Crystal Structures and Mutagenesis of PPP-family ser/thr Protein Phosphatases Elucidate the Selectivity of Cantharidin and Novel Norcantharidin-based Inhibitors of PP5C *

Debasish Chattopadhyay^{1,2}, Mark R. Swingle^{1,3}, Edward A. Salter^{1,4}, Eric Wood^{3,4}, Brandon D'Arcy³, Catherine Zivanov^{3,4}, Kevin Abney³, Alla Musiyenko³, Scott F. Rusin⁵, Arminja Kettenbach^{5,6}, Larry Yet⁴, Chad E. Schroeder⁶, Jennifer E. Golden⁶, Wade H. Dunham^{7,8}, Anne-Claude Gingras^{7,8}, Surajit Banerjee¹⁰, David Forbes⁴, Andrzej Wierzbicki⁴ and Richard E. Honkanen^{3,¶}

¹*These authors contributed equally to this work*

²*Department of Medicine, University of Alabama Birmingham, Birmingham, AL 35294,*

³*Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile AL 36688, USA,*

⁴*Department of Chemistry, University of South Alabama, Mobile AL 36688, USA,*

⁵*Department of Biochemistry, Geisel School of Medicine at Dartmouth, Lebanon, NH 03756, USA,*

⁶*Norris Cotton Cancer Center, Geisel School of Medicine at Dartmouth, Lebanon, NH 03756*

⁷*Department of Medicinal Chemistry, University of Kansas Specialized Chemistry Center, Lawrence, KS, 66047, USA,*

⁸*Centre for Systems Biology, Lunenfeld-Tanenbaum Research Institute at Mount Sinai Hospital, Toronto, Ontario, M5G 1X5, Canada*

⁹*Department of Molecular Genetics, University of Toronto, Toronto, Ontario, M5S 1A8, Canada, and*

¹⁰*Northeastern Collaborative Access Team (NE-CAT) Cornell University, Lemont, IL USA.*

Running title: Crystal Structure of PP5C-inhibitor complexes

[¶]*To whom correspondence should be addressed: Richard E. Honkanen, Dept. Biochemistry and Molecular Biology, University of South Alabama, Mobile, AL 36688, USA. Tel. 1 251-460-6859; E-mail rhonkanen@southalabama.edu*

Keywords: Phosphatase, inhibitors, screening, crystal structures, PP5C

Abbreviations used: FLINT, fluorescence intensity; MBP, maltose binding protein; His-MBP, hexahistidine-tagged maltose binding protein; TEV, tobacco etch virus; DTT, dithiothreitol; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; DiFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; MPD, 2-Methyl-2,4-pentanediol; PEG, polyethylene glycol. PP, protein phosphatase, PP followed by a number indicates the type (i.e. PP1), Genbank gene IDs are in italics (e.g. *PPP5C*). R indicates regulatory subunit (e.g. PP4R3A, protein phosphatase 4 regulatory subunit 3A); C indicates catalytic subunit (encoded by a separate gene (i.e. PP1C, PP2AC, PP4C) or PP5C catalytic domain if the catalytic subunit is encoded in one gene along with regulatory or targeting subunits and expressed as a single polypeptide chain (e.g. PP3/PP2B/calcineurin, PP5C and PP7C). SMEK1, suppressor of MAPK/ERK kinase 1; SMEK2, suppressor of MAPK/ERK kinase 2; PP4R4/KIAA1622, protein phosphatase 4 regulatory subunit 4.

Abstract

Cantharidin is a natural toxin and an active constituent in a traditional Chinese medicine used to treat tumors. Cantharidin acts as a semi-selective inhibitor of PPP-family ser/thr protein phosphatases. Despite sharing a common catalytic mechanism and marked structural similarity with PP1C, PP2AC and PP5C, human PP4C was found to be insensitive to the inhibitory activity of cantharidin. To explore the molecular basis for this selectivity, we synthesized and tested novel C5/C6-derivatives designed from quantum-based modeling of the interactions revealed in the co-crystal structures of PP5C in complex with cantharidin. Structure-activity relationship studies and analysis of high-resolution (1.25 Å) PP5C-inhibitor co-crystal structures reveal close contacts between the inhibitor bridgehead oxygen and both a catalytic metal ion and a non-catalytic phenylalanine residue, the latter of which is substituted by tryptophan in PP4C. Quantum chemistry calculations predicted that steric clashes with the bulkier tryptophan side chain in PP4C would force all cantharidin-based inhibitors into an unfavorable binding mode, disrupting the strong coordination of active site metal ions observed in the PP5C co-crystal structures, thereby rendering PP4C insensitive to the inhibitors. This prediction was confirmed by inhibition studies employing native human PP4C. Mutation of PP5C (F446W) and PP1C (F257W), to mimic the PP4C active site, resulted in markedly suppressed sensitivity to cantharidin. These observations provide insight into the structural basis for the natural selectivity of cantharidin and provide an avenue for PP4C deselection. The novel crystal structures also provide insight for interactions that provide increased selectivity of the C5/C6 modifications for PP5C versus other PPP-family phosphatases.

1. Introduction

Although reversible phosphorylation was once viewed strictly as an eukaryotic adaptation to cope with the complex signaling environment of “sophisticated” organisms, it is now clear that protein phosphorylation actually represents a primitive and important form of post-translational modification that is common to bacteria, archaea, and eukarya [1]. Indeed, many archaea express ser/thr phosphatases that share ~30-35% identity with human PPP-family phosphatases (PPPsases). This places PPPases among the most primitive and structurally-conserved enzymes on earth.

The revelation that PPPases are evolutionarily-primitive enzymes helps to explain the abundance and structural diversity of natural compounds targeting these enzymes (Figure 1A). Okadaic acid, a complex polyether produced by marine dinoflagellates (e.g. *Prorocentrum sp.* and *Dinophysis sp.*), was the first natural toxin shown to act as a PPPase inhibitor [2]. Soon thereafter, microcystin-LR, a toxic cyclic heptapeptide produced by fresh water cyanobacteria (*Microcystis sp.*) and nodularin, a structurally related compound produced by marine cyanobacteria (*Nodularia sp.*), were shown to inhibit the same phosphatases [3,4]. Analysis of extracts from marine sponges and red algae identified calyculins, dragmacidins and thysiferin 23-acetate [5–8]. Extracts from soil bacteria (*Streptomyces sp.*) yielded fostriecin, phostriecin, sultricin [9–11], phospholine [12], leustroducsins [13], phoslactomycins [14], cytostatin [15], tautomycin [16] and tautomycetin [17]. Cantharidin is produced by ~1500 species of blister beetles [18]. Although the aforementioned compounds are structurally diverse (Figure 1), they act by inhibiting a subset of the PPP-family of phosphatases (PPPsases). The toxin-sensitive PPPases include PP1C (*PPP1C*), PP2AC (*PPP2AC*), PP4C (*PPP4C*), PP5C (*PPP5C*) and PP6C (*PPP6C*). These PPPases share considerable structural similarity and a common catalytic mechanism [19–22]; still, the binding affinity and inhibitory activity of the natural compounds can differ substantially among the sensitive enzymes. Notably, fostriecin, which entered Phase I clinical trials as a novel antitumor agent, is a potent inhibitor of PP2AC (IC₅₀ ~2 nM) and a weak inhibitor of PP1C and PP5C (IC₅₀ = 50-70 μM)[23,24]. PP2BC (*PPP3C*) and PP7C (*PPP7C*) share a common catalytic mechanism with the other PPP-family phosphatases. However, both

PP2BC and PP7C have unique region in their catalytic domains and are insensitive to inhibition by the natural toxins [18, 19, 20].

While some argue PPP-targeting toxins evolved to prevent pressure from grazing, others contend they evolved as a means of primitive chemical warfare used to prevent invasion by similar species in competition for a niche. Most investigators agree it is reasonable to conclude that PPP-inhibitors appeared very early in evolution as a means of chemical defense [25]. Because mammals rarely graze on or compete with cyanobacteria, soil bacteria, marine dinoflagellates or beetles, the toxicity of the primitive natural compounds in humans likely resides in the residual similarity between human enzymes and the PPPases of the primitive predators/competitors which the toxins evolved to act upon. A better understanding of the interactions between the natural toxins and human PPPases should aid our understanding of toxicity and provide insight for the development of novel inhibitors that have greater specificity for individual family members. Highly-selective inhibitors of PPP-family phosphatases will serve as powerful probes to aid the understanding of the roles of PPPases in biology and disease. Specific inhibitors may also prove useful as lead compounds for drug development. The studies provided here further elucidate the molecular basis for the inhibitory activity and natural selectivity of cantharidin. In addition, we identify a scaffold for the development of compounds that retain potency against PP5C but have essentially no inhibitory activity against PP4C.

2. Materials and methods

2.1. Vector construction

The constructs allowing expression of human PP1C (*PPP1CA*), PP5C (*PPP5C*) and PP6C (*PPP6C*) were created using standard cloning methods as previously described [22,24,26,27]. Expression and purification of PP5C was performed essentially as described previously [22]. For PP1C, the N-terminal maltose binding protein fusion to PP1C was expressed in BL21(DE3) Tuner pLacI cells (Novagen, Darmstadt, Germany) and purified using heparin-sepharose affinity chromatography. Following digestion with enterokinase (New England Biolabs, Ipswich, MA, USA), PP1C was separated from MBP by anion exchange chromatography on Q-sepharose HP (GE Life Sciences, Marlborough, MA, USA). The PP5C (F446W) mutant was produced by gene synthesis (1097 bp; GeneArt, Life Technologies, IL USA) and expressed from the pDest-HisMBP [28] expression vector, producing a hexahistidine-tagged maltose binding fusion protein (His-MBP). The fusion protein was purified using a HiTrap Chelating column (GE Life Sciences) and treated with TEV protease (Life Technologies, IL USA) to cleave off the His-MBP fusion partner. The free mutant PP5C catalytic domain was then purified using HiTrap Q columns (GE Life Sciences) essentially as described for PP5C. Phosphatase preparations were divided into aliquots and stored at -80 °C. Working dilutions were prepared and stored at -20 °C in tris buffer containing 50% glycerol, 2 mM DTT, 0.1 mg/mL BSA and 2 mM MnCl₂. pDEST-HisMBP was a gift from Dr. David Waugh (Addgene plasmid # 11085, Cambridge, MA, USA). The coding sequence of *PPP6C* (PP6C) was amplified using PCR and cloned in-frame into p3XFLAG-CMV10 as described in Rusin et al (27), and sequence verified. PP6C (W256F) was generated by site-directed mutagenesis and verified by sequencing. HEK293T cells stably expressing 3XFLAG-PP6C or 3XFLAG-PP6C (W256F) were generated. PP6C was purified by FLAG-affinity chromatography, eluting with FLAG peptide in 50 mM Tris pH7.5 and 150 mM NaCl, aliquoted, and stored at -80°C as previously described (27). The absence of contaminating PPPs in the 3XFLAG-PP6C or 3XFLAG-PP6C (W256F) purifications was confirmed by mass spectrometry as described by Rusin et al. (27).

2.2 Cell Culture

HEK-293 cells were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM (Dulbecco's Modified Eagle's Medium; Invitrogen, IL, USA) supplemented with 10% Fetal Bovine Serum and L-Glutamine (4.0 mM) at 37 °C in 5% CO₂.

2.3 DiFMUP fluorescence intensity (FLINT) biochemical assays

DiFMUP (ThermoFisher, IL, USA) based assays were conducted in 96 well plates according to procedures described previously [26]. For the current studies, reactions were performed in the following buffer: 30 mM HEPES, pH 7.0, 1 mM MnCl₂ (0.1 mM for PP5C), 1 mM DTT, 1 mM sodium ascorbate, 0.01% triton X-100, 0.1 mg/ml BSA (Sigma-Aldrich, St. Louis, MO, USA). Reactions were carried out at room temperature (PP1C and PP5C) or 30°C (PP6C). End-point reads (ex=360 nm, em=450 nm) were taken after reactions were stopped at 30 min by the addition of ½ volume of 300 mM phosphate pH 10.

2.3 Radiolabeled Phosphatase assays: Preparation of Phosphohistone Substrate and Determination of Phosphatase Activity

To validate findings obtained with FLINT assays, [³²P/³³P]-labeled phosphohistone or glycogen phosphorylase was produced. [³²P]-Phosphohistone, was prepared by the phosphorylation of bovine brain histone (type-2AS) with cAMP-dependent protein kinase (PKA) from rabbit muscle in the presence of cAMP and [³²P]ATP as described previously [20,24]. In some experiments [³³P]ATP was used. [³³P]-labeled phosphorylase was prepared by phosphorylation of rabbit muscle glycogen phosphorylase b with rabbit muscle phosphorylase kinase in the presence of [³³P]ATP essentially as described previously [29]. In some experiments [³²P]ATP was used. Protein phosphatase assays with phosphoprotein substrates were performed in the same buffer system as used for the FLINT-based assays. Phosphatase activity was measured by the quantification of [^{32/33}P]-labeled orthophosphate liberated from substrate using established protocols [3,24].

2.4 PP6C Aurora Phosphatase assay

Aurora A is a known substrate for PP6, and western analysis using Aurora A (Sigma-Aldrich) and Aurora A-pThr288 (Cell Signaling, Danvers, MA, USA) specific antibodies were used to measure PP6C activity as described previously [27].

2.5 Immunoprecipitation-based PP4C assays

The coding sequences of human *PPP4C* (PP4C) and *PPP4R2* (PP4R2) were PCR amplified from the pcDNA3-based vectors described in Gingras *et al.* [30], cloned in-frame in the EcoRI and XhoI sites of the vector pcDNA5-FRT-FLAG [31], and sequence-verified. Flp-In T-REx HEK293 cells (Invitrogen) cells stably expressing tetracycline inducible FLAG-PP4C or FLAG-PP4R2 were generated as described previously [31]. PP4R2-FLAG expressing cells were grown in 150 mm culture dishes until ~60-70% confluent. Tetracycline (1 µg/mL) was added to induce expression. After 24 h the cells were washed with phosphate buffered saline, scraped from the dish and collected by centrifugation. The cell pellet was weighed and resuspended in lysis buffer (pellet/buffer ratio of 1:4): 50 mM HEPES, 100 mM KCl, 0.1% NP40, 1 mM PMSF, 1 mM DTT, protease inhibitors (P8340; Sigma-Aldrich) and 10% glycerol, followed by three freeze/thaw cycles to complete lysis. The crude lysate was then clarified by centrifugation (20,000 x G for 20 min at 4°C). Anti-FLAG M2 magnetic beads (Sigma-Aldrich) equilibrated with wash buffer (50 mM HEPES, 100 mM KCl, 0.1% NP40, 10% glycerol; Sigma-Aldrich)

were added to the clarified lysate for 4 h at 4°C with gentle agitation. The tube was placed in a magnetic field for 1 min to collect beads. Fluid was then removed and the beads were gently washed 3 times with 1 mL of wash buffer, with magnetic separation performed between each wash. After the final wash step, anti-FLAG beads were collected using magnets and resuspended in either 2X SDS-PAGE loading buffer for western analysis or 1.33X phosphatase assays buffer (1X = 30 mM HEPES, pH 7.0, 1 mM MnCl₂, 1 mM DTT, 1 mM sodium ascorbate, 0.01% triton X-100, 0.1 mg/ml BSA; Sigma-Aldrich) for dose-response studies. Phosphatase activity was measured using DiFMUP as described above with the following modifications. The reaction was started with the addition of substrate (100 µM DiFMUP), and stopped by using magnets to collect the antibodies followed by transferring the supernatant to a well of a 96 well plate containing 100 µL of stop buffer (300 mM phosphate pH 10; Sigma-Aldrich). Fluorescence was measured using EX 360 nm, EM 450 nm on a plate reader.

2.6 Homology Modeling

The protein sequence of human serine/threonine protein phosphatase 4 catalytic domain (UniProtKB ID P60510) was submitted to the SWISS-MODEL server (swissmodel.expasy.org) for homology model building [32–34]. BLAST [35] identified the experimentally-determined PP2AC structure [36] as a suitable single template with 68% sequence identity ([36];PDB entry 4I5L, chain F) for the successful model build.

2.7 Quantum Chemical Modeling

For quantum calculations, a smaller, 40-residue "cluster" model of the PP4C catalytic site (charge = +2) was adapted from the above-described homology model using Gaussview (v5.0, Semichem, Inc.). Where the peptide bonds were broken, carboxylates were terminated as aldehydes and amines were unprotonated. Acidic/basic residue side chains were protonated/deprotonated as appropriate for pH 7; all histidines in the model were chosen to be neutral, with the exception of His115, which was protonated, as its basicity is presumably enhanced by the proximal carboxylate of Asp85. The positions of all hydrogen atoms were determined by a semi-empirical PM6 optimization as implemented in Gaussian09 [37]. The bis-acid form of norcantharidin (**3**) (charge = -2) was manually docked into an initial position within the PP4C catalytic site by overlaying the PP5C-cocrystal structure ([63]; PDB entry 3H61). A two-level hybrid ONIOM [38] optimization was then carried out on the charge-neutral complex using Gaussian09 under the default SCRF option for water as solvent. The high-level antiferromagnetic wavefunction (appropriate for the Mn^{II}Mn^{II} system) was determined by the B3LYP DFT method [39] with the 6-31g(d) basis set [40–42]. The low-level wavefunction was determined by the semi-empirical PM6 method [43]. Only the inhibitor was allowed to move during the optimization, along with the Trp257, Arg86, and Tyr262 sidechains and the atoms of the His238/Gln239 peptide bond linkage.

Likewise, a 42-residue cluster model of the PP5C catalytic site, prepared from PDB entry 3H61 [63] and previously defined as model II [44], was used in two-level ONIOM optimizations in which only the inhibitor was allowed to move. A similar 42-residue cluster ONIOM model with a somewhat smaller "high-level" region was also used in PP5C/inhibitor optimizations.

2.8 Protein crystallography

PP5C crystals were grown using the hanging drop vapor diffusion technique at 16 °C. The reservoir solution contained 25% MPD (Hampton Research, Aliso Viejo, CA, USA) , 6% PEG (Fluka, IL, USA), 10 mM Tris, pH 8.0 (Sigma-Aldrich). Crystals of the (**4**) complex were grown

using the streak-seeding technique. Drops containing 2 μ L of protein-inhibitor-mixture (1:5 molar ratio) and 2 μ L of reservoir solution were pre-equilibrated for 24 h against the reservoir chamber at 16° C. Seeds prepared from crystals of PP5C were introduced into pre-equilibrated drops and sealed over the reservoir chamber. Crystals of the **(5)** complex were obtained by soaking PP5C crystals overnight at 16 °C in the reservoir solution containing 100 μ M **(2/2e)**. Crystals of both complexes were frozen directly into liquid nitrogen for data collection. Intensity data were collected on ADSC Q315 3X3 CCD detector at the Advanced Photon Source on NE-CAT beamline 24-ID-E. A total of 220 images (1° /frame) were collected at a crystal-to-detector distance of 220 mm. Data were processed with XDS [45] and SCALA [46] of the CCP4 program suite [47] as part of the RAPD data-collection strategy at NE-CAT (<https://rapd.nec.aps.anl.gov/rapd>).

Crystals of the PP5C-**(4)** complex belong to the monoclinic space group $P2_12_12_1$ with 1 molecule in the asymmetric unit. Crystals of the PP5C-**(5)** complex belong to the monoclinic space group $P1$ and contain 4 molecules in the asymmetric unit. Crystal structures of both complexes were solved by molecular replacement using atomic coordinates for one molecule of PP5C (from [22]; PDB entry 1s95) using the Phenix program suite [48]. For the structure of the PP5C-**(4)** complex, Phenix was used in the initial rounds of refinement, followed by refinement using REFMAC5 [49]. REFMAC5 was also used for refinement of the PP5C-**(5)** complex structure. After 3 cycles of refinement and initial manual map fitting using Coot [50], the inhibitor was unambiguously placed in the difference electron density map and refined. Water molecules were also fitted in the model using the ‘water-pick’ subroutine in Coot and refined. During refinement of the PP5C-**(5)** structure, automatically generated local non-crystallographic symmetry was applied. In the final stage of refinement we used the TLS and restrained refinement option in REFMAC5 [49]. TLS parameters were generated using the TLS Motion Determination (TLSMD) server (<http://skuld.bmsc.washington.edu/tlsmd/>) [51]. Details of data collection and structure refinement parameters are reported in Table 2. Final coordinates and structure factors have been deposited at the Protein Data Bank with PDB IDs 4zx2 (PP5C-**(4)** complex) and 4zvz (PP5C-**(5)** complex).

2.9 Chemical synthesis and analysis

General Experimental Considerations. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were obtained as solutions in CDCl_3 (Cambridge Isotope Laboratories, Andover, MA, USA). Chemical shifts were reported in parts per million (ppm, $\square$) and referenced to $\square$ 7.27 (^1H NMR) and $\square$ 77.00 (^{13}C NMR) when using CDCl_3 . Infrared spectra were reported in wavenumbers (cm^{-1}). Distillations were performed using a Kugelrohr ball-tube distillation apparatus. TLC analyses were performed on aluminium backed TLC plates with a fluorescent indicator (Sigma-Aldrich). Detection was conducted by UV absorption (254 nm) and charring with 10% KMnO_4 in water. High purity silica gel (40-60 $\square$ m, 60A) was used for all chromatographic separations. All chemicals used for synthetic procedures were reagent grade or better. Celite 545, silica gel (40-60 μ m, 60A) and tetrahydrofuran (anhydrous) were purchased from Acros Organics (New Brunswick, NJ, USA). Molecular hydrogen was supplied by Airgas (Mobile, AL, USA). Cambridge Isotope Laboratories (Andover, MA, USA) was the supplier of the deuterated chloroform. Fisher Scientific (Fair Lawn, NJ, USA) was the supplier for ethyl acetate, hydrochloric acid, magnesium sulfate (anhydrous), and sodium chloride. The thin-layer chromatography plates (TLC Silica gel 60 F254 aluminum sheets) was supplied by Merck KGaA (Billerica, MA, USA). Sigma-Aldrich was the supplier for the Pd on carbon (10%), 1-iodopropane, 3-furancarboxaldehyde, benzyl bromide, maleic anhydride, potassium permanganate, sodium borohydride, sodium hydride (60% oil dispersion), *t*-butyl methyl ether, and tetrabutylammonium iodide. VWR Analytics (Radnor, PA, USA) supplied the solvents

benzene, hexanes, and methylene chloride. Solutions were concentrated in vacuo with a rotary evaporator and the residue was purified using a silica gel column unless specified otherwise. The method and synthesis of novel compounds is provided as supplemental data.

3. Results

3.1 Structure-activity relationship (SAR) studies

Cantharidin (2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic anhydride) is known to strongly inhibit the catalytic activity of PP1C, PP2AC, PP5C and PP6C (IC_{50} ~ 0.2-8 μ M), while having little or no inhibitory activity against PP2BC/calcineurin (PP3C) or PP7C (IC_{50} >1mM) [18,20,52–54]. Determining the activity of inhibitors against PP4C has proven difficult, because to date the successful heterologous expression of active human PP4C in prokaryotes has not been successful. In addition, due to its structural similarity with PP2AC (~65% identity), PP4C has proven extremely difficult to separate using conventional chromatographic and biochemical techniques. To overcome this difficulty, a stable HEK293-derived cell line expressing FLAG-PP4R2 (a known regulatory protein that binds PP4C but not PP2AC) was generated. Anti-FLAG antibodies were then used to immunoprecipitate FLAG-PP4R2, which retains native PP4C with catalytic activity that can be easily measured with the miniaturized FLINT-assay. Dose-response studies conducted with cantharidin confirmed the strong inhibition of PP5C and revealed little inhibition of PP4C (Figure 2A). Under identical conditions PP4C retained sensitivity to tautomycin, a larger natural inhibitor with a different binding motif (data not shown). Based on western analysis, the PP4C that co-precipitates with FLAG-PP4R2 is completely free of contaminating PP2AC (Figure 2B)

3.2 Structure-activity relationships (SAR) of cantharidin derivatives

Quantum chemical modeling was used to explore the use of cantharidin as a scaffold for the development of novel compounds with increased selectivity against human PP5C. These models predicted that large *endo* C6 modifications and *exo* C5 or *exo* C6 modifications would not be well tolerated. However, *endo* C5 modifications, which should not be well tolerated for PP1C or PP2AC, would be possible for PP5C. To test modeling predictions, several 7-oxabicyclo[2.2.1]heptene/heptane-2,3-dicarboxylic anhydride/acid derivatives were synthesized and tested in head-to-head assays using PP1C and PP5C. For all derivatives tested, the reduction of the C5-C6 double bond (i.e., reduction of 7-oxabicyclo heptane to 7-oxybicyclo heptane) increased potency ~10 fold, but did not increase selectivity between PP1C and PP5C (Illustrated in Figure 3A). The addition of a methyl group to C5 or C6 [(1)/(1e)] resulted in a minor deselection for PP1C. As predicted, the addition of a propoxymethyl moiety at C5 (2e) was tolerated and increased the strength of inhibition against PP5C, yielding a ~5-10 fold deselection for PP1C (Figure 3B).

3.3 Crystal structure of PP5C in complex with (4); 5-methyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid

The structure of PP5C streaked seeded with the (1)/(1e) racemate was solved at a resolution of 1.25 Å (Table 2). As observed in the structure of PP5C bound to the substrate analogue orthophosphate ion (23), the active site of PP5C, identified from the positions of two closely apposed metal ions, is located at the base of a shallow depression on the surface formed by residues from four loops connecting secondary structural elements in the protein: β 4- α D, α G- α H, β 10- β 11, and β 12- β 13. Each metal ion is coordinated by six ligands in slightly distorted

octahedral geometry (Figure 4A), presenting the metals at the surface in a manner suitable for interaction with substrates. M1 is coordinated by Asp271, Asn303, His352, His427, a water/hydroxide molecule (W1), and an oxygen atom of the orthophosphate ion bound in the active site. M2 is coordinated by Asp242, His244, Asp271, a phosphate oxygen, W1, and a terminally ligated water (W2). The close proximity of the metals (3.3 Å apart) is facilitated by bridging coordination by both a carboxylate oxygen of Asp271 and the water/hydroxide (W1) that is likely the catalytic nucleophile in the phosphomonoester hydrolysis reaction. In conjunction with three conserved residues (Arg275, His304, and Arg400) the two metal ions create a positively charged, shallow binding site for the substrate's dianionic phosphoryl moiety. All six metal-coordinating residues, as well as the three phosphoryl-coordinating residues, are absolutely conserved in PPP-family phosphatases. Superposition of available structures of PPP-family phosphatases, crystallized with various ligands under a variety of conditions, reveals a core active site that is apparently quite rigid, with most of the conserved residues (both sidechain and backbone) adopting essentially the same three-dimensional spatial arrangement in all structures (data not shown). The major exceptions to this apparent structural rigidity are the sidechains of a tyrosine (Tyr451 in PP5C) and an arginine (Arg275 in PP5C). Flexibility is especially apparent for the arginine, which is observed in a variety of conformations in different PPPase structures. The flexibility of Arg275 and Tyr451 is discussed further below in the context of PP5C complexes.

Although the anhydrides (**1/1e**) were streak seeded as a racemic mixture with PP5C for co-crystallization, the structure of the inhibitor complex reveals exclusively the bis-acid (**4**) bound at the active site (Figure 4B), indicating hydrolysis of the anhydride occurred either in solution or while bound to the enzyme. In the PP5C-(**4**) complex, the two metal ions (M1, M2) occupy the same positions as in the PP5C-phosphate complex. However, the coordination geometry differs due to the multiple strong interactions between the metal ions and (**4**) (Figures 4B, C, D and E). M2 maintains an octahedral coordination sphere with three ligands provided by PP5C residues (Asp242, His244, and Asp271) and three ligands provided by the inhibitor (the O7 bridgehead oxygen and both the 2- and 3-carboxy groups). M1 changes to a trigonal bipyramidal coordination geometry, with four ligands provided by PP5C residues (Asp271, Asn303, His352, and His427) and one by the 3-carboxy group of (**4**). Comparing the substrate analogue complex to the PP5C-(**4**) structure reveals that the bridging water/hydroxide is replaced by the 3-carboxy group of (**4**) and that the water molecule terminally ligated to M2 is replaced by the O7 bridgehead oxygen. Also, one of the metal coordination sites occupied by a phosphate oxygen in the substrate analogue complex is substituted by the 2-carboxy group of (**4**), and (**4**) forms hydrogen bonds (via the 2- and 3-carboxy groups) with three conserved substrate-coordinating residues: Arg275, His304, and Arg400. Thus, coordination sites for the substrate phosphoryl moiety and the possible nucleophiles in the hydrolysis reaction are occupied by the inhibitor in the PP5C-(**4**) complex. The side chains of Arg275 and Tyr451 adopt conformations slightly farther away from the active site metals in order to accommodate the inhibitor (Figure 4). Hydrophobic contacts between the inhibitor and the sidechains of Val429, Phe446, and Tyr451 further stabilize the complex (Figures 4 and 5). PP5C selection of the C6-substituted enantiomer (**4**) over (**4e**) is attributed to the occupation of the small hydrophobic space bounded by Val429, Met455, and Tyr451 by the new methyl group (Figures 4 and 5). While rotation of the methyl substituent precludes accurate placement of its hydrogens, inference of hydrogen positions is possible for rigid groups such as the oxabicyclo ring of (**4**). The C1 and C4 hydrogens make very close contacts (less than the sum of van der Waals radii) with oxygen atoms in the protein: hydroxyl of Tyr451 and the backbone carbonyl of His427, respectively. The orientations of the C-H bonds relative to the oxygens ($r(\text{H}^{\cdots}\text{O}) \leq 2.6 \text{ Å}$; $\angle(\text{C,H,O}) = 146.5^\circ$ and 123.6° , respectively) suggest favorable electrostatic interactions (i.e., weak hydrogen bonds). A weak hydrogen bond is also observed between the O7 bridgehead oxygen of (**4**) and an aromatic ring hydrogen of

Phe446 [$r(\text{H}\cdots\text{O}) = 2.5 \text{ \AA}$; $a(\text{C,H,O}) = 144.7^\circ$]. The C2, C3, and *endo* C5 hydrogens face away from the active site and do not make any very close contacts with the enzyme.

3.4 PP5C co-crystal structure with (5); 5-propoxymethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid

The structure of PP5C soaked with the racemate of anhydrides (2)/(2e) was solved at a resolution of 2.00 Å (Table 2). The asymmetric unit contained 4 molecules of PP5C with the inhibitor visible in all four, but with the propoxymethyl moiety disordered beyond the oxygen atom, which is best localized in the complex with chain D. As above, hydrolysis of the anhydride and enantioselection occurred with co-crystallization. In contrast to (4), however, co-crystallization of (2)/(2e) selected the C5-substituted enantiomer (5). The structures of the inhibitor cores and enzyme active sites in the PP5C-(5) and PP5C-(4) complexes are virtually identical except that the 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid scaffold in PP5C-(5) has a longer O7 $\cdots$ Metal distance (2.5 Å versus 2.3 Å). As a novel feature, the propoxymethyl oxygen affords a hydrogen bond to the backbone amide H of Val429 ($r(\text{O}\cdots\text{N}) = 2.9 \text{ \AA}$), displacing a water present in the PP5C-(3), -(4) and -orthophosphate complexes. Also, we infer, without well-defined positions of the terminal atoms, that the propoxymethyl group generally follows the sidechains of Val429 and Glu428 for hydrophobic stability (Figure 5B). As shown, the rotational conformations about the new propoxymethyl's first two leading bonds differ from the usual *trans*: $d(\text{C6,C5,C,O}) = -101.8^\circ$ and $d(\text{C5,C,O,C}) = -151.2^\circ$. However, $d(\text{C5,C,O,C})$ cannot be determined with precision due to weak electron density for the last carbon in the dihedral grouping and attendant uncertainty in the position of that atom - inspection of the crystal structure suggests a sterically-allowed range of about 60° (from approximately 155° to -145°). While the C α hydrogen of Glu428 and the sidechain of Val429 are obstacles for extension at C5, sufficient flexibility in the propoxymethyl group (hence the observed disorder in the crystal) evidently permits alternate conformations to achieve the aforementioned novel favorable interactions while avoiding the severe penalties of steric clashes. Hence, the C5-substituted enantiomer binds to PP5C. For chain extension beyond one carbon at the C6 position, Tyr451 appears to present a severe steric problem which is not overcome.

3.5 PP4C insensitivity

Based on shared homology (~65% identity) and data provided in the reported crystal structures for PP2AC, we constructed a practical computational model of the PP4C-norcantharidin complex (see Procedures). The side view of the PP5C-(3) [the bis-acid form of norcantharidin] complex (Figure 6) reveals that in PP5C, Phe446 (Figure 6B, green) abuts the bound inhibitor (Figure 6C, 2.50 Å) and provides a stabilizing interaction. PP4C, however, contains a tryptophan (Trp257) in this position, and according to the homology model, a steric clash between Trp257 and the inhibitor may perturb the high-affinity binding mode it adopts in PP5C-norcantharidin. The optimized quantum-based ONIOM model of the PP4C-norcantharidin complex (see Procedures) bears this out. Notably, the steric conflict leads to disruption of the O7-Mn²⁺ coordination [$r(\text{O7}\cdots\text{Mn}^{2+}) = 4.31 \text{ \AA}$ versus 2.30 Å] (Figure 6D). All other contacts with the metals except one (2.13 Å) are also weakened, but to a lesser extent. This argues that the insensitivity of PP4C to compounds containing the 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid/anhydride-based core is due to conflict with Trp257.

To test this prediction and to ensure that the observed inability to inhibit PP4C was not an artifact of the immunoprecipitation-based assay, we next constructed point mutations in PP1C and PP5C, replacing the endogenous phenylalanine (F) with tryptophan (W) [PP1C (F257W); PP5C (F446W)]. Human PP1C, PP5C and mutants can be expressed in an active form in *E coli*

and have similar activity to the native enzymes (Figure 2S). As illustrated in Figure 7, the F257W mutation of PP1C produced a decrease in sensitivity to cantharidin. For PP5C, in which cantharidin fits tightly in the catalytic pocket, the F446W mutation ablated inhibition, as observed with native PP4C. Human FLAG-PP6C fusion protein retains catalytic activity, and like PP4C, PP6C also contains a tryptophan (W256) in the cantharidin-binding site. Mutation of PP6C (W256F), to mimic the PP5C high affinity binding site, increased PP6C sensitivity to cantharidin (Figure 7). Together these studies indicate that the non-catalytic phenylalanine allows for high affinity cantharidin binding in PP5C.

4. Discussion

The studies presented here reveal that cantharidin has minimal inhibitory activity against PP4C, and C5/C6 derivatives built upon the 7-oxybicyclo[2.2.1] heptane scaffold have increased selectivity for PP5C versus PP1C. In addition to providing further structural insight into the important contacts between PP5C and the natural beetle toxin (62), our studies build upon previous studies of cantharidin analogues tested against PP1C and PP2AC, which provide substantial insight into the structure-activity relationships for modifications that affect PP1C, PP2AC and cell toxicity [55–61]. Using the numbering shown in Figure 1, cantharidin derivatives with either the aldehyde or bis-acids are known to function as inhibitors of PP1C or PP2AC, and modifications at either of the carboxylic acids attached to C2 or C3 (but not both) are tolerated (but rarely improved the strength of inhibition). Modifications of C1 or C4 are not well tolerated for the development of inhibitors for PP1C or PP2AC, and decoration at C5 or C6 is not tolerated for inhibitors of PP2AC [57,61]. SAR studies with PP5C indicate C1/C4 modifications are also not tolerated in the development of PP5C specific inhibitors. As observed for PP1C and PP2A, modification of the C3 carboxylic acid was tolerated by PP5, but this strategy did not yield compounds with increased selectivity. In contrast, modifications of C5 and C6 were tolerated, providing a novel avenue for the development of PP5C selective inhibitors.

The high-resolution crystal structures of PP5C-(**4**) and PP5C-(**5**) complexes provide details of inhibitor binding (Figures 4 and 5). For all 7-oxybicyclo[2.2.1] heptane/heptene based compounds tested, reduction of the C5-C6 double bond (heptene to heptane) produced a ~10-fold increase in potency but failed to provide an advantage related to selectivity (Table 1; Figure 3). This improvement was predicted to occur due to the trigonal planar geometry of the heptene C5 projecting the C5 hydrogen toward the amide hydrogen of Val429. Quantum-mechanical modeling of a PP5C complex, in which (**3**) is substituted with its unsaturated C5-C6 analogue indicates that the heptene-based analogues are consequently forced higher in the cavity than corresponding heptane-based compounds, resulting in two substantially longer O $\cdots$ Mn²⁺ interaction distances [2.51 and 2.42 Å versus 2.23 and 2.21 Å in PP5C-(**3**)] and weaker binding affinity.

Consistent with the predictions of the models, *endo*-modifications of C5/C6 retained strength of inhibition for PP5C and provided modest deselection for PP1C. As seen in the PP5C-(**4**) complex (Figure 4E), the side chains of Arg275 and Tyr451 move away from the positions adopted in the orthophosphate complex in order to accommodate the inhibitor. Similar flexibility of the equivalent arginine is also observed in other PPPases. For example, the conformations of Arg96 of PP1C in the nodularin ([64]; PDB: 3E7A) and okadaic acid ([65]; PDB: 1JK7) complexes differ significantly from that observed in the orthophosphate complex ([66]; PDB: 4MOV).

Aqueous-phase quantum calculations (B3LYP/6-311+g(d,p)) indicate that the lowest energy conformation of (**5**) is with the methoxypropyl oxygen *trans* to C6 [$d(C6,C5,C,O)=-180^\circ$], and when bound to PP5C, a steric conflict between it (or any 2nd atom of a longer sidechain at C5) with the C α hydrogen of Glu428 should occur. A second conformation with $d(C6,C5,C,O)=-$

72.4° (O roughly *trans* to C4) is inherently ~0.4 kcal/mol higher in energy and would clash with Val429 upon binding. Therefore, the strength of PP5C-(**5**) binding was unexpected. In the computational model of the bound inhibitor, which exhibited some scaffold strain [$d(C3,C2,C6,C5) = -9.4^\circ$], the sidechain adopted a conformation with $d(C6,C5,C,O) = -173.9^\circ$ to establish a new H-bond between the sidechain oxygen and the backbone amide H of Val429 [$r(O\cdots N) = 3.10 \text{ \AA}$]. The crystal structure shows that the new H-bond is established; however, the steric conflict and the consequential scaffold strain is actually bypassed by a combination of torsion angles in the propoxymethyl sidechain, namely, $d(C6,C5,C,O) = -101.8^\circ$ (intermediate of the two aqueous-phase conformations mentioned above) and $d(C5,C,O,C) = -151.2^\circ$ (Figure 6B). For C6-substituted inhibitors, including (**2**), Tyr451 poses an even more severe barrier for any 2nd atom of a sidechain. Consequently, PP5C preferentially accommodates longer chain extensions at C5 over C6, including (**2e**) over (**2**).

PP1C, PP2AC, PP4C, PP5C and PP6C share a common catalytic mechanism, and all 9 amino acids that either coordinate the metal ions, orient the incoming substrate phosphoryl moiety for nucleophilic attack by activated hydroxide, or are necessary for efficient protonation of the leaving group in the transition state, are conserved. The PP5C-(**4**) and PP5C-(**5**) structures reveal close contact between the bridgehead oxygen of the inhibitors and the side chain of Phe446 (Figures 4). Although the crystal structure of PP4C has not been reported, the primary amino acid sequence of PP2AC and PP4C share ~65% identity, allowing us to construct a model of PP4C based on shared homology provided in the reported structures for PP2AC (see Procedures). The side view of the PP5C-(**3**) [the bis-acid form of norcantharidin] complex (Figure 6 B-C) reveals that, as discussed above, three carboxylate oxygens and the bridgehead oxygen are coordinated to the metals (M1 and M2; spheres). In PP5C, Phe446 (Figure 6B) abuts the bound inhibitor (Figure 6C, 2.50 Å), forming a weak hydrogen bond. This Phe is conserved with PP1C, PP2AC, and PP2BC. However, PP4C and PP6C contain a tryptophan (Trp257 and Trp256, respectively) in this position. Since Trp257 in PP4C (Phe446 in PP5C) is locked by enclosure from Asp54 (Asp242), Ala259 (Ala448), Leu240 (Val429) and the peptide bond with the adjacent Gln239 (Glu428), a steric clash is produced by Trp257; notably, in our optimized quantum-based computational model of the PP4C-(**3**) complex, the inhibitor's bridgehead oxygen (O7) is pushed to a distance from M1 that no longer contributes significantly to binding (4.31 Å, Figure 6D) and all other contacts with the metals except one (2.13 Å) are also negatively impacted, but to a lesser extent. This argues that 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid/ anhydride based compounds should be poor inhibitors of PP4C, which is consistent with inhibition assay data (Figures 2 and 7).

Like PP4C, PP2BC is not sensitive to inhibition by cantharidin [18], yet the non-catalytic Phe (Phe306 in PP2B) is conserved. This suggests the catalytic pocket of PP2BC contains additional conflicts that prevent strong cantharidin binding. PP2BC differs from PP1-PP6 in the respect that it contains a six amino acid (TTGFPS) insert four amino acids upstream of Phe306 and a unique hydrophobic cluster (LDVY) immediately downstream of a conserved active site Tyr (Tyr 311). When cantharidin is positioned into the catalytic pocket of PP2BC, retaining the orientation observed in PP5-cocrystal structures, modeling suggest that Tyr311 in PP2BC produces a steric clash with cantharidin at both C2 and C6 (Figure 6F). It is predicted that the tight packing of Tyr311 by adjacent hydrophobic cluster (LDV) prevents Tyr311 from moving to accommodate the inhibitor. This may account for the weak inhibitory actions of cantharidin for this enzyme. However, this hypothesis needs to be tested experimentally.

Acknowledgments: *This work was supported in part by the Abraham Mitchell Cancer Research Fund (to AW and REH), Mayer Mitchell Annual Award for Excellence in Cancer Research (to

REH). National Institute of Health Molecular Libraries Probe Production Centers Network (NIH/MLPCN) GrantID: U54HG005031 (to the KU Specialized Chemistry Center), a grant from the American Cancer Society Research Grant IRG-82-003-30 (to ANK), grants from NIH; RO3MH05702, R21NS071553 (to REH); and support from the Canadian Cancer Society Research Institute (to ACG). Computational modeling was made possible, in part, by a grant of high-performance computing resources and technical support from the Alabama Supercomputing Authority. Some of this work is based upon research conducted at the Northeastern Collaborative Access Team beamlines, which are funded by the NIH (P41 GM103403). This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

Conflict of interest: The authors declare no conflict of interest with the contents of this article.

Author contribution: MRS and REH conceived and coordinated the study and wrote the paper. DC, SB and MRS collected the data and determined the X-ray structures. EW, CZ, CK, MRS conducted and analyzed the data from phosphatase assays, and assisted in the chemical synthesis of compounds. WHD and ACG conceived and developed the PP4C expression vectors and cell lines. EAS and AW conducted the quantum chemical modeling and were instrumental in inhibitor design. SFR and AK produced and provided PP6C and PP6C mutants proteins. BD'A, KA, AM, MRS, SFR purified the proteins and conducted phosphatase inhibition studies. DF, LY, JEG and WHD designed and guided the synthesis of the novel chemical inhibitors. EAS, JG, ACG, DC, DF, KA, LY and AW, assisted in writing and or review of the paper. REH, AW, JG, ACG, DC and AK obtain funding for the studies. All authors reviewed and approved the manuscript.

REFERENCES

- [1] P.J. Kennelly, Protein Ser/Thr/Tyr phosphorylation in the Archaea, *J. Biol. Chem.* 289 (2014) 9480–9487. doi:10.1074/jbc.R113.529412.
- [2] A. Takai, C. Bialojan, M. Troschka, J.C. Rüegg, Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin, *FEBS Lett.* 217 (1987) 81–84.
- [3] R.E. Honkanen, J. Zwiller, R.E. Moore, S.L. Daily, B.S. Khatra, M. Dukelow, et al., Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases, *J. Biol. Chem.* 265 (1990) 19401–19404.
- [4] R.E. Honkanen, M. Dukelow, J. Zwiller, R.E. Moore, B.S. Khatra, A.L. Boynton, Cyanobacterial nodularin is a potent inhibitor of type 1 and type 2A protein phosphatases, *Mol. Pharmacol.* 40 (1991) 577–583.
- [5] M. Suganuma, H. Fujiki, H. Furuya-Suguri, S. Yoshizawa, S. Yasumoto, Y. Kato, et al., Calyculin A, an inhibitor of protein phosphatases, a potent tumor promoter on CD-1 mouse skin, *Cancer Res.* 50 (1990) 3521–3525.
- [6] H. Ishihara, B.L. Martin, D.L. Brautigan, H. Karaki, H. Ozaki, Y. Kato, et al., Calyculin A and okadaic acid: inhibitors of protein phosphatase activity, *Biochem. Biophys. Res. Commun.* 159 (1989) 871–877.
- [7] null Capon, null Rooney, null Murray, null Collins, null Sim, null Rostas, et al., Dragmacidins: new protein phosphatase inhibitors from a southern australian deep-water marine sponge, *spongosorites* sp, *J. Nat. Prod.* 61 (1998) 660–662. doi:10.1021/np970483t.
- [8] S. Matsuzawa, T. Suzuki, M. Suzuki, A. Matsuda, T. Kawamura, Y. Mizuno, et al., Thyriferyl 23-acetate is a novel specific inhibitor of protein phosphatase PP2A, *FEBS Lett.*

- 356 (1994) 272–274.
- [9] S.W. Mamber, W.G. Okasinski, C.D. Pinter, J.B. Tunac, Antimycotic effects of the novel antitumor agents fostriecin (CI-920), PD 113,270 and PD 113,271, *J. Antibiot. (Tokyo)*. 39 (1986) 1467–1472.
 - [10] C.P. Burke, N. Haq, D.L. Boger, Total synthesis, assignment of the relative and absolute stereochemistry, and structural reassignment of phostriecin (aka Sultriestin), *J. Am. Chem. Soc.* 132 (2010) 2157–2159. doi:10.1021/ja9097252.
 - [11] H. Ohkuma, N. Naruse, Y. Nishiyama, T. Tsuno, Y. Hoshino, Y. Sawada, et al., Sultriestin, a new antifungal and antitumor antibiotic from *Streptomyces roseiscleroticus*. Production, isolation, structure and biological activity, *J. Antibiot. (Tokyo)*. 45 (1992) 1239–1249.
 - [12] T. Ozasa, K. Tanaka, M. Sasamata, H. Kaniwa, M. Shimizu, H. Matsumoto, et al., Novel antitumor antibiotic phospholine. 2. Structure determination, *J. Antibiot. (Tokyo)*. 42 (1989) 1339–1343.
 - [13] T. Kohama, R. Enokita, T. Okazaki, H. Miyaoka, A. Torikata, M. Inukai, et al., Novel microbial metabolites of the phoslactomycins family induce production of colony-stimulating factors by bone marrow stromal cells. I. Taxonomy, fermentation and biological properties, *J. Antibiot. (Tokyo)*. 46 (1993) 1503–1511.
 - [14] S. Fushimi, S. Nishikawa, A. Shimazu, H. Seto, Studies on new phosphate ester antifungal antibiotics phoslactomycins. I. Taxonomy, fermentation, purification and biological activities, *J. Antibiot. (Tokyo)*. 42 (1989) 1019–1025.
 - [15] M. Amemiya, T. Someno, R. Sawa, H. Naganawa, M. Ishizuka, T. Takeuchi, Cytostatin, a novel inhibitor of cell adhesion to components of extracellular matrix produced by *Streptomyces* sp. MJ654-NF4. II. Physico-chemical properties and structure determination, *J. Antibiot. (Tokyo)*. 47 (1994) 541–544.
 - [16] M. Hori, J. Magae, Y.G. Han, D.J. Hartshorne, H. Karaki, A novel protein phosphatase inhibitor, tautomycin. Effect on smooth muscle, *FEBS Lett.* 285 (1991) 145–148.
 - [17] X.C. Cheng, T. Kihara, X. Ying, M. Uramoto, H. Osada, H. Kusakabe, et al., A new antibiotic, tautomycetin, *J. Antibiot. (Tokyo)*. 42 (1989) 141–144.
 - [18] R.E. Honkanen, Cantharidin, another natural toxin that inhibits the activity of serine/threonine protein phosphatases types 1 and 2A, *FEBS Lett.* 330 (1993) 283–286.
 - [19] R.E. Honkanen, T. Golden, Regulators of serine/threonine protein phosphatases at the dawn of a clinical era?, *Curr. Med. Chem.* 9 (2002) 2055–2075.
 - [20] M. Swingle, L. Ni, R.E. Honkanen, Small-molecule inhibitors of ser/thr protein phosphatases: specificity, use and common forms of abuse, *Methods Mol. Biol. Clifton NJ*. 365 (2007) 23–38. doi:10.1385/1-59745-267-X:23.
 - [21] Y. Shi, Serine/threonine phosphatases: mechanism through structure, *Cell*. 139 (2009) 468–484. doi:10.1016/j.cell.2009.10.006.
 - [22] M.R. Swingle, R.E. Honkanen, E.M. Ciszak, Structural basis for the catalytic activity of human serine/threonine protein phosphatase-5, *J. Biol. Chem.* 279 (2004) 33992–33999. doi:10.1074/jbc.M402855200.
 - [23] L.H. Lê, C. Erlichman, L. Pillon, J.J. Thiessen, A. Day, N. Wainman, et al., Phase I and pharmacokinetic study of fostriecin given as an intravenous bolus daily for five consecutive days, *Invest. New Drugs*. 22 (2004) 159–167. doi:10.1023/B:DRUG.0000011792.13160.b0.
 - [24] M.R. Swingle, L. Amable, B.G. Lawhorn, S.B. Buck, C.P. Burke, P. Ratti, et al., Structure-activity relationship studies of fostriecin, cytostatin, and key analogs, with PP1, PP2A, PP5, and(beta12-beta13)-chimeras (PP1/PP2A and PP5/PP2A), provide further insight into the inhibitory actions of fostriecin family inhibitors, *J. Pharmacol. Exp. Ther.* 331 (2009) 45–53. doi:10.1124/jpet.109.155630.
 - [25] A. Rantala, D.P. Fewer, M. Hisbergues, L. Rouhiainen, J. Vaitomaa, T. Börner, et al., Phylogenetic evidence for the early evolution of microcystin synthesis, *Proc. Natl. Acad.*

- Sci. U. S. A. 101 (2004) 568–573. doi:10.1073/pnas.0304489101.
- [26] L. Ni, M. Swingle, A.C. Bourgeois, R.E. Honkanen, High yield expression of serine/threonine protein phosphatase type 5, and a fluorescent assay suitable for use in the detection of catalytic inhibitors, *Assay Drug Dev. Technol.* 5 (2007) 645–653. doi:10.1089/adt.2007.079.
- [27] S.F. Rusin, K.A. Schlosser, M.E. Adamo, A.N. Kettenbach, Quantitative phosphoproteomics reveals new roles for the protein phosphatase PP6 in mitotic cells, *Sci. Signal.* 8 (2015) rs12. doi:10.1126/scisignal.aab3138.
- [28] S. Nallamsetty, B.P. Austin, K.J. Penrose, D.S. Waugh, Gateway vectors for the production of combinatorially-tagged His6-MBP fusion proteins in the cytoplasm and periplasm of *Escherichia coli*, *Protein Sci. Publ. Protein Soc.* 14 (2005) 2964–2971. doi:10.1110/ps.051718605.
- [29] T. Fellner, P. Piribauer, E. Ogris, Altering the holoenzyme composition and substrate specificity of protein phosphatase 2A, *Methods Enzymol.* 366 (2003) 187–203.
- [30] A.-C. Gingras, M. Caballero, M. Zarske, A. Sanchez, T.R. Hazbun, S. Fields, et al., A novel, evolutionarily conserved protein phosphatase complex involved in cisplatin sensitivity, *Mol. Cell. Proteomics MCP.* 4 (2005) 1725–1740. doi:10.1074/mcp.M500231-MCP200.
- [31] M.J. Kean, A.L. Couzens, A.-C. Gingras, Mass spectrometry approaches to study mammalian kinase and phosphatase associated proteins, *Methods San Diego Calif.* 57 (2012) 400–408. doi:10.1016/j.ymeth.2012.06.002.
- [32] K. Arnold, L. Bordoli, J. Kopp, T. Schwede, The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling, *Bioinforma. Oxf. Engl.* 22 (2006) 195–201. doi:10.1093/bioinformatics/bti770.
- [33] T. Schwede, J. Kopp, N. Guex, M.C. Peitsch, SWISS-MODEL: An automated protein homology-modeling server, *Nucleic Acids Res.* 31 (2003) 3381–3385.
- [34] N. Guex, M.C. Peitsch, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling, *Electrophoresis.* 18 (1997) 2714–2723. doi:10.1002/elps.1150181505.
- [35] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, et al., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.* 25 (1997) 3389–3402.
- [36] N. Wlodarchak, F. Guo, K.A. Satyshur, L. Jiang, P.D. Jeffrey, T. Sun, et al., Structure of the Ca²⁺-dependent PP2A heterotrimer and insights into Cdc6 dephosphorylation, *Cell Res.* 23 (2013) 931–946. doi:10.1038/cr.2013.77.
- [37] M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, et al., Gaussian 09, Gaussian, Inc., Wallingford, CT, USA, 2009.
- [38] S. Dapprich, I. Komáromi, K.S. Byun, K. Morokuma, M.J. Frisch, A new ONIOM implementation in Gaussian98. Part I. The calculation of energies, gradients, vibrational frequencies and electric field derivatives1, *J. Mol. Struct. THEOCHEM.* 461–462 (1999) 1–21. doi:10.1016/S0166-1280(98)00475-8.
- [39] A.D. Becke, Density- functional thermochemistry. III. The role of exact exchange, *J. Chem. Phys.* 98 (1993) 5648–5652. doi:10.1063/1.464913.
- [40] R. Ditchfield, W.J. Hehre, J.A. Pople, Self- Consistent Molecular- Orbital Methods. IX. An Extended Gaussian- Type Basis for Molecular- Orbital Studies of Organic Molecules, *J. Chem. Phys.* 54 (1971) 724–728. doi:10.1063/1.1674902.
- [41] W.J. Hehre, R. Ditchfield, J.A. Pople, Self—Consistent Molecular Orbital Methods. XII. Further Extensions of Gaussian—Type Basis Sets for Use in Molecular Orbital Studies of Organic Molecules, *J. Chem. Phys.* 56 (1972) 2257–2261. doi:10.1063/1.1677527.
- [42] V.A. Rassolov, J.A. Pople, M.A. Ratner, T.L. Windus, 6-31G* basis set for atoms K

- through Zn, *J. Chem. Phys.* 109 (1998) 1223–1229. doi:10.1063/1.476673.
- [43] J.J.P. Stewart, Optimization of parameters for semiempirical methods V: modification of NDDO approximations and application to 70 elements, *J. Mol. Model.* 13 (2007) 1173–1213. doi:10.1007/s00894-007-0233-4.
- [44] E.A. Salter, R.E. Honkanen, A. Wierzbicki, Modeling the antiferromagnetic MnII MnII system within the protein phosphatase-5 catalytic site, *J. Mol. Model.* 21 (2015) 2556. doi:10.1007/s00894-014-2556-2.
- [45] W. Kabsch, XDS, *Acta Crystallogr. D Biol. Crystallogr.* 66 (2010) 125–132. doi:10.1107/S0907444909047337.
- [46] P. Evans, Scaling and assessment of data quality, *Acta Crystallogr. D Biol. Crystallogr.* 62 (2006) 72–82. doi:10.1107/S0907444905036693.
- [47] M.D. Winn, C.C. Ballard, K.D. Cowtan, E.J. Dodson, P. Emsley, P.R. Evans, et al., Overview of the CCP4 suite and current developments, *Acta Crystallogr. D Biol. Crystallogr.* 67 (2011) 235–242. doi:10.1107/S0907444910045749.
- [48] P.D. Adams, P.V. Afonine, G. Bunkóczi, V.B. Chen, I.W. Davis, N. Echols, et al., PHENIX: a comprehensive Python-based system for macromolecular structure solution, *Acta Crystallogr. D Biol. Crystallogr.* 66 (2010) 213–221. doi:10.1107/S0907444909052925.
- [49] G.N. Murshudov, P. Skubák, A.A. Lebedev, N.S. Pannu, R.A. Steiner, R.A. Nicholls, et al., REFMAC5 for the refinement of macromolecular crystal structures, *Acta Crystallogr. D Biol. Crystallogr.* 67 (2011) 355–367. doi:10.1107/S0907444911001314.
- [50] P. Emsley, K. Cowtan, Coot: model-building tools for molecular graphics, *Acta Crystallogr. D Biol. Crystallogr.* 60 (2004) 2126–2132. doi:10.1107/S0907444904019158.
- [51] J. Painter, E.A. Merritt, Optimal description of a protein structure in terms of multiple groups undergoing TLS motion, *Acta Crystallogr. D Biol. Crystallogr.* 62 (2006) 439–450. doi:10.1107/S0907444906005270.
- [52] M.R. Swingle, R.E. Honkanen, Development and validation of a robust and sensitive assay for the discovery of selective inhibitors for serine/threonine protein phosphatases PP1 α (PPP1C) and PP5 (PPP5C), *Assay Drug Dev. Technol.* 12 (2014) 481–496. doi:10.1089/adt.2014.603.
- [53] T.D. Prickett, D.L. Brautigan, The α 4 regulatory subunit exerts opposing allosteric effects on protein phosphatases PP6 and PP2A, *J. Biol. Chem.* 281 (2006) 30503–30511. doi:10.1074/jbc.M601054200.
- [54] X. Huang, R.E. Honkanen, Molecular cloning, expression, and characterization of a novel human serine/threonine protein phosphatase, PP7, that is homologous to Drosophila retinal degeneration C gene product (rdgC), *J. Biol. Chem.* 273 (1998) 1462–1468.
- [55] M.E. Hart, A.R. Chamberlin, C. Walkom, J.A. Sakoff, A. McCluskey, Modified norcantharidins; synthesis, protein phosphatases 1 and 2A inhibition, and anticancer activity, *Bioorg. Med. Chem. Lett.* 14 (2004) 1969–1973. doi:10.1016/j.bmcl.2004.01.093.
- [56] A. McCluskey, C. Walkom, M.C. Bowyer, S.P. Ackland, E. Gardiner, J.A. Sakoff, Cantharimides: a new class of modified cantharidin analogues inhibiting protein phosphatases 1 and 2A, *Bioorg. Med. Chem. Lett.* 11 (2001) 2941–2946.
- [57] A. Thaqi, J.L. Scott, J. Gilbert, J.A. Sakoff, A. McCluskey, Synthesis and biological activity of Delta-5,6-norcantharimides: importance of the 5,6-bridge, *Eur. J. Med. Chem.* 45 (2010) 1717–1723. doi:10.1016/j.ejmech.2010.01.004.
- [58] T.A. Hill, S.G. Stewart, C.P. Gordon, S.P. Ackland, J. Gilbert, B. Sauer, et al., Norcantharidin analogues: synthesis, anticancer activity and protein phosphatase 1 and 2A inhibition, *ChemMedChem.* 3 (2008) 1878–1892. doi:10.1002/cmdc.200800192.
- [59] M. Tarleton, J. Gilbert, J.A. Sakoff, A. McCluskey, Synthesis and anticancer activity of a series of norcantharidin analogues, *Eur. J. Med. Chem.* 54 (2012) 573–581.

doi:10.1016/j.ejmech.2012.06.010.

- [60] I.-J. Tseng, S.-Y. Sheu, Y.-T. Chen, C.-Y. Huang, C.-T. Lin, P.-Y. Lin, A simple procedure for preparation of N-thiazol, thiadiazol, pyridyl and sulfanylamidocantharidinimines analogues and evaluation of their cytotoxicities against human HL-60, MCF7, Neuro-2a and A549 carcinoma cell, *Chem. Pharm. Bull. (Tokyo)*. 60 (2012) 1453–1457.
- [61] L. Deng, J. Dong, W. Wang, Exploiting protein phosphatase inhibitors based on cantharidin analogues for cancer drug discovery, *Mini Rev. Med. Chem.* 13 (2013) 1166–1176.
- [62] Schrodinger LLC, The PyMOL Molecular Graphics System, Version 1.5.0.1, n.d.
- [63] I. Bertini, V. Calderone, M. Fragai, C. Luchinat, E. Talluri, Structural basis of serine/threonine phosphatase inhibition by the archetypal small molecules cantharidin and norcantharidin, *J. Med. Chem.* 52 (2009) 4838–4843. doi:10.1021/jm900610k.
- [64] M.S. Kelker, R. Page, W. Peti, Crystal structures of protein phosphatase-1 bound to nodularin-R and tautomycin: a novel scaffold for structure-based drug design of serine/threonine phosphatase inhibitors *J. Mol. Biol.* 385 (2009) 11-21
- [65] J.T. Maynes, K.S. Bateman, M.M. Cherney, A. K. Das, H.A. Luu, C.F. Holmes, M.N. James, Crystal structure of the tumor-promoter okadaic acid bound to protein phosphatase-1. *J. Biol. Chem.* 276 (2001) 44078-44082
- [66] M.S. Choy, M. Hieke, G.S. Kumar, G.R. Lewis, K.R. Gonzalez-Dewhitt, R.P. Kessler, B.L. Stein, M. Hessenberger, A.C. Nairn, W. Peti, R. Page, Understanding the antagonism of retinoblastoma protein dephosphorylation by PNUTS provides insights into the PP1 regulatory code. *Proc. Natl. Acad. Sci. USA* 111 (2014) 4097-4102

Figure 1. Structures inhibitors. A) Natural toxins known to act as strong inhibitors of PP1C, PP2AC, and PP5C. B) Novel norcantharidin based inhibitors numbered below. IC₅₀ values for novel compounds are provided in Table 1.

Figure 2. Inability of cantharidin to inhibit the activity of native human PP4C. A) Concentration response curves for the inhibition of PP4C and PP5C activity by cantharidin. The inhibition of native human PP4C and recombinant human PP5C by cantharidin was compared head-to-head. Highly purified PP5C (filled squares) and PP4C purified from HEK293T-derived cells by co-immunoprecipitation with FLAG-PP4R2 (diamonds) were assayed over a range of inhibitor concentrations as described in Procedures. The data shown for PP4C represent 4 separate experiments (each indicated by a separate line connecting the symbols for each dataset) in which PP4C activity was measured in quadruplicate for each concentration of cantharidin shown (symbols and error bars denote mean and +/- SD, respectively). B) Western analysis of anti-FLAG immunoprecipitates demonstrating the PP4C used in A was not contaminated with PP2AC. Flp-In T-Rex HEK293T cells stably expressing the indicated FLAG- tagged constructs were treated with tetracycline to induce expression of FLAG-PP4C or FLAG-PP4R2 and the cells were lysed after 24 h. Immunoprecipitation with anti-FLAG (M2) conjugated agarose beads was then performed on lysates. Immune complexes were resolved by SDS-PAGE followed by transfer to Immobilon (PVDF). Co-precipitation of native PP4C or PP2AC protein was detected by immunoblotting (IB) for native PP4C or PP2AC with anti-PP4C (lanes 1 and 2) or PP2AC (lanes 3-5) specific antibodies. CL = representative crude HEK293-cell lysate prior to IP. HC and LC represent heavy chain and light chain of the antibody used for IP, which is detected by the anti-PP2A antibody used.

Figure 3. Inhibitory effect of **(2)**/**(2e)** racemate, **(6)**, and **(7)**, and on the activities of purified PP1C (triangles) and PP5C (squares). A) Reduction of C5-C6 double bond increases the potency against both PP1C and PP5C. B) The addition of a propoxymethyl group provides a modest increase in the strength of inhibition and selectivity for PP5C compared to PP1C. Inhibition assays were conducted using highly purified PP1C or PP5C and using DiFMUP as a substrate (as described in Procedures). Each point represents the mean +/- SD (n=4). IC₅₀ values are provided in Table1.

Figure 4. Detailed representation of contacts within the active site of PP5C, and a comparison of the structures of **(4)** and **(5)** bound to PP5C. A) Active site contacts in the absence of inhibitor with bound substrate analogue, phosphate (from [22] PDB code 1s95). Hydrogen bonds are shown as yellow dotted lines and metal-ligand bonds are shown as solid lines. The green dotted line between the hydroxyl (W¹) ion and phosphorus atom (P) represents a close contact (3.0 Å) suggestive of a near-attack configuration between phosphorus and the nucleophile in the enzyme-catalyzed phosphomonoester hydrolysis reaction. B) Active site contacts with bound inhibitor **(4)**. Conventional hydrogen bonds are shown as yellow dotted lines and metal-ligand bonds are shown as solid lines. Hydrophobic contacts between the inhibitor and the side-chains of Val429, Phe446, and Tyr451 are represented as blue dotted lines (drawn to the β carbon of Val429 and the aromatic ring centroids, respectively). The small blue spheres represent aromatic ring centroids. Red dotted lines represent weak C-H...O hydrogen bonds. Figure was prepared with PyMol [62]. C) Fo-Fc omit map at the location of the inhibitor [**(4)** left; **(5)** right] contoured at +3σ with the bound inhibitor structure from the refined model displayed as sticks. D) Stick representation showing the coordination sphere of each metal ion in the **(4)** left, and **(5)** right, complex. Residues are labeled, and catalytic metals are shown as spheres (labeled M1 and M2, respectively). E) Comparison Stereo diagram showing superposition of amino acid residues near

the active site in the phosphate complex (PDB code 1s95; [22]) and the **(4)** complex (present structure). Amino acid residues, phosphate, and **(4)** are shown as sticks. Carbon atoms are shown as cyan in the phosphate complex and green in the **(4)** complex. The metal-coordinated waters in the phosphate complex are shown as magenta spheres (W1 and W2). Metal ions are shown as ecru spheres.

Figure 5. Selection of C5- versus C6-substituted enantiomers by PP5C cocrystallization. A) PP5C selects the C6-methyl enantiomer **(4)** from the C5/C6 racemate. Space-filling representations show that the hydrophobic space at right, bounded by Val429, Met455, and Tyr451, is just large enough to accommodate the C6-methyl group (red). The water associated with Val429's backbone amide at left (dashed line) tolerates the presence of the new methyl group at C6, but would be displaced by a methyl moiety at the C5 position. B) In reversal of preference, PP5C selects the C5-propoxymethyl enantiomer **(5)** from the C5/C6 racemate. Space-filling representations show navigation of the C5-propoxymethyl moiety (red) between the C α hydrogen of Glu428 (circle) and Val429. The ether oxygen of the new sidechain displaces a water molecule and H-bonds with Val429's backbone amide (dashed line). Here, the terminus of the propoxymethyl sidechain is shown to follow along Glu428 in one possible orientation, but the atom positions are undetermined by x-ray diffraction analysis. A propoxymethyl group at the C5 position would unfavorably encounter Tyr451 or Met455 at right.

Figure 6. Quantum-based modeling predictions of a steric clash between Trp257 of PP4C and the bis-acid form of norcantharidin **(3)**. A) Surface-filled diagram of the PP5C-**(3)** complex ([63];PDB code 3H61) with **(3)** shown as a stick figure tightly bound within the catalytic site of PP5C (small depression, with catalytic metals shown as spheres). B) Side view of the PP5C-**(3)** complex with inhibitor displayed as opaque sticks with a partially-transparent molecular surface. The carboxylates of **(3)** are coordinated to the metal ions (spheres). Phe446 (green surface) of PP5C abuts the bound inhibitor. The superposition of Trp257 (surface) from a homology model of PP4C (described in Procedures) indicates that binding to PP4C will be disrupted by a steric clash. C) Close-up view of the PP5C-**(3)** complex showing key O $\cdots$ M1 distances ($\text{\AA}$). The bridge oxygen (O7) of **(3)** interacts with one metal ion and is in contact with H $^{\epsilon}$ of Phe446. D) Optimized PP4C-**(3)** model system. The larger Trp257 of PP4C disrupts favorable interactions between **(3)** and the M1 M2 system, as compared to the PP5C-**(3)** complex; all carboxylate O $\cdots$ metal interaction distances ($\text{\AA}$) are longer but one, and the O7 $\cdots$ M1 distance is too far ($> 4 \text{ \AA}$) to contribute significantly to binding. E) Sequence similarity of the PPases within the catalytic core domain. Absolutely conserved residues of the compared PPases are indicated in boldface. Trp distinguishes PP4C and PP6C from the other PPases (highlight). F) Model of a theoretical steric clash between Tyr311 of PPBC and carbons C2 and C6 of cantharidin and norcantharidin derived inhibitors.

Figure 7. Mutation of PP1C, PP5C and PP6C alters sensitivity to inhibition by cantharidin. A) Substitution of tryptophan with phenylalanine in PP6C [PP6C (W256F); open circles] increases sensitivity to inhibition by cantharidin. B) In contrast, the substitution of phenylalanine (F) with tryptophan (W) in PP1C or PP5C [PP1C (F257W); PP5C (F446W)] reduces or ablates sensitivity to inhibition to cantharidin, respectively. Recombinant human PP1C, PP1C (F257W), PP5C and PP5C (F446W) were expressed in *E. coli*, and purified according to the same protocol. The activity was measured in the presence of the indicated amount of cantharidin as described in in Figure 2 and Procedures. Enzyme titrations curves are shown in Fig.2S. The data is expressed as the mean $\pm$ SD; n=4.

Table 1. Inhibition of PP1C and PP5C.

Compound	Name	Enzyme inhibition (IC ₅₀ μM +/- SE)	
		PP1C	PP5C
Cantharidin:	2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic anhydride	0.6 +/- 0.05	0.2 +/- 0.03
Norcantharidin	7-oxabicyclo [2.2.1] heptane-2,3-dicarboxylic anhydride	8.9 +/- 0.5	1.0 +/- 0.08
(1) (1e)	*5-methyl-7-oxabicyclo [2.2.1]heptane-2,3-dicarboxylic anhydride	6.0 +/- 0.8	1.0 +/- 0.6
R= CH ₂ - CH ₂ -CH ₃ (2) (2e)	*5-[(propoxy)methyl]-7-oxabicyclo[2.2.1] heptane-2,3-dicarboxylic anhydride	3.7 +/- 0.3	0.7 +/- 0.4
(6)	*5-[(phenylmethoxy)methyl]-7-oxabicyclo [2.2.1] heptane-2,3-dicarboxylic anhydride	7.0 +/- 0.3	7.0 +/- 0.5
(7)	*5-[(phenylmethoxy)methyl]-7-oxabicyclo [2.2.1]hept-5-ene-2,3-dicarboxylic anhydride	125 +/- 24.7	213 +/- 57
(8)	3-[(2,4-dimethoxyphenyl)amino]carbonyl]-7-oxabicyclo[2.2.1] heptane-3-carboxylic acid	18.9 +/- 0.9	4.16 +/- 0.2

* These compounds were assayed as racemic mixtures and present as bis-acids in the active site.

Table 2

<i>Data collection</i>	PP5C-(4) complex (4zx2)	PP5C-(5) complex (4zvz)
Resolution (Å)	91.83-1.19 (1.25-1.19)	91.03 – 2.00 (2.11 – 2.00)
Completeness (%)	94.8 (72.9)	97.9 (96.8)
Number of unique reflections	107757 (11838)	76635 (11083)
Redundancy	4.6 (3.3)	2.4 (2.4)
Rmerge	0.060 (0.594)	0.086 (0.505)
Rpim	0.031 (0.373)	0.063 (0.381)
CC(1/2)	0.999 (0.642)	0.986 (0.640)
I/σ(I)	18.3 (1.9)	11.7 (2.1)
<i>Crystal data</i>		
Space group	P2 ₁ 2 ₁ 2 ₁	P1
Unit cell parameters		
<i>a, b, c</i> (Å)	40.60, 90.83, 94.83	40.858, 80.520, 91.165
α, β, γ (°)	90, 90, 90	88.15, 87.33, 86.70
Matthews co-efficient	2.36	2.10
Solvent content (%)	47.9	41
<i>Refinement</i>		
Resolution range (Å)	40.96-1.23 (1.262-1.230)	91.03-2.00 (2.05-2.00)
Number of reflections	95453 (6121)	72774 (5362)
Completeness (%)	97.95 (90.05)	97.86 (96.67)
Rcryst	0.163 (0.244)	0.206 (0.284)
Rfree	0.175 (0.272)	0.235 (0.315)
Overall B-value (Å ²) from Wilson plot)	10.25	33.29
Number of protein atoms	2701	10084
Number of water molecules	353	284
Number of metal ions	2	8+1
Number of ligand atoms	14	86
Root mean square deviation		
Bond distance (Å)	0.006	0.005
Bond angle (°)	1.253	1.094
<i>Map and model</i>		
Correlation coefficient Fo-Fc	0.966	0.948
Correlation coefficient Fo-Fc Free	0.964	0.930
<i>Ramachandran Plot</i>		
Favored + Allowed+ disallowed (%)	96.8 + 3.2+0	96+4+0

A

B

B

A

B

C

D

E

E

PP1Ca	.RAHQVVEDGYEFFAKRQ.....LVTLF	SAPNYCGEFDNAGA.
PP2ACa	.RAHQLVMEGYNWCHDRN.....VVTIF	SAPNYCYRCGNQAA.
PP2Ba	.RAHEAQDAGYRMYRKSQTTGFPSLITIF	SAPNYLDVYNNKAA.
PP4C	.RAHQLVMEGYKWHFNET.....VLTVW	SAPNYCYRCGNVAA.
PP5C	.RSEHVKAEGYEVAHGGR.....CVTVF	SAPNYCDQMGNKAS.
PP6C	.RAHQLVHEGYKFMFDEK.....LVTVW	SAPNYCYRCGNIASIM
	R H GY T SA NY N	

↑

F

A

B

B

A

B

C

D

E

E

PP1Ca .**RA**HQVV**EDGY**EFFAKRQ.....LV**T**LF**SAPNY**CGEFD**N**AGA.
 PP2ACa .**RA**HQLVME**GY**NWCHDRN.....VV**T**IF**SAPNY**CYRCG**N**QAA.
 PP2Ba .**RA**HEAQDAG**GY**RMYRKSQTTGFPSLI**T**IF**SAPNY**LDVYNN**K**AA.
 PP4C .**RA**HQLVME**GY**KWHFNET.....VL**T**VW**SAPNY**CYRCG**N**VAA.
 PP5C .**RS**HEVKAE**GY**EVAHGGR.....CV**T**VF**SAPNY**CDQMGN**K**AS.
 PP6C .**RA**HQLVHE**GY**KFMFDEK.....LV**T**VW**SAPNY**CYRCG**N**IASIM

R H GY T SA NY N

↑

F