

Optimization of a Cyclic Peptide Inhibitor of Ser/Thr Phosphatase PPM1D (Wip1)

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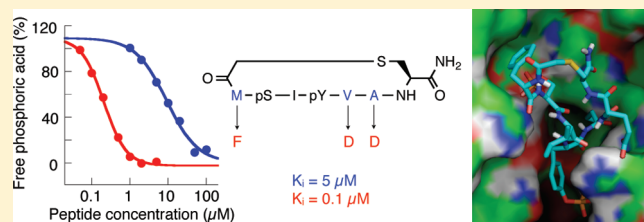
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S Supporting Information

ABSTRACT: PPM1D (PP2C δ or Wip1) was identified as a wild-type p53-induced Ser/Thr phosphatase that accumulates after DNA damage and classified into the PP2C family. It dephosphorylates and inactivates several proteins critical for cellular stress responses, including p38 MAPK, p53, and ATM. Furthermore, PPM1D is amplified and/or overexpressed in a number of human cancers. Thus, inhibition of its activity could constitute an important new strategy for therapeutic intervention to halt the progression of several different cancers. Previously, we reported the development of a cyclic thioether peptide with low micromolar inhibitory activity toward PPM1D. Here, we describe important improvements in the inhibitory activity of this class of cyclic peptides and also present a binding model based upon the results. We found that specific interaction of an aromatic ring at the X1 position and negative charge at the X5 and X6 positions significantly increased the inhibitory activity of the cyclic peptide, with the optimized molecule having a K_i of 110 nM. To the best of our knowledge, this represents the highest inhibitory activity reported for an inhibitor of PPM1D. We further developed an inhibitor selective for PPM1D over PPM1A with a K_i of 2.9 μ M. Optimization of the cyclic peptide and mutagenesis experiments suggest that a highly basic loop unique to PPM1D is related to substrate specificity. We propose a new model for the catalytic site of PPM1D and inhibition by the cyclic peptides that will be useful both for the subsequent design of PPM1D inhibitors and for identification of new substrates.



Kinases and phosphatases are important regulators of protein function in biological systems and thus constitute good targets for the development of new drugs. While the human genome encodes 518 kinases,¹ there are estimated to be only 147 phosphatases; of those, only 40 are serine/threonine phosphatases.^{1–3} The PP2C family in humans consists of seven monomeric serine/threonine phosphatases.^{4,5} This includes PPM1D (also called PP2C δ or Wip1), which was first identified as an enzyme that is induced by wild-type p53 after DNA damage.⁶ Consistent with other members of the PP2C family, PPM1D is a monomeric enzyme that requires divalent cations, either Mn²⁺ or Mg²⁺, for catalytic activity and is insensitive to okadaic acid.⁷ This phosphatase is composed of two major domains: a highly conserved N-terminal phosphatase domain and a less-conserved, noncatalytic domain at the C-terminus.⁷ The known substrates of PPM1D include several proteins critical for cellular stress responses, namely, p38 MAPK,⁸ Chk1,⁹ Chk2,^{10–12} ATM,¹³ and p53.⁹ Dephosphorylation of each of these proteins by PPM1D results in its inactivation. PPM1D is amplified and/or overexpressed in a number of human cancers, such as breast cancer,^{14–16} neuroblastoma,¹⁷ medulloblastoma,¹⁸ ovarian clear cell adenocarcinoma,¹⁹ and

pancreatic adenocarcinoma.²⁰ In addition, PPM1D-null mice show a dramatic tumor-resistant phenotype.²¹ Thus, inhibition of PPM1D activity could constitute an important new strategy for therapeutic intervention to halt the progression of several different cancers.

PPM1D dephosphorylates phosphoserine (pS) or phosphothreonine (pT) as a part of two different peptide motifs: pT-X-pY²² and pS/pT-Q.²³ In a study of the pT-X-pY motif, we observed that PPM1D preferentially dephosphorylates pT from a diphosphorylated sequence compared to a monophosphorylated one and that amino acids adjacent to the motif do not significantly affect the substrate specificity.²⁴ Additionally, it was found that substitution of pS for the pT in the pT-X-pY sequence from p38 MAPK resulted in PPM1D inhibition. This result raised the possibility that a pS-substituted peptide could be developed as an effective inhibitor of PPM1D phosphatase activity. After extensive optimization, a cyclic thioether peptide

Received: December 7, 2010

Revised: April 28, 2011

Published: April 29, 2011

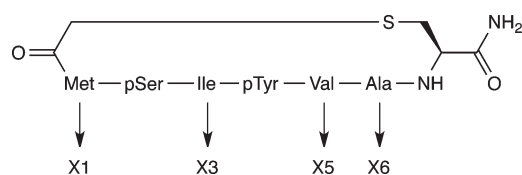


Figure 1. Schematic representation of the cyclic thioether peptide. The thioether bond was formed between the acylated N-terminal residue and the cysteine side chain at the C-terminus.

with the M-pS-I-pY-VAC sequence was identified with a K_i of approximately $5 \mu\text{M}$ (Figure 1).

Combining this result with mutagenesis studies of the protein and a NMR solution structure of the cyclic peptide, we were able to propose a structural model of the complex at the active site.²⁴ For this, we developed a homology model of PPM1D from the crystal structure of the related PPM1A (PP2C α) protein in humans.²⁵ Although this model incorporated the pS and pY residues of the cyclic peptide in key charge–charge interactions with the protein, it did not provide obvious roles for the Met, Ile, and Ala residues. We suggested that at least some of them may interact with a relatively long loop adjacent to the active site (residues 237–268) that is unique to PPM1D. Unfortunately, because of its absence in the PPM1A template, the loop could not be included in our homology model. Although the precise start of the insertion is uncertain, a subsegment of it (residues 245–268) has been dubbed the “B-loop” by Chuman et al.²⁶ because of the preponderance of positively charged amino acids. Interestingly, the B-loop sprouts from a conserved subdomain (residues 165–194 of PPM1A) that has been designated a flap, presumably because of its different conformations observed in the crystal structures of both eukaryotic and prokaryotic homologues^{27–33} (Figure S1 of the Supporting Information). It has been postulated that movement of the flap regulates enzymatic activity through modulation of the binding of a third metal ion, substrate recognition, and/or steric availability to the catalytic site. Thus, the fact that the B-loop is part of the flap subdomain in PPM1D supported the idea that it plays a role in regulation.

In this work, we describe the development of a new cyclic peptide with significantly enhanced inhibitory activity. While the pS-I-pY core residues were maintained, the side chains at the other positions were extensively modified to identify an optimized molecule with a K_i for PPM1D of approximately 100 nM, reflecting a 50-fold improvement over that of the starting peptide. Combining the results of the optimization with the results of strategic mutations in the B-loop allowed further refinement of the model of the PPM1D catalytic domain. We also describe improvements obtained in selectivity for PPM1D over PPM1A by modification of the pS residue of the cyclic peptide. These results further demonstrate the possibility of developing selective inhibitors for this neglected class of phosphatase targets.

EXPERIMENTAL PROCEDURES

Chemical Reagents. Resins and *N*- α -Fmoc-protected amino acids, including phosphorylated amino acids and Fmoc succinimide, were purchased from Novabiochem (San Diego, CA). *N*- α -Fmoc-protected unusual amino acids, including L-2-amino-4-phosphono-4,4-difluorobutyric acid (F₂Pab), were obtained from Anaspec (San Jose, CA). Chloroacetic anhydride, solvents,

and the amino acid standard solution for amino acid analysis were obtained from Sigma-Aldrich (Milwaukee, WI).

Peptide Synthesis. Peptides were synthesized by the solid-phase method utilizing 9-fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl or *tert*-butoxycarbonyl (Boc)/benzyl chemistry. Trityl (Trt) and *p*-methoxybenzyl groups were utilized as Cys side chain protecting groups for Fmoc and Boc chemistry, respectively. Peptides were assembled on NovaPEG Rink amide resin or Wang resin for Fmoc chemistry or MBHA resin for Boc chemistry. The amino group of *O*-sulfo-L-serine was protected by an Fmoc group.³⁴ Phosphorylated Ser and Tyr were incorporated as Fmoc-Ser[PO(OBzl)OH]-OH and Fmoc-Tyr(PO₃H₂)-OH, respectively. In the synthesis of peptides 46 and 48, *O*-sulfo-L-serine and homoserine (Hse) were assembled as Boc-Ser(SO₃H)-OH and Fmoc-Hse(Trt)-OH on MBHA resin, respectively. The coupling reactions were conducted by means of the HBTU-HOBt method. To introduce a phosphate group onto the hydroxyl group of the Hse residue in peptide 48, the trityl group was removed by treatment with a trifluoroacetic acid/dichloromethane/triisopropylsilane mixture (1/94/5, v/v) after the amino group of the Phe residue at position X1 was protected with a Boc group. Phosphorylation was achieved using dibenzyl-*N,N'*-diisopropylphosphoramidate and anhydrous *tert*-butylhydroperoxide. The N-terminus of each peptide was chloroacetylated using chloroacetic anhydride for downstream cyclization. Cleavage of the peptide from the resin was achieved with a trifluoroacetic acid/water/triisopropylsilane mixture (92.5/5/2.5, v/v) or a trifluoromethanesulfonic acid/trifluoroacetic acid/water/triisopropylsilane mixture (10/82.5/5/2.5, v/v) for 2 h at room temperature. After the resin had been removed by filtration, the filtrate was concentrated by flushing with nitrogen gas and crude peptides were precipitated with diethyl ether. The crude peptides were then dissolved in 1% triethylamine-containing water (pH 8–9, approximately 3 mM) and stored at room temperature overnight to allow cyclization to occur. The cyclization reaction was quenched by acidification using acetic acid. Crude peptides were purified using reversed-phase high-performance liquid chromatography (RP-HPLC) on a preparative C4 column (BioAdvantage Pro 300, Thomson Liquid Chromatography) with a water/acetonitrile solvent system containing trifluoroacetic acid. Purified peptides were characterized by matrix-associated laser desorption ionization time-of-flight mass spectrometry (MALDI micro MX, Waters) and RP-HPLC on an analytical C18 column (Eclipse XDB-C18, Agilent). The purity of all peptides was found to be >95%.

Amino Acid Composition Analysis and Determination of Concentration. Amino acid composition analysis was conducted using the phenylthiocarbamyl method.^{35,36} Peptide stock solutions in water ($\sim 5 \text{ mM}$) were transferred to a glass tube for hydrolysis, resulting in $\sim 10 \text{ nmol}$ per glass tube. The peptide was hydrolyzed with a 6 N hydrogen chloride solution at 105 °C in a sealed tube for 24 h. After cooling, the solution was evaporated with nitrogen gas and a water bath. A solution (10 μL) of ethanol, water, and triethylamine (2/1/1, v/v) was added to each tube. After the solution had been removed in vacuo, a phenyl isothiocyanate-containing solution (ethanol/water/triethylamine/phenyl isothiocyanate, 7/1/1/1, v/v) was added to the lyophilized sample. The tube was left for 30 min at room temperature. Subsequently, the sample was redried in vacuo to remove excess reagent. The dried sample was dissolved in 1 mL of 60 mM sodium acetate buffer (pH 6.0), and 10% of the total was analyzed by RP-HPLC as follows: BioAdvantage Pro 300 C18 5 μm (4.6 mm \times 250 mm) column; solution A, 60 mM sodium acetate buffer (pH 6.0);

solution B, acetonitrile; gradient, 5 to 50% B from 0 to 30 min. Identification and quantitation of each amino acid in the solution were performed on the basis of retention times and peak area integration as compared with that of an amino acid standard solution. The peptide concentration was calculated on the basis of a standard curve made by the peptide stock solution described above.

Protein Expression and Purification. The cloning and purification of the human PPM1D catalytic domain (residues 1–420) containing an N-terminal His tag were performed as previously described.²² The plasmid containing the catalytic domain was used for construction of mutants using the Quick-Change protocol as described by the manufacturer (Stratagene, La Jolla, CA). The sequences of the oligonucleotides used for PPM1D R243A were as follows: 5′-GAGTGAATGAACAAGTCTGGGGTTAACGCTGTAGTTTGGAACGACCTCGA CTC-3′ and 5′-GAGTCGAGGTCGTTTCCAACTACAGCGTTAACCCAGACTTGTTTATTACACTC-3′. An HpaI restriction enzyme site was created in the oligonucleotides (underlined) to facilitate screening of mutant clones. For PPM1D K247A, the following oligonucleotides were used: 5′-CTGGGTGAATCGTGTAGTTTGGGCCCCGACCTCGACTCACTCACAATGGACCTG-3′ and 5′-CAGGTCCATTGTGAGTGA GTCGAGGTCGGGCCCCAACTACACGATTACCCCCAG-3′. An ApaI site (underlined) was created in the oligonucleotides for mutant screening.

Individual mutant plasmids were cotransformed with pACYC-Duet (Novagen, Madison, WI) expressing Skp and DsbC proteins to improve the solubility of the proteins in BL21-star competent cells (Invitrogen, Carlsbad, CA). Transformed colonies were selected on LB plates containing ampicillin (100 µg/mL) and chloramphenicol (15 µg/mL). Cells were grown (800 mL) to midlog phase in Circle Grow (Q-Biogen, Irvine, CA) at 37 °C before being cooled to 16 °C. After incubation for approximately 15 min, cells were induced with 0.5 mM isopropyl 1-thio- α -D-galactopyranoside for 16 h. Cells were harvested and the His-tagged mutant PPM1D proteins purified by metal affinity chromatography, as described for the wild-type protein.²² The activity of the purified protein was confirmed by measuring the kinetic properties for dephosphorylation of a substrate peptide from ATM [[S1981pS] human ATM(1976–1986)-GY].

Phosphatase Activity. Phosphatase activities of wild-type and mutant PPM1D were measured by a malachite green/molybdate-based assay (Millipore), as described previously.²² A substrate peptide [[S1981pS] human ATM(1976–1986), AFEEG-pS-QSTTIGY, or [T180pT, Y182pY] human p38 α MAPK(175–185), TDDEM-pT-G-pY-VAT] was incubated with 60 ng of PPM1D in 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.02% 2-mercaptoethanol, 40 mM NaCl, and 30 mM MgCl₂ for 7 min at 30 °C, and the amount of free phosphoric acid released from the substrate was detected by measuring the absorbance at 650 nm from the molybdate–malachite green–phosphate reaction complex. The kinetic parameters K_m and V_{max} were determined with a range of peptide concentrations.

The inhibitory activity of the cyclic peptides was measured as previously described.²² PPM1D was incubated with 30 µM substrate peptide [[S1981pS] human ATM(1976–1986) or [T180pT, Y182pY] human p38 α MAPK] and a cyclic peptide under the same conditions used for the phosphatase activity assay described above. For most assays, peptides were included at 10 µM, with some peptides tested also at 1 µM. Data were expressed as means \pm the standard deviation. Statistical analysis of the inhibitory activity was performed by a Student's *t* test with a *p* < 0.05 statistical

significance. The determination of the apparent inhibitory constant K_i was performed with a range of cyclic peptide concentrations. For most peptides, the concentration was determined from the powder weight of the cyclic peptide; the peptide concentration of peptides 1 and 37 was calculated using amino acid composition analysis.

PPM1A phosphatase activity was measured using the malachite green/molybdate assay described above. Cyclic peptides were incubated with 12 or 24 ng of PPM1A under the same conditions described above. For assays of PPM1A inhibition, cyclic peptide 40 (50 µM) was used as the substrate.

Inhibition Mechanism. The mechanism of inhibition of cyclic thioether peptide 37 was estimated from the initial velocities of dephosphorylation measured at different concentrations of cyclic peptide and [S1981pS] human ATM(1976–1986) peptide, as described above. The observed initial velocities were graphed on double-reciprocal plots and fitted by the Lineweaver–Burk equation (eq 1) using GraphPad Prism 4 (GraphPad Software, La Jolla, CA).

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

where *V* is the reaction velocity, K_m is the Michaelis–Menten constant, V_{max} is the maximum reaction velocity, and [*S*] is the substrate concentration.

Molecular Modeling. The molecular model of PPM1D was updated from that used previously.²⁴ A new sequence alignment with the PPM1A structural template is presented in Figure S2 of the Supporting Information, which also depicts the seven regions of conservation defined by Chuman et al.²⁶ The only changes to the previous model were to regions I and VII, which now make all regions consistent with the model of Chuman et al.²⁶ Although changes to the model in region I involve residues that comprise the active site, the sequence changes are conservative and the impact on the predictive properties of the model is minimal. Specifically, in the previous model, PPM1D residue Arg76 was at the position of the functionally important, phosphate-binding Arg33 residue of PPM1A.^{25,37} In the current model, this is replaced with PPM1D residue Arg18. The change also added metal-binding PPM1D residue Glu22, which substitutes for PPM1A residue Glu37. However, this residue is on the opposite side of the active site from which the substrates approach and bind. Addition of PPM1D residues Lys19 and Tyr20 to positions proximal to (above) the metal center is significant, as they are featured in the optimization of the cyclic peptide inhibitor (see Results).

Models of the cyclic peptide inhibitor bound to PPM1D were energy-minimized with the CHARMM software package.³⁸ The backbone of the cyclic peptide was the same as in the structure determined by NMR in our previous study.²⁴ Residue topology files for the different side chains tested on the inhibitor were developed manually using the “all22_prot” force field of MacKerell et al.³⁹ Because our focus was on the interactions with the substrate or inhibitors, we did not attempt to model the inserted loops and deletions of the PPM1D homology model distant from the active site.

Structural similarity searches of the Protein Data Bank⁴⁰ were conducted with the VAST conformational search engine.⁴¹ Structural alignments, conformational adjustments, and figures were generated with the UCSF Chimera molecular modeling software package.⁴² Sequence alignments and figures were made with BioEdit Sequence Alignment Editor.⁴³

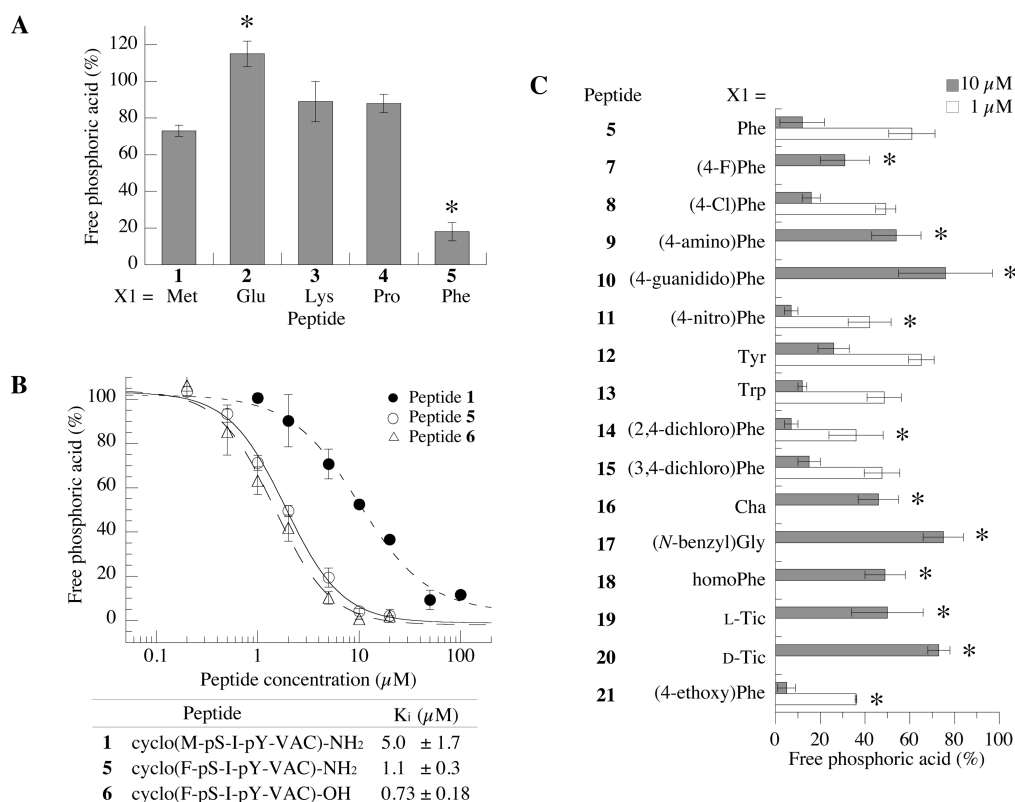


Figure 2. An aromatic residue at position X1 of the cyclic peptide is important for inhibitory activity. (A) Inhibitory activity of cyclic peptides (10 μ M) substituted at position X1 in the presence of [S1981pS] human ATM(1976–1986) peptide (30 μ M) as a substrate (* p < 0.05 as compared with peptide 1). (B) Concentration dependence of inhibition by cyclic peptides: peptide 1, cyclo(M-pS-I-pY-VAC)-amide (●); peptide 5, cyclo(F-pS-I-pY-VAC)-amide (○); peptide 6, cyclo(F-pS-pY-I-VAC)-OH (△). (C) Inhibitory activity of cyclic peptides used at 10 and 1 μ M, as indicated (* p < 0.05 as compared with peptide 5).

Circular Dichroism (CD) Spectroscopy. The CD spectra of wild-type and mutant PPM1D were recorded on a JASCO (Easton, MD) J-715 spectropolarimeter with a cylindrical cell with a 1 mm path length at room temperature. The CD cell was washed with an aqueous NaOH solution before each new measurement to remove any material that might have adhered to the inner surface. Sample solutions [1.8–2.1 μ M PPM1D in 50 mM Tris-HCl, 30 mM MgCl₂, and 5% glycerol (pH 7.5)] were prepared 5 min before measurement. All spectra shown are the average of eight repeated measurements obtained by collecting data from 260 to 190 nm at 0.2 nm intervals with a response time of 2 s for each point. The results are expressed as the mean residue ellipticity.

RESULTS

Phe Substitution at Position X1 Increases the Inhibitory Activity of the Cyclic Peptide against PPM1D. Previously, we identified a cyclic thioether peptide (M-pS-I-pY-VAC, peptide 1) (Figure 1) that functions as an inhibitor of the Ser/Thr phosphatase PPM1D.²⁴ In that peptide, only the X3 position between the pS and pY residues was optimized. Here, we explored the structure–activity relationship for each of the other positions of the peptide with the goals of improving the inhibitory activity and learning more about PPM1D substrate specificity. Initially, position X1 (Figure 1) was varied by replacement of the Met of peptide 1 with different amino acids, and then the inhibitory activities of the analogues were determined. Inhibitory activity was calculated from the ability of the cyclic peptide to suppress dephosphorylation

of pS in a peptide derived from ATM (residues 1976–1986 with phosphorylation of Ser1981) using a malachite green/molybdate assay to detect the amount of free phosphoric acid in solution. Performing this assay in the presence of 10 μ M peptide 1, we observed a 30% decrease in PPM1D dephosphorylation activity (Figure 2A), resulting in a K_i value for peptide 1 of 5.0 \pm 1.7 μ M (Figure 2B). This value is 5-fold reduced compared with that found in the previous report of this inhibitor.²⁴ However, in that study, the peptide concentration was calculated from pY absorbance at 280 nm with 640 M^{−1} cm^{−1} as the molar extinction coefficient.²⁴ As reported, the molar extinction coefficients for pY range from 600 to 700 M^{−1} cm^{−1} at pH 7.0,⁴⁴ and they are strongly dependent on pH.⁴⁵ In this study, we determined the cyclic peptide concentration from the dry weight, which by amino acid composition analysis we found to be more accurate. The concentration of peptide 1 determined in this way was approximately 6-fold higher than the concentration calculated from the molar extinction coefficient used previously, resulting in overestimation of the K_i value. Because of the difficulty associated with purifying large quantities of PPM1D, each new inhibitor was tested at 10 μ M, with some also used at 1 μ M for improved discrimination of the inhibitors.

In our original model of peptide 1 bound to PPM1D, the Met side chain of peptide 1 was proximal to Arg76.²⁴ Thus, we initially hypothesized that mutation of the Met residue to an acidic residue would allow formation of a charge–charge interaction that could improve the inhibitory activity of the cyclic peptide. In contrast to the hypothesis, a peptide in which Glu was substituted

at position X1 (peptide 2) abrogated inhibitory activity of the peptide at 10 μ M and also functioned as a weak substrate for PPM1D (Figure 2A). Further substitution at the X1 position with a Lys residue (peptide 3) or a Pro residue (peptide 4) also did not significantly alter the inhibitory activity compared to that of peptide 1. In contrast, a Phe-substituted analogue (peptide 5) significantly decreased PPM1D phosphatase activity in the presence of 10 μ M peptide (Figure 2A). From measurements at several concentrations of peptide 5, we determined that the K_i value of peptide 5 was 1.1 ± 0.3 μ M (Figure 2B), \sim 5-fold more potent than the original peptide 1. A second cyclic peptide terminating in a carboxylic acid group (peptide 6) rather than an amide as in peptide 5 showed an inhibitory activity [$K_i = 0.73 \pm 0.18$ μ M (Figure 2B)] similar to that of peptide 5, indicating that a negative charge at the extreme C-terminus does not affect inhibitory activity.

To further explore the role of the Phe residue in the X1 position for inhibitory activity, we synthesized a series of peptides containing Phe derivatives and determined their inhibitory activities (Figure 2C). Substitution of position X1 with halogenated phenylalanine derivatives (peptides 7, 8, 14, and 15) did not significantly change the inhibitory activity compared with that of peptide 5. Likewise, substitution of the Phe at position X1 with 4-nitrophenylalanine (peptide 11), Tyr (peptide 12), Trp (peptide 13), and 4-ethoxyphenylalanine (peptide 21) did not greatly improve the inhibitory activity relative to that of peptide 5. These results indicate that addition of a functional group to the 3- and/or 4-position of the aromatic ring does not affect the interaction between the cyclic peptide and PPM1D. In contrast, peptides 9 and 10, containing basic functional groups on the Phe (4-amino-phenylalanine and 4-guanidophenylalanine, respectively), showed decreased inhibitory activities at 10 μ M (Figure 2C). These results indicate that a basic functional group at position X1 disrupts the interaction of the cyclic peptide with PPM1D.

To determine if the position of the aromatic ring of the Phe had an effect on inhibitory activity, we substituted position X1 with *N*-benzylglycine (peptide 17), homophenylalanine (peptide 18), and *L*- and *D*-Tic [(*S*)- and (*R*)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, respectively] (peptides 19 and 20, respectively) (structures in Figure S3 of the Supporting Information). The inhibitory activities of these peptides were reduced relative to that of peptide 5 at 10 μ M (Figure 2C). Peptide 16, containing β -cyclohexylalanine (Cha), which has a cyclohexyl ring instead of the aromatic ring of Phe, also exhibited decreased activity as compared with that of the Phe-substituted peptide, with approximately 50% inhibition of dephosphorylation observed in the presence of 10 μ M peptide 16. Thus, these results suggest that a simple phenyl ring at position X1 of the peptide is important for inhibition, but additional groups attached to the aromatic ring do not further enhance activity.

The Chirality of the Ile Residue at Position X3 Affects PPM1D Inhibition. Our previous work showed that the Ile residue at position X3 is critical for inhibition.²⁴ However, the importance of the stereochemistry of the Ile was not investigated. Therefore, *D*-Ile- and *L*-allo-Ile-substituted peptides (22 and 23, respectively) (Figure 3A) were synthesized, and their inhibitory activity was measured. *D*-Ile peptide 22 showed greatly reduced inhibitory activity (Figure 3B). In contrast, the K_i value of *L*-allo-Ile analogue peptide 23 was 1.1 ± 0.3 μ M, similar to that of peptide 5, which has *L*-Ile (Figure 3B). These results indicate that the chirality at the β -position of the Ile is not important for inhibition while that of the α -position is.

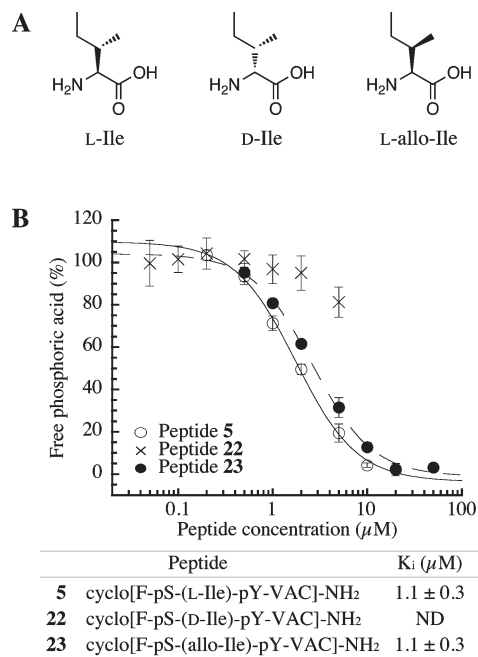


Figure 3. The enantiomer of the Ile at position X3 is important for inhibition of PPM1D phosphatase activity. (A) Chemical structure of isoleucine analogues. (B) Concentration dependence of the inhibition of PPM1D by cyclic peptide [F-pS-(L-Ile)-pY-VAC]-NH₂ [peptide 5 (○)], [F-pS-(D-Ile)-pY-VAC]-NH₂ [peptide 22 (×)], and [F-pS-(L-allo-Ile)-pY-VAC]-NH₂ [peptide 23 (●)] in the presence of [S1981pS] human ATM-(1976–1986) peptide (30 μ M) as a substrate.

Negative Charges at Positions X5 and X6 Are Preferred for Inhibition. Figure 4 shows the inhibitory activities of X5- and X6-substituted cyclic peptides in which the Val and Ala residues were modified to aromatic, acidic, basic, neutral, and aliphatic amino acids. In position X5, the substitution of Val in the original peptide with Lys (peptide 25) or Phe (peptide 26) reduced the inhibitory activity of the cyclic peptide (Figure 4A). Substitution with Leu (peptide 27), Met (peptide 28), Ile (peptide 29), or Cha (peptide 30) did not have any significant effects (Figure 4A). In contrast, a peptide containing Glu (peptide 24) at position X5 exhibited improved inhibitory activity, decreasing PPM1D phosphatase activity by >90 and $>60\%$ at 10 and 1 μ M, respectively.

Substitutions at position X6 with hydrophobic amino acids, as in peptides 33 (Phe), 34 (Leu), and 35 (Met), either did not have any significant effect or significantly reduced the inhibitory activity [peptide 34 (Figure 3B)], while incorporation of Lys at position X6 (peptide 32) also significantly reduced inhibitory activity (Figure 4B). In contrast, increased inhibitory activity was observed when a Glu residue was introduced at position X6 (peptide 31), with PPM1D dephosphorylation activity decreasing by 95 and 80% when the cyclic peptide was used at concentrations of 10 and 1 μ M, respectively. Combined with the results of the screen at position X5, these data suggest that an acidic residue is preferred at positions X5 and X6 for PPM1D inhibition. Indeed, a cyclic peptide containing Gln at position X6 (peptide 36) did not have improved inhibitory activity relative to that of peptide 5 (Figure 4B). Moreover, the improvement associated with introduction of an acidic residue was moderately larger in the X6 position than in the X5 position.

Finally, we determined the effect of having acidic residues at positions X5 and X6 on inhibitory activity. As expected, inclusion

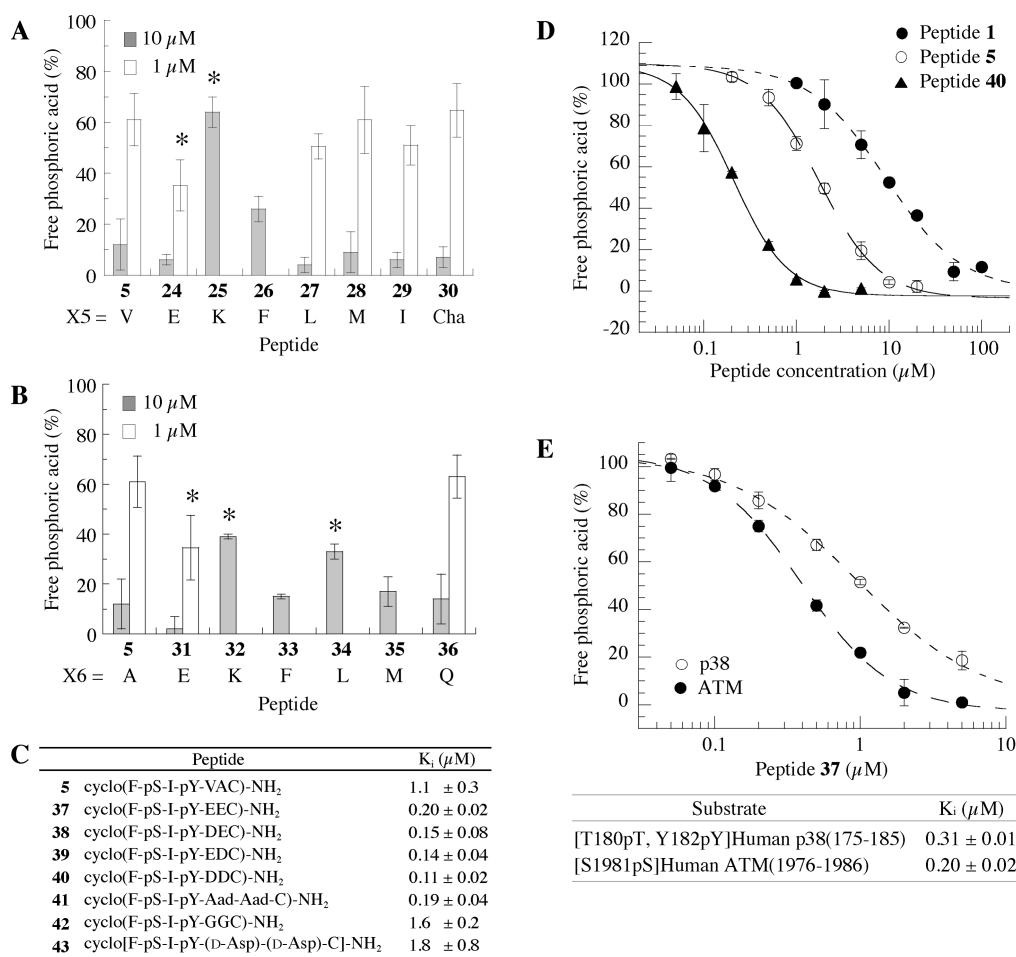


Figure 4. Acidic residues at positions X5 and X6 increase the inhibitory activity of the cyclic peptides. Inhibitory activity of cyclic peptide F-pS-I-pY-X5-X6-C in the presence of [S1981pS] human ATM(1976–1986) peptide (30 μ M) as a substrate. Inhibitory activities of (A) cyclo(F-pS-I-pY-X5-AC)-amide and (B) cyclo(F-pS-I-pY-V-X6-C)-amide peptides were measured at 10 μ M (gray bar) and 1 μ M (white bar) (* p < 0.05 as compared with that of peptide 5). (C) K_i values of cyclic thioether peptides substituted with acidic residues at positions X5 and X6. (D) Concentration dependence of cyclic thioether peptides (M-pS-I-pY-VAC)-amide [peptide 1 (●)], (F-pS-I-pY-VAC)-amide [peptide 5 (○)], and (F-pS-I-pY-DDC)-amide [peptide 40 (▲)]. (E) Inhibitory activity of cyclic peptide 37 in the presence of different Wip1 substrates: (○) [T180pT, Y182pY] human p38(175–185) and (●) [S1981pS] human ATM(1976–1986).

of Glu residues at both positions X5 and X6 further increased the inhibitory activity of the peptide (peptide 37). The K_i value of peptide 37 was 0.20 ± 0.02 μ M, an improvement of 4-fold relative to that of peptide 5 (Figure 4C). A similar K_i value for this peptide was observed in the presence of a different substrate derived from human p38 MAPK (residues 175–185, [T180pT, Y182pY]) (Figure 4E), demonstrating that PPM1D inhibition was not dependent on the substrate motif. To evaluate whether any acidic residue would improve the inhibitory activity or if a Glu residue was specifically required, we tested peptides in which X5 or X6 was individually substituted with Asp instead of Glu (peptide 38 or 39, respectively), X5 and X6 were both Asp (peptide 40), and X5 and X6 were both homoglutamic acid (Aad, peptide 41). All peptides containing an acidic group at position X5, position X6, or both showed similar inhibitory activities (Figure 4C), although peptide 40, containing two Asp residues, did show slightly improved activity relative to that of peptide 37, which has two Glu residues. The K_i value for peptide 40 was found to be 110 nM, increased 10- and 50-fold relative to those of peptides 5 and 1, respectively (Figure 4D). In contrast, when positions X5

and X6 were substituted with Gly residues (peptide 42), the inhibitory activity was not affected compared with that of peptide 5 (Figure 4C). Thus, a negative charge at positions X5 and X6 is critical, although the carbon chain length of the side chain with the acidic residues does not seem to affect inhibitory activity.

To investigate if the stereochemistry affects the inhibitory activity, we synthesized a cyclic peptide containing D-Asp at positions X5 and X6 (peptide 43) and estimated its inhibitory activity (Figure 4C). The K_i value of peptide 43 was found to be 1.8 ± 0.8 μ M, approximately 20-fold lower than that of peptide 40, containing L-Asp at positions X5 and X6 (Figure 4C). Thus, the chirality at positions X5 and X6 affects the inhibitory activity; however, the effect is not as large as that observed at position X3.

Binding of the Cyclic Peptides to PPM1D Is Competitive with Substrate. Having identified an optimized inhibitor, we next investigated its mechanism of inhibition to improve our understanding of its binding site on PPM1D. Figure 5 shows the double-reciprocal plot of PPM1D dephosphorylation of [S1981pS] human ATM(1976–1986) in the absence or presence of cyclic peptide inhibitor 37. The inverse of V , the rate of the

PPM1D-mediated reaction, is shown on the vertical axis, while the inverse of the substrate concentration is plotted on the abscissa. Fitting of the data using the Lineweaver–Burk equation provided the maximum reaction velocity V_{\max} from the inverse of the intercept of the vertical axis and the Michaelis constant K_m ,

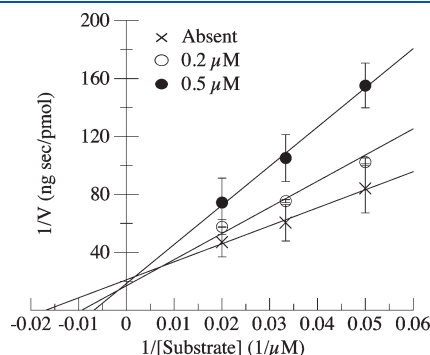


Figure 5. Cyclic peptide that is a competitive inhibitor of PPM1D. Double-reciprocal plot of PPM1D phosphatase activity vs [S1981pS] human ATM(1976–1986) peptide concentration during the inhibition of PPM1D with different concentrations of cyclic thioether peptide (F-pS-I-pY-EEC)-amide (peptide 37): (x) 0, (○) 0.2, and (●) 0.5 μM .

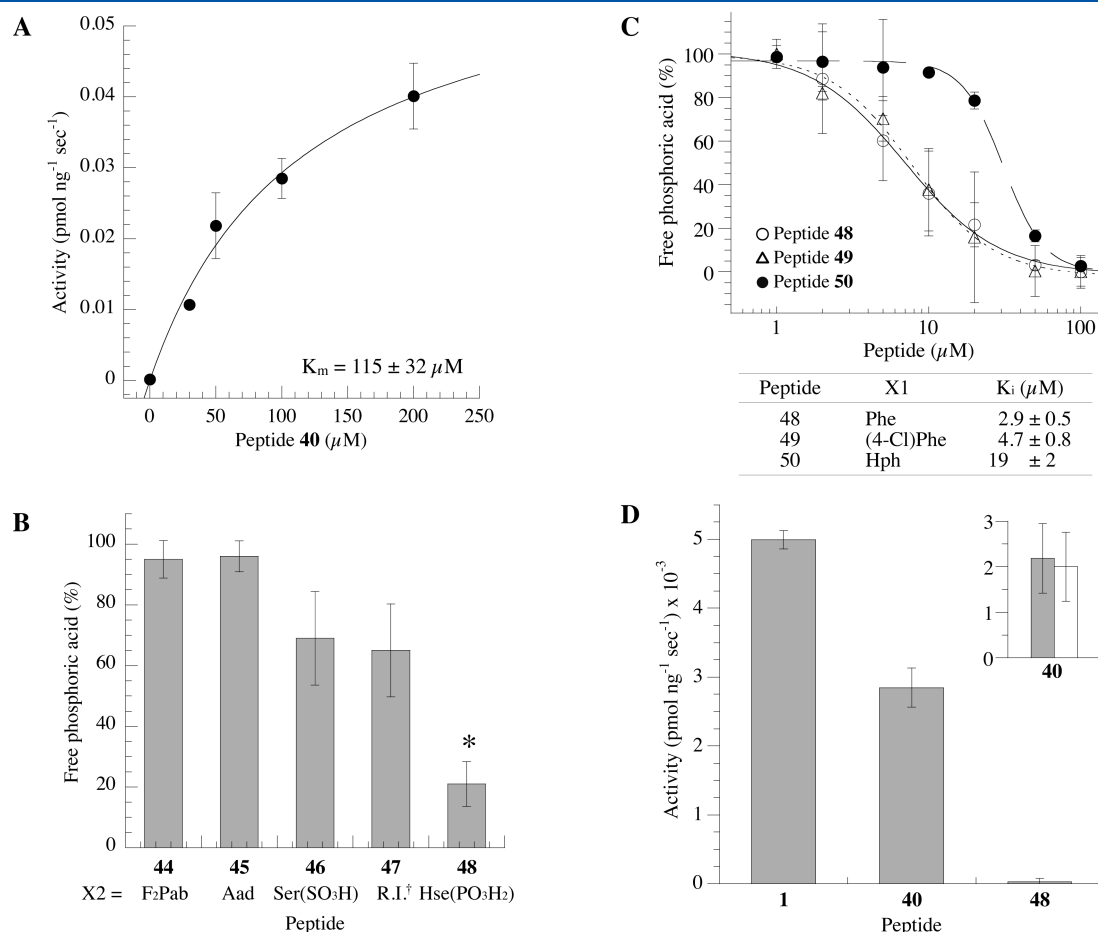


Figure 6. Peptide 48 is a selective inhibitor of PPM1D. (A) Dephosphorylation of peptide 40 by PPM1A. (B) Inhibitory activity of cyclic peptides (25 μM) substituted at position X2 in the presence of [S1981pS] human ATM(1976–1986) peptide (30 μM) as a substrate (* $p < 0.05$ as compared with a no-inhibitor control). R.I. denotes the retro-inverse peptide. (C) Concentration dependence of cyclic thioether peptides 48 (○), 49 (△), and 50 (●). (D) Phosphatase activity of PPM1A against cyclic peptides 1, 40, and 48 at 100 μM . The inset shows PPM1A phosphatase activity against peptide 40 (50 μM) in the presence (white bar) or absence (gray bar) of peptide 48 (50 μM).

which indicates the binding affinity of the substrate for PPM1D, from the inverse of the intercept on the abscissa. The slope of the best fit line is equal to the value of V_{\max}/K_m . The data show that the value of V_{\max}/K_m changed at different cyclic peptide concentrations, whereas the V_{\max} value was independent of the cyclic peptide concentration. These results indicate that the presence of the cyclic peptide decreased the substrate binding affinity in PPM1D but not the rate of reaction. Thus, these results demonstrate that the cyclic peptide is a competitive inhibitor of PPM1D and, as such, binds to the catalytic site.

Substitution of pS with Phosphohomoserine Confers Specificity toward PPM1D over PPM1A. As specific inhibitory activity is critical for drug development, we assessed whether peptide 40 was specific for PPM1D. Previously, we found that peptide 1 was recognized as a substrate by PPM1A (PP2C α) ($K_m = 11 \mu\text{M}$).²⁴ Similarly, PPM1A dephosphorylated 40 with a K_m of $115 \pm 32 \mu\text{M}$, 10-fold lower than that of peptide 1 (Figure 6A). We synthesized a series of peptides containing nonhydrolyzable mimetics of phosphoserine to try to identify a peptide that would not be recognized by PPM1A as either a substrate or an inhibitor. We first substituted the pS with L-2-amino-4-phosphono-4,4-difluorobutyric acid (F₂Pab), in which the bridging oxygen of the phosphate group in pS is replaced with a CF₂ moiety. In contrast to previous results in which a

F₂Pab-substituted peptide was partially active against PPM1D (5-fold weaker than the parent peptide),²⁴ peptide 44, cyclo(F-F₂Pab-I-pY-DDC)-amide, did not exhibit inhibitory activity against PPM1D at 25 μ M (Figure 6B). Peptide 45, containing Aad, also did not have any inhibitory activity toward PPM1D (Figure 6B). Similarly, peptides containing Ser(SO₃H) in place of pS (peptide 46) and the retro-inverse peptide 47, cyclo[(D-Asp)-(D-Asp)-(D-pY)-(D-I)-(D-pS)-(D-F)-C]-amide, showed no significant inhibitory activity toward PPM1D at 25 μ M (Figure 6B). In contrast,

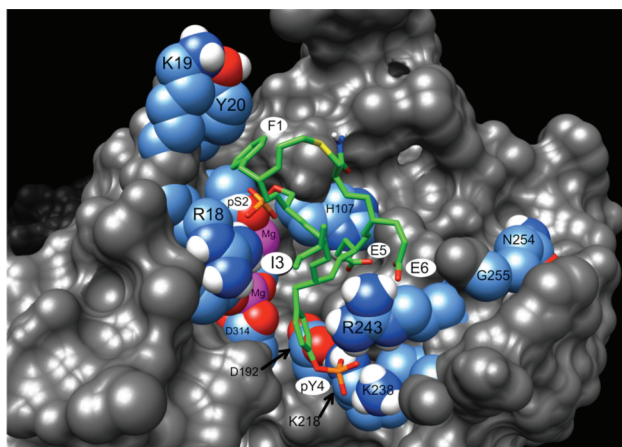


Figure 7. Model of cyclic peptide inhibitor 37 bound to the active site of PPM1D. Residues discussed in the text are labeled. Highlighted residues of the protein are colored cyan, blue, red, and white, for carbon, nitrogen, oxygen, and hydrogen, respectively. The metal ions are colored magenta. The unique colors of the cyclic peptide are green, yellow, and orange, for carbon, sulfur, and phosphorus, respectively.

when the pS was substituted with phosphorylated homoserine (pHse, peptide 48), the resultant peptide exhibited good inhibitory activity toward PPM1D, with a K_i of 2.9 ± 0.5 μ M (Figure 6B,C). Importantly, peptide 48 did not exhibit any activity, either as a substrate or as an inhibitor, toward PPM1A ($K_m > 100$ μ M) (Figure 6D). Thus, although the K_i value of peptide 48 was approximately 30-fold reduced in comparison to that of peptide 40, it was highly selective. One possible reason for the decreased inhibitory activity of peptide 48 is that the longer side chain of pHse may disrupt or weaken critical stabilizing interactions between PPM1D and other residues on the cyclic peptide. To determine if this was occurring, we examined cyclic peptides containing (4-Cl)Phe {peptide 49, cyclo[(4-Cl)Phe-Hse(PO₃H₂)-I-pY-DDC]-amide} or homophenylalanine {Hph, peptide 50, cyclo[Hph-Hse(PO₃H₂)-I-pY-DDC]-amide} with the hypothesis that the increased length of the Phe side chain would improve inhibitory activity. While the K_i value of peptide 49 was to be found 4.7 ± 0.8 μ M, similar to that of peptide 48, peptide 50 exhibited decreased inhibitory activity ($K_i = 19 \pm 2$ μ M) (Figure 6C). These results suggest that the orientation of the aromatic ring at position X1 of peptide 48 is still critical for inhibition, as described previously.

Molecular Modeling. To understand the physical basis for the enhanced binding affinity, we modeled one of the best cyclic peptides (37) bound to the active site of PPM1D. We knew from our previous experimental and computer docking studies with substrates and peptide inhibitor 1 that the interaction is dominated by electrostatic interactions involving the two phosphate groups.^{22,24} Specifically, the phosphate group of the pS residue interacts with the metal center and Arg18, and the phosphate of pY interacts with the side chains of Lys218 and Lys238 (this latter confirmed by mutagenesis studies²⁴). Thus, we maintained these

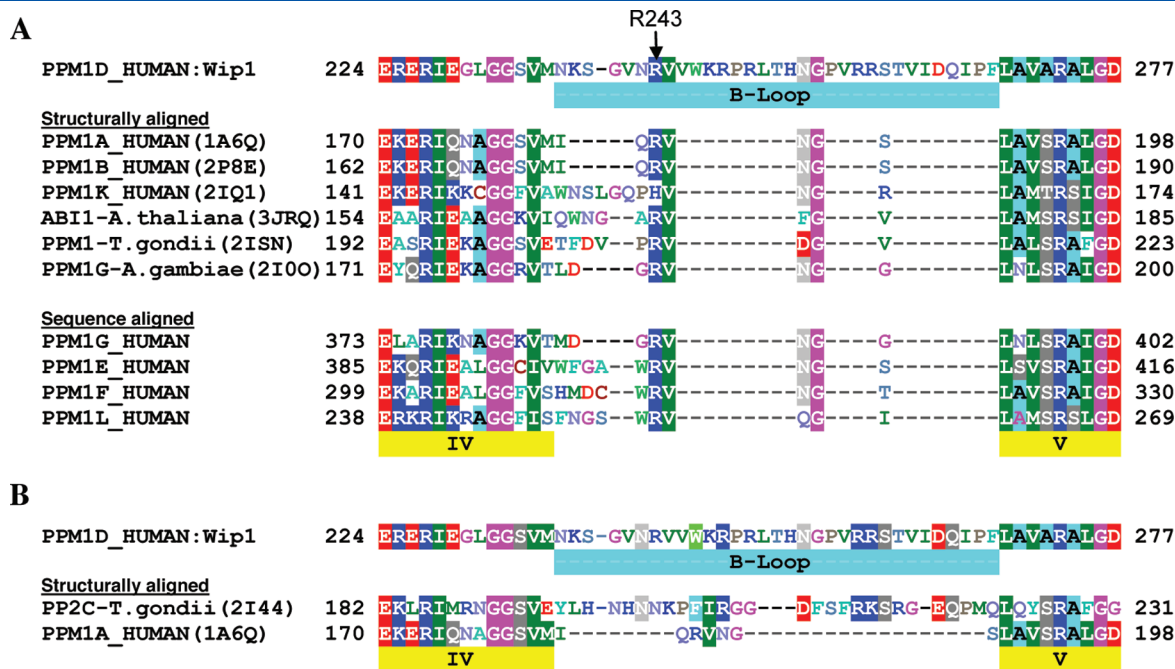


Figure 8. Putative alignment of the B-loop region of PPM1D. (A) Comparison with PPM1A and structurally aligned PP2C homologues with similar flap subdomain conformations, and with human PP2C proteins with unknown three-dimensional structure. References for the structures are given in Table S1 of the Supporting Information. The Swiss-Prot database (<http://expasy.org/sprot/>) accession numbers for PPM1D and the other human sequences are O15297 for PPM1D_HUMAN, P35813 for PPM1A_HUMAN, O75688 for PPM1B_HUMAN, Q8WY54 for PPM1E_HUMAN, P49593 for PPM1F_HUMAN, O15355 for PPM1G_HUMAN, Q8N3J5 for PPM1K_HUMAN, and Q5SGD2 for PPM1L_HUMAN. (B) Comparison with the structurally aligned sequences of PPM1A and PP2Ctg.

interactions as the core of the updated model. As one can see in Figure 7 (and the alternate view in Figure S4 of the Supporting Information), this locates the phenylalanine at position X1 of peptide 37 (F1) next to the nonpolar regions of Lys19 and Tyr20 of PPM1D, thus forming a stabilizing, hydrophobic interaction. This explains why, as demonstrated in Figure 2, highly charged side chains at X1 lead to a reduced level of inhibition. The fact that Tyr, Trp, and some derivatized Phe residues were also favored, and not proline and cyclohexane (peptide 16), suggests that the stabilization is enhanced by aromatic, π – π interactions. This is also consistent with the inhibitor being selective for PPM1D over PPM1A, because the corresponding residues in the latter are the nonaromatic Val34 and Glu35. This conformation also buries the side chain of the X5 peptide residue (E5) atop PPM1D His107. This would explain why, as shown in Figure 4A, the best inhibition was obtained with negatively charged residues at this position. Given the electrostatic influence of the carboxylate group, the histidine can be expected to be protonated, thus forming a stabilizing salt bridge.

Unfortunately, the effects of substitutions at positions X3 and X6 (i.e., preference for an isoleucine and a negatively charged residue, respectively) are more complicated to understand. Given the conformation of the bound cyclic peptide, these two side chains project away from the conserved, catalytic core of PPM1D and either are solvent-exposed or interact with the unique B-loop insert of the flap subdomain.^{24,43} In an attempt to find a structural template for the B-loop, we searched the Protein Data Bank⁴⁰ for all structures similar to that of PPM1A, the prototypical PP2C family member.⁴¹ The results were clustered into 14 unique structures by visual inspection (Table S1 of the Supporting Information). These were then superimposed to identify the range of conformational changes and sequence insertions and/or deletions in the flap subdomain. The results are displayed in Figure 8A, which aligns the sequence of PPM1D with that of PPM1A and the subset of structures close enough to be structurally aligned, and with homologous human sequences that lack known structure. The most prominent feature in the B-loop region is the conservation of a RV motif (residues Arg243 and Val244 in PPM1D) among all the sequences. It should be noted that human PPM1H (SwissProt entry Q9ULR3) and PPM1J (SwissProt entry Q5JR12) proteins also have an insertion in the flap region with RV motifs, but the sequences are too divergent to align unambiguously. While this procedure failed to provide a clear template for the B-loop, it did highlight the importance of the RV motif, so as an initial hypothesis, we chose the simplest option of keeping the RV residues of PPM1D structurally aligned with that of PPM1A. As one can see in Figure 7, this places Arg243 in an ideal position to interact with the X6 residue (E6) of the cyclic peptide. This explains the preference for a negatively charged side chain at this position of the cyclic peptide and the weaker inhibitory effect of positively charged residues (Figure 4B). Although more distant, this position of Arg243 also allows for interaction with the pY side chain of the cyclic peptide. In contrast, structurally aligning the similarly conserved Asn254 and Gly255 residues of PPM1D positions them too far from the active site to appear relevant to regulation. We also attempted automated computer modeling of the B-loop to investigate how it might fold over the active site and interact with the I3 peptide side chain. Unfortunately, this was unsuccessful, and we are still unable to credibly position these additional B-loop residues in the model.

Finally, of all the homologous structures identified, only PP2Ctg from *Taxoplasma gondii* (Protein Data Bank entry 2I44)³¹ had a

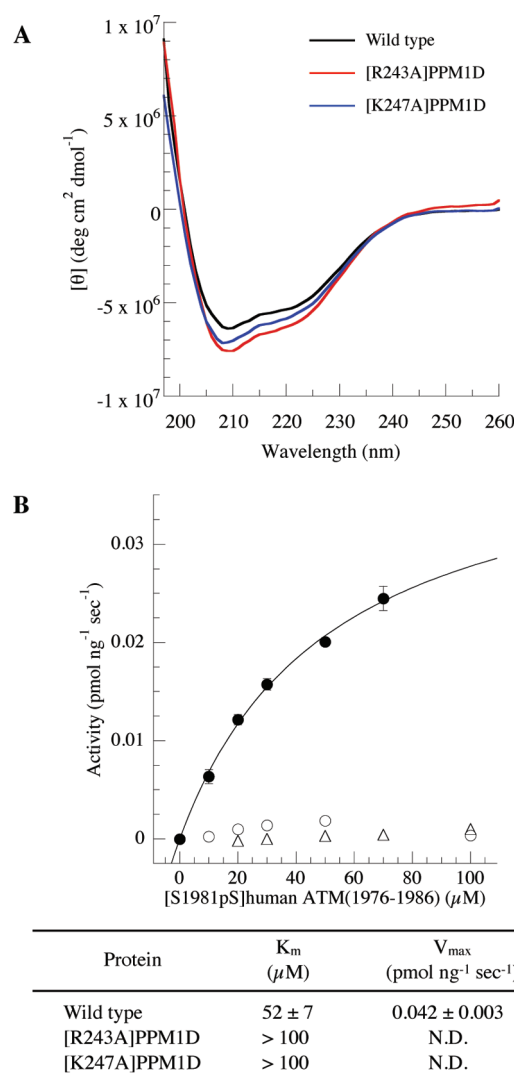


Figure 9. Characterization of the structure and activity of PPM1D mutants. (A) CD spectra of wild-type and mutant PPM1D: wild type (black), [R243A]PPM1D (red), and [K247A]PPM1D (blue). The protein concentration ranged from 1.8 to 2.1 μM in 50 mM Tris-HCl, 30 mM MgCl_2 , and 5% glycerol (pH 7.5). (B) K_m and V_{\max} values for wild-type and mutant PPM1D. Phosphatase activities were measured using [S1981pS] human ATM(1976–1986) substrate peptide: (●) wild type, (○) [R243A]PPM1D, and (△) [K247A]PPM1D.

B-loop insertion similar to that of PPM1D. This is shown in Figure 8B, which aligns the flap subdomain of PPM1D with that of the structurally aligned PP2Ctg and PPM1A. In addition to being similar in length, they have the highlighted similarity of the WXR(246–248), RRS(258–260), and DQ(264–265) subsequences of PPM1D. However, because the overall degree of conservation is relatively low and it would reposition the highly conserved RV residues away from the location in the PPM1A structure, we disfavored the use of PP2Ctg as a template for the B-loop. Nonetheless, the structural overlay of PP2Ctg and PPM1A shown in Figure S5 of the Supporting Information provides a good, general hypothesis for how the B-loop emerges from the conserved flap subdomain.

Arg243 and Lys247 in PPM1D Are Crucial for Phosphatase Activity. The model of PPM1D in complex with the cyclic peptides, combined with the increased inhibitory activity of cyclic peptides containing acidic groups at positions X5 and X6, suggests

the importance of the basic residues in the B-loop for PPM1D activity. Among these amino acids, Arg243 is proposed by the model to interact with the acidic amino acid at position X6; Lys247, located proximal to Arg243, may also interact with acidic amino acids in the cyclic peptide. To investigate if such interactions were occurring, we individually mutated these residues to alanine and looked at the effect of the mutations on PPM1D activity. We first used CD spectroscopy to ensure that the mutations did not alter the PPM1D structure. The CD spectrum of wild-type PPM1D was characterized by double minima of negative ellipticity at approximately 200 and 225 nm and positive ellipticity at shorter wavelengths in the presence of Mg^{2+} . The two mutants, [R243A]PPM1D and [K247A]PPM1D, had very similar CD spectra (Figure 9A), indicative of similar structures for the wild-type and mutant proteins. Next, we estimated the phosphatase activities of the two mutants using the human ATM substrate peptide. Surprisingly, both mutants showed no phosphatase activity. These results demonstrate that Arg243 and Lys247 are crucial for PPM1D catalytic activity, likely through recognition of the phosphorylated substrate.

DISCUSSION

In this study, the activity of a cyclic peptide inhibitor of PPM1D was improved 50-fold relative to those of previously reported inhibitors of this type.²⁴ Alteration of specific side chains in the cyclic peptide resulted in marked improvements in activity and also revealed new structural information about the catalytic site and substrate specificity of PPM1D. At position X1, an aromatic residue (especially Phe) generally improves the inhibitory activity. At position X3, only the L-enantiomer of the Ile α -carbon leads to good inhibitory activity, while either enantiomer at the β -position is tolerated. At positions X5 and X6, a negatively charged residue increases the inhibitory activity dramatically. Combined, the optimal cyclic peptide inhibited PPM1D with a K_i of 110 nM.

Previously, we found that a Leu at position X3 converts the cyclic peptide into a substrate for PPM1D, whereas Val- and Cha-substituted analogues had inhibitory activities similar to those of the Ile-substituted analogue.²⁴ Branching at the β -position is required for inhibitory activity, possibly by providing steric hindrance that prevents the pS side chain from being appropriately positioned in the binding pocket for hydrolysis. Furthermore, hydrophobic interactions in this region appear to be required, as a Gly-substituted linear peptide is a much weaker inhibitor than the Ile-substituted linear peptide.²⁴ The observation that the peptides had similar activities indicates that the methyl group at the β -position does not bind in one specific pocket of PPM1D.

As described above, substitution of acidic residues at positions X5 and X6 greatly improved inhibitory activity, showing a 10-fold increase relative to that of peptide 5 (Figure 4C,D). This observation is consistent with previous research on PPM1D substrate specificity, which reported that mutation of two Glu residues to alanines in the substrate sequence of ATM led to a 3-fold decrease in K_m relative to that of the wild-type sequence.²³ A separate study investigating p53 substrate peptides reported that the introduction of acidic residues around p53 Ser15 resulted in high substrate affinity for PPM1D.²⁶ Combined, these results demonstrate the importance of negative charge in the substrate or inhibitor for binding to PPM1D, likely through formation of electrostatic interactions with the enzyme. PPM1D is the only known PP2C family member to have a highly basic loop inserted into the flap subdomain, which may confer a preference for acidic

substrates. Our molecular model suggests that Arg243 could interact with the Glu at position X6 on the cyclic peptide if it has the same position as in the PPM1A template. The interaction of the Glu at X6 is further suggested by the similar inhibitory activities of other peptides containing an L-acidic residue at this position and the poor inhibitory activities of those with basic residues. Thus, the uniqueness of the B-loop makes it an interesting target for the development of a highly specific inhibitor.

As PPM1D is a member of a family of phosphatases, it is important to understand the selectivity of our inhibitors. We previously demonstrated that peptide 1 had no activity toward PP2A but was a substrate for PPM1A.²⁴ Likewise, although peptide 40 exhibited significantly improved PPM1D inhibition, it was dephosphorylated by PPM1A, albeit 10-fold less than peptide 1. Therefore, to obtain an inhibitor selective for PPM1D, we synthesized five different peptides, four containing a pS mimetic and the fifth a retro-inverse peptide. This process proved to be difficult, as four of the five showed weak to no PPM1D inhibitory activity. Only peptide 48, in which pS was substituted with pHse, was found to be active against PPM1D, although its inhibitory activity was approximately 30-fold decreased relative to that of peptide 40 (Figure 6C). This peptide was not dephosphorylated by PPM1A, nor was it an inhibitor of it. These results indicate that the inhibitory activity of PPM1D is strongly dependent on the carbon chain length of the pS side chain. The dramatic changes in PPM1A dephosphorylation that resulted from relatively minor chemical variations likely reflect changes to the conformation of the complex that determine whether the phosphate bond is positioned correctly for hydrolysis by the enzyme's metal center. Thus, we show it is possible to develop a selective inhibitor of PPM1D, but detailed structural information will likely be required to further improve the inhibitory activity.

For quite a while, the structure of PPM1A from Das et al.²⁵ was the only one available for this class of enzymes. However, recently published related structures have revealed that the so-called "flap sub-domain" adjacent to the active site is likely not a static unit but rather changes conformation to accommodate the binding of different substrates^{27–30,32,33} (Figure S1 of the Supporting Information). This makes accurate modeling of substrate and inhibitor binding much more difficult. This is especially true for PPM1D, because it has the added complication of the relatively long B-loop. Despite this difficulty, we can surmise a number of structural features important for activity from the available data. The first is the conservation of the Arg243-Val244 sequence of PPM1D in all eukaryotic PP2C sequences and some prokaryotic ones (Figure 8A). The second is the correspondence of His107 in PPM1D with His62 in PPM1A (Figure S2 of the Supporting Information), which has been implicated as the acid that protonates the leaving group oxygen and is thus crucial for the phosphatase activity. A corresponding histidine residue is conserved in most of the other eukaryote homologues (except PP2CH/J) and in some prokaryotic ones. In the model of the PPM1D–inhibitor complex, a negatively charged residue at the cyclic peptide X5 position forms a stabilizing salt bridge and sterically blocks His107 of PPM1D, thus enhancing inhibition.

As the B-loop is unique to PPM1D in the PP2C family, it is important to know how it confers the unique substrate specificity of this enzyme and, thus, its physiological functions. The elimination of activity observed here for the R243A and K247A point mutations is consistent with the results of Chuman et al.,²⁶ who substituted B-loop residues 245–268 with the corresponding residues from the PPM1A sequence (NGS). In this case, both the K_m and the

k_{cat} of the mutant were approximately 3-fold reduced relative to those of wild-type PPM1D. The difference between complete abrogation and 3-fold reduction may reflect the different substrates used (ATM vs p53 phosphopeptides) and their differing patterns of negatively charged residues. This raises the question of the purpose of these and the other positively charged residues in the B-loop. Presumably, they function to attract and correctly position the highly negatively charged mono- and diphosphorylated substrates for catalysis. Fundamental to this understanding is knowing exactly where the B-loop starts in the flap subdomain sequence. As one can see in Figure 8, PPM1D and the other proteins display a range of insertions before the RV motif compared to PPM1A. Thus, it is uncertain whether the highly conserved RV residues are located at the same position in the structure as in PPM1A. If the locations are conserved, as in the model presented here and as defined by Chuman et al.,²⁶ then the arginines corresponding to Arg243 likely play similarly important roles in all the other PP2C proteins that share this motif. If not, then Arg243 plays a unique role in PPM1D along with Lys247 and potentially some or all of the other positively charged residues in the B-loop. Further research is needed to discover how these residues co-ordinate in a possibly mobile flap subdomain mechanism.

Here, we report a cyclic thioether peptide (F-pS-I-pY-DDC-amide) characterized by good inhibitory activity ($K_i = 110$ nM). To date, only a few compounds have been reported as PPM1D inhibitors,^{26,43,46,47} and the cyclic peptides presented here show the highest inhibitory activity, to the best of our knowledge. We further identified a second cyclic peptide (F-pHse-I-pY-DDC-amide) that showed selectivity for inhibition of PPM1D over PPM1A. Although the two phosphoric acid groups on the cyclic peptide severely limit the cellular bioavailability of the compounds, their substitution with non-phosphorus-based mimetics, such as a tetrazole scaffold, a sulphydantoin scaffold, or a pyridine-based scaffold,⁴⁸ could be an effective way to overcome this issue. In addition, although the Lipinski rules suggest that a cyclic peptide would have poor absorption or permeation into a cell because of its high molecular weight, use of a drug delivery system, such as nanoparticles, including liposomes,^{49–52} and cell-penetrating peptide conjugation,^{53–55} could overcome this issue. In the absence of detailed structural information, the findings in this study will be valuable in designing the next generation of small molecule inhibitors of PPM1D and in understanding the substrate specificity of PPM1D as a serine/threonine phosphatase.

■ ASSOCIATED CONTENT

Supporting Information. Figures S1–S6 show the full alignment of PPM1A and PPM1D along with additional views of the PPM1D model, and Table S1 contains Protein Data Bank entries and references for proteins used in modeling. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Funding Sources

This research was supported by the Intramural Research Program of the National Cancer Institute and National Institute of

Diabetes and Digestive and Kidney Diseases, National Institutes of Health and federal funds from the National Cancer Institute, National Institutes of Health, under HHSN 261 2008 00001E.

■ ABBREVIATIONS

Aad, homoglutamic acid; Boc, *tert*-butoxycarbonyl; CD, circular dichroism; Cha, β -cyclohexylalanine; Fmoc, 9-fluorenylmethoxycarbonyl; F₂Pab, L-2-amino-4-phosphono-4,4-difluorobutyric acid; Hph, L-homophenylalanine; Hse, L-homoserine; pS, phosphoserine; pT, phosphothreonine; RP-HPLC, reversed-phase high-performance liquid chromatography; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Trt, trityl.

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