

Gottlin EB, Xu X, Epstein DM, Burke SP, Eckstein JW, Ballou DP, and Dixon JE (1996) Kinetic analysis of the catalytic domain of human cdc25B. *J Biol Chem* **271:**27445–27449.

Guan KL, Broyles SS, and Dixon JE (1991) A Tyr/Ser protein phosphatase encoded by vaccinia virus. *Nature* **350:**359–362.

Guan KL and Dixon JE (1990) Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia*. *Science* **249:**553–556.

Hendriks WJ, Elson A, Harroch S, and Stoker AW (2008) Protein tyrosine phosphatases: functional inferences from mouse models and human diseases. *FEBS J* **275:**816–830.

Jeffrey KL, Camps M, Rommel C, and Mackay CR (2007) Targeting dual-specificity phosphatases: manipulating MAP kinase signaling and immune responses. *Nat Rev Drug Discov* **6:**391–403.

Jiang ZX and Zhang ZY (2008) Targeting PTPs with small-molecule inhibitors in cancer treatment. *Cancer Metastasis Rev* **27:**263–272.

Johnston PA, Foster CA, Shun TY, Skoko JJ, Shinde S, Wipf P, and Lazo JS (2007) Development and implementation of a 384-well homogeneous fluorescence intensity high-throughput screening assay to identify mitogen-activated protein kinase

phosphatase-1 dual-specificity protein phosphatase inhibitors. *Assay Drug Dev Technol* **5:**319–332.

Joseph JW, Jensen MV, Ilkayeva O, Palmieri F, Alarcon C, Rhodes CJ, and Newgard CB (2006) The mitochondrial citrate/isocitrate carrier plays a regulatory role in glucose-stimulated insulin secretion. *J Biol Chem* **281:**35624–35632.

Koksal AC, Nardozzi JD, and Cingolani G (2009) Dimeric quaternary structure of the prototypical dual-specificity phosphatase VH1. *J Biol Chem* **284:**10129–10137.

Krstic D, Krinulovic K, Spasojevic-Tisma V, Joksic G, Momic T, and Vasic V (2004) Effects of digoxin and gitoxin on the enzymatic activity and kinetic parameters of Na+/K+-ATPase. *J Enzyme Inhib Med Chem* **19:**409–415.

Lai JP, Bao S, Davis IC, and Knoell DL (2009) Inhibition of the phosphatase PTEN protects mice against oleic acid-induced acute lung injury. *Br J Pharmacol* **156:** 189–200.

Maehama T and Dixon JE (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273:**13375–13378.

Maehama T, Taylor GS, Slama JT, and Dixon JE (2000) A sensitive assay for phosphoinositide phosphatases. *Anal Biochem* **279:**248–250.

Mark JK, Aubin RA, Smith S, and Hefford MA (2008) Inhibition of mitogen-activated protein kinase phosphatase 3 activity by interdomain binding. *J Biol Chem* **283:** 28574–28583.

Milburn JL Jr, Hirose H, Lee YH, Nagasawa Y, Ogawa A, Ohneda M, BeltrandelRio H, Newgard CB, Johnson JH, and Unger RH (1995) Pancreatic β-cells in obesity. Evidence for induction of functional, morphologic, and metabolic abnormalities by increased long chain fatty acids. *J Biol Chem* **270:**1295–1299.

Murphy MP and Smith RA (2007) Targeting antioxidants to mitochondria by conjugation to lipophilic cations. *Annu Rev Pharmacol Toxicol* **47:**629–656.

Myers MP, Stolarov JP, Eng C, Li J, Wang SI, Wigler MH, Parsons R, and Tonks NK (1997) PTEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc Natl Acad Sci USA* **94:**9052–9057.

Pagliarini DJ, Wiley SE, Kimple ME, Dixon JR, Kelly P, Worby CA, Casey PJ, and Dixon JE (2005) Involvement of a mitochondrial phosphatase in the regulation of ATP production and insulin secretion in pancreatic β-cells. *Mol Cell* **19:**197–207.

Pagliarini DJ, Worby CA, and Dixon JE (2004) A PTEN-like phosphatase with a novel substrate specificity. *J Biol Chem* **279:**38590–38596.

Patterson KI, Brummer T, O'Brien PM, and Daly RJ (2009) Dual-specificity phosphatases: critical regulators with diverse cellular targets. *Biochem J* **418:**475–489.

Pulido R and Hooft van Huijsduijnen R (2008) Protein tyrosine phosphatases: dual-specificity phosphatases in health and disease. *FEBS J* **275:**848–866.

Ronnebaum SM, Ilkayeva O, Burgess SC, Joseph JW, Lu D, Stevens RD, Becker TC, Sherry AD, Newgard CB, and Jensen MV (2006) A pyruvate cycling pathway involving cytosolic NADP-dependent isocitrate dehydrogenase regulates glucose-stimulated insulin secretion. *J Biol Chem* **281:**30593–30602.

Ross MF, Da Ros T, Blaikie FH, Prime TA, Porteous CM, Severina II, Skulachev VP, Kjaergaard HG, Smith RA, and Murphy MP (2006) Accumulation of lipophilic dications by mitochondria and cells. *Biochem J* **400:**199–208.

Severina II, Vyssokikh MY, Pustovidko AV, Simonyan RA, Rokitskaya TI, and Skulachev VP (2007) Effects of lipophilic dications on planar bilayer phospholipid membrane and mitochondria. *Biochim Biophys Acta* **1767:**1164–1168.

Song M, Park JE, Park SG, Lee do H, Choi HK, Park BC, Ryu SE, Kim JH, and Cho S (2009) NSC-87877, inhibitor of SHP-1/2 PTPs, inhibits dual-specificity phosphatase 26 (DUSP26). *Biochem Biophys Res Commun* **381:**491–495.

Streuli M, Krueger NX, Tsai AY, and Saito H (1989) A family of receptor-linked protein tyrosine phosphatases in humans and *Drosophila*. *Proc Natl Acad Sci USA* **86:**8698–8702.

Tabernero L, Evans BN, Tishmack PA, Van Etten RL, and Stauffacher CV (1999) The structure of the bovine protein tyrosine phosphatase dimer reveals a potential self-regulation mechanism. *Biochemistry* **38:**11651–11658.

Vang T, Miletic AV, Arimura Y, Tautz L, Rickert RC, and Mustelin T (2008) Protein tyrosine phosphatases in autoimmunity. *Annu Rev Immunol* **26:**29–55.

Yi T and Lindner D (2008) The role and target potential of protein tyrosine phosphatases in cancer. *Curr Oncol Rep* **10:**114–121.

Yip KW, Ito E, Mao X, Au PY, Hedley DW, Mocanu JD, Bastianutto C, Schimmer A, and Liu FF (2006) Potential use of alexidine dihydrochloride as an apoptosis-promoting anticancer agent. *Mol Cancer Ther* **5:**2234–2240.

Zhang JH, Chung TD, and Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high-throughput screening assays. *J Biomol Screen* **4:**67–73.

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