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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

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Summary

Protein Phosphatase 1 is ubiquitously distributed throughout all tissues and regulates many diverse pathways ranging from cell cycle progression to carbohydrate metabolism. Many naturally occurring, molecular toxins modulate PP1 activity, though the exact mechanism of this differential regulation is not understood. A detailed elucidation of these interactions is crucial for understanding the cellular basis of phosphatase function and signaling pathways, but, more importantly, they can serve as the basis for highly specific therapeutics, i.e. against cancer. We report the crystal structures of PP1 in complex with Nodularin-R at 1.63 Å and Tautomycin at 1.70 Å resolution. The PP1:Nodularin-R complex was used to demonstrate the utility of our improved PP1 production technique which produces highly active, soluble PP1. Tautomycin is one of the few toxins that reportedly preferentially binds PP1>PP2A. Therefore, the PP1:tautomycin structure is the first complex structure with a toxin with preferred PP1 specificity. Furthermore, since tautomycin is a linear non-peptide based toxin, our reported structure will critically aid in the design of lead compounds for novel PP1 specific pharmaceuticals.

Keywords

Protein phosphatase 1; nodularin-R; tautomycin; crystal structure; molecular toxins

The serine/threonine phosphatase Protein Phosphatase 1 (PP1) is tightly regulated by its interaction with more than 180 known targeting proteins,¹⁻³ proteins that localize PP1 to distinct regions of the cell, and inhibitor proteins, proteins that bind and block the PP1 active site. This large diversity of binding partners is consistent with PP1's distribution throughout the human body and its regulatory role in cellular processes as diverse as cell cycle progression, protein synthesis, muscle contraction, carbohydrate metabolism, transcription and neuronal signaling.

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In addition, large numbers of natural molecular toxins from different organisms have been identified that are capable of directly inhibiting the catalytic activity of PP1.⁴ Interest in understanding the molecular mechanism of action of these naturally occurring toxins is two-fold. First, they are an important and essential tool for dissecting phosphatase-based cell functions and signaling pathways. Second, and most importantly, they can be used as lead-compounds for the development of phosphatase-based therapeutics, especially for cancer treatments.⁵ Natural products, such as molecular toxins, have been shown to be powerful potential chemical leads for drug discovery.⁶ Unfortunately, PP1 toxins are often much larger (~500-1000 Da) than commonly used drugs (~100-300 Da). Thus, only sub-fragments of the toxin, such as those that interact at the active site, would typically be used as lead compounds.⁷ These sub-fragments can be readily identified from the 3-dimensional structures of the toxin:PP1 complexes.

The three most widely studied classes of PP1 toxin inhibitors are: 1) cyclic hepta-peptide microcystins⁸ (MCLR, freshwater marine cyanobacteria, *Microcystis sp.* and *Nodularia sp.*), 2) okadaic acid⁹ (OA, polyether fatty acids from the marine dinoflagellates *Prorocentrum sp.* and *Dinophysis sp.*) and 3) calyculin A¹⁰ (octamethyl polyhydroxylated fatty acids from marine sponges). Other identified but less-extensively studied toxins include: 1) nodularins¹¹ (cyclic penta-peptides from brackish water and marine cyanobacteria), 2) tautomycin¹² (from the soil bacteria *Streptomyces sp.*), 3) fostriecin¹³ and 4) cantharidin¹⁴ (blister beetles, ~1500 species). Finally, a new PP1 inhibitor, belizeanic acid, was recently extracted from the dinoflagellate *Prorocentrum belizeanum*.¹⁵ Because the biochemically best studied ser/thr phosphatases PP1, PP2A (Protein phosphatase 2A) and, to some extent, PP2B (calcineurin), have highly similar active sites, these toxins also inhibit PP2A and PP2B (PP1, and PP2A and PP2B form the PPP subfamily of protein phosphatases). However, these do so with affinities that differ from those of PP1. The molecular basis of these differences is not well understood.

The 3-dimensional structures of PP1:okadaic acid,¹⁶ PP1:calyculin A,¹⁷ PP1:microcystin-LR,¹⁸ PP1:dihydromicrocystin-LA,¹⁹ PP1:nodularin-V¹⁹ (motuporin) and PP2Ac:microcystin-LR^{20,21} have been reported. All of these toxins are large (500-1000 Da) and consistently show superior potency against PP2A. However, because of the similarities in the active sites of PPPs, it is possible to identify structural features of toxin inhibition which are common to the PPP phosphatase family. These common features include: 1) the binding of the molecular toxins at the PPP active sites, 2) the interaction of the molecular toxins with the toxin-sensitive β 12- β 13-loop²² (residues 268-281 of PP1), and 3) the interaction of the molecular toxins within the hydrophobic groove, one of three functionally important grooves on the Ser/Thr phosphatase surface. Because so many structural features of the Ser/Thr phosphatase:inhibitor interaction are similar among the Ser/Thr phosphate family, this also means that there is no inhibitor currently available that is selective for a particular PPP, such as PP1 or PP2A. This has hindered efforts to understand essential biological signaling cascades specific to each individual phosphatase and also for rational drug design.

In this report, we describe the co-crystal structures of PP1:nodularin-R and PP1:tautomycin. The structure of the PP1:nodularin-R (Figure 1(a)) co-complex enabled us to both develop a novel route of PP1 production, which results in PP1 identical to previously studied PP1, however at ~10-times higher expression yields, and to provide a direct comparison with the recently published PP1:nodularin-V structure. It is also the highest-resolution structure of PP1 to-date reported, at 1.63 Å. However the emphasis of this work is on the anticipated structure of the PP1:tautomycin complex. Tautomycin (Figure 1(b)) is one of the few reported molecular toxins with preferred PP1 specificity.²³ Furthermore, since tautomycin is a linear aliphatic inhibitor of PP1, and not a cyclic peptide like microcystins and nodularins, it forms a much needed lead compound for novel PP1-specific drug design.

Experimental Details

Here we describe a novel route for the production of soluble and active PP1 (~10 times increase compared with previously reported efforts; details are described in Table caption 1). Soluble, active PP1 α (human), comprised of residues 7-300 fused to an N-terminal His₆-tag and tobacco etch mosaic virus (TEV) protease site, was expressed in *Escherichia coli* by co-expression with the GroEL-ES chaperone. After an initial Ni-NTA purification step, PP1 activity was confirmed using the pNPP assay; K_M of $2.76 \pm 0.12 \mu\text{M}$ and k_{cat} of 13.8 s^{-1} values are in excellent agreement with previously published results.²⁴ The PP1:toxin complexes were formed by adding either nodularin or tautomycin in a 1:1 molar ratio. The complexes were further purified by enzymatic His₆-tag removal, followed by anion exchange (Mono Q, GE Healthcare) and size exclusion (Superdex 75 26/60, GE Healthcare) chromatography.

PP1:nodularin-R complex crystals (P2₁2₁2₁; a=65.5 Å, b=77.2 Å, c=132.2 Å) were formed under oil from 20% PEG 3350 and 0.2 M NaI and contained two monomers per asymmetric unit. The structure was determined to 1.63 Å by molecular replacement using the PP1:Microcystin-LR (PDBid: 1fjm)¹⁸ structure as the search model. Initial $3F_o-2F_c$ maps were of high quality and facilitated building of residues 7-299 of monomers A and B. The final model ($R_{\text{cryst}}=14.3\%$, $R_{\text{free}}=16.9\%$) is composed of 586 protein residues, four Mn⁺² atoms and two nodularin molecules. Superposition of monomer A onto monomer B gives root-mean-square deviations (rmsd) of 0.35 Å. The model stereochemistry is excellent (Table 1) with 99.8% of the residues in the favored region. One residue (0.2%), P24, in monomer B, is in the disallowed region; however, it is involved in a tight turn and has complete main chain and side chain density.

PP1:tautomycin complex crystals (P2₁2₁2₁; a=61.8 Å, b=78.5 Å, c=130.8 Å) were obtained under oil from 0.1 M Tris pH 8.0, 30% PEG 6K and 1 M LiCl and contained two monomers per asymmetric unit. The structure was determined to 1.70 Å by molecular replacement using the PP1:nodularin-R (PDBid: 3e7a) structure as the search model. Initial $3F_o-2F_c$ maps were of high quality and enabled rapid building of residues 7-299 of monomers A and B. The final model ($R_{\text{cryst}}=15.4\%$, $R_{\text{free}}=17.8\%$) is composed of 588 residues, four Mn⁺² atoms and two tautomycin molecules. Consistent with published results, the diacid tautomer of tautomycin was bound in the active site.^{25,26} Superposition of monomer A onto monomer B gives rmsd values of 0.3 Å for all atoms. The model stereochemistry is excellent (Table 1) with 99.7% of the residues in the favored region. Two residues (0.3%), R23 and P24 in monomer B, are in the disallowed region; however, both have complete main chain and side chain density.

PP1 and the PP1:Nodularin-R Co-Complex

The catalytic subunit of PP1 consists of 10 α -helices and three β -sheets comprised of 14 β -strands. The active site lies at the bifurcation point of three predicted substrate binding grooves: the hydrophobic, C-terminal and acidic grooves. Within the active site, two very strong electron-density peaks were assigned as Mn⁺² ions, due to the presence of 1 mM MnCl₂ in all buffers during expression, purification and crystallization. As assessed using the DALI²⁷ server, the tertiary structure of PP1 in the PP1:nodularin-R and PP1:tautomycin complex structures are highly similar to those of previously published PP1 complexes. To date, all reported PP1 structures are remarkably similar, despite being: 1) crystallized in disparate crystallization conditions, 2) forming crystals in different space groups and, 3) being crystallized with different ligands. This lack of conformational change in PP1 itself highlights the importance of PP1 binding proteins and molecular toxin ligands for regulating activity of PP1.^{1,3}

An overlay of all known PP1:toxin structures highlights the well-recognized and intriguing similarity of all reported 3-dimensional structures of PP1. Differences are observed only in

two distinct regions (supporting information, Figure S1). The first major difference among the PP1 molecular toxin structures occurs in the PP1 β 12- β 13 loop, which has been proposed to confer inhibitor specificity.^{22,28} Specifically, a large movement of the β 12- β 13 is the hallmark of the PP1:microcystin-LR interaction. This rearrangement, which occurs only upon binding microcystin-LR, is due to covalent alkylation of the C273 sulfhydryl group by a dehydroalanine residue.²⁹

The second major difference among PP1 molecular toxin structures, observed when PP1 is bound to calyculin A or nodularin-V, involves movement of the loop region comprising residues D194-G199. As expected, nodularin-R binding does not change the active site of PP1 and, in this region of PP1, it forms interactions identical to the nodularin-V structure and the closely structurally related MCLR structure.¹⁸ Nodularin-R is anchored to the active site by an extensive hydrogen bonding network (Figure 2(b)); an additional ligplot-derived drawing and table are shown in supporting information Figure S2(a) and Table S1). The variable residue of nodularin, arginine, makes no contacts to PP1, as also observed for the variable residue, V, of the nodularin-V structure. In addition, in both PP1:nodularin complexes, the *N*-methyldehydrobutyrine residue is ~4.4 Å away from C273 in the β 12- β 13 loop. This distance is too far to form a covalent adduct similar to that present in the MCLR structure, clearly illustrating why a unique β 12- β 13 loop configuration is seen only in the PP1:MCLR structure and not in the PP1:nodularin-R, or any other PP1:molecular toxin structures.

As previously reported, the hydrophobic 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6,-dienoic acid “Adda” residue of nodularin-R binds to the hydrophobic groove (Figures 2(a) and (b)). However, the interactions of nodularin-V and nodularin-R with the PP1 hydrophobic groove and especially the D194-G199 loop are different (Figure 3(a)). This differential binding is likely due to the presence of two β -mercaptoethanol (BME) molecules in the PP1:nodularin-V structure. One BME molecule shifts the D194-G199 loop ~4 Å away from the hydrophobic Adda residue relative to the loop position in the nodularin-R structure. The second BME molecule is bound directly beneath the Adda moiety, shifting the position of Adda’s aromatic ring 2.65 Å closer to α -helix 4 in PP1. This shift results in a decreased number of hydrophobic contacts between nodularin-V and PP1 as compared to the PP1:nodularin-R structure. Thus, the observed movement of the PP1 D194-G199 loop in the PP1:nodularin-V structure is likely a crystallization artifact. Because BME is not expected to interact with PP1 *in vivo*, the nodularin-R interaction observed in our PP1:nodularin-R structure, which does not result in a change in the conformation of the D194-G199 loop, is most likely the biologically relevant conformation.

The PP1:Tautomycin Co-Complex

Tautomycin is one of the few known molecular toxin PP1 inhibitors that show a small preferred selectivity for PP1 versus PP2A (~1.5-4 times more selective).²³ As indicated by its name, in solution tautomycin exists in an equilibrium between two tautomers: a diacid and anhydride. Multiple studies have shown that the active tautomer for PP1 inhibition is the diacid form.^{25,26} The tautomycin molecule can be divided into two functional fragments (Figure 1(b)): 1) a hydrophilic moiety (C18-C7’) that contains the tautomeric center in the diacid form and, as we show here, binds the active site of PP1, and 2) a hydrophobic bicyclic ketal moiety (C1-C17) that makes contacts with the hydrophobic groove of PP1.

The first functional fragment of tautomycin, which contains the tautomeric center, binds the PP1 active site. The tautomycin diacid is anchored via one water mediated and six direct hydrogen bonds with PP1 active site residues R96, R221, V250 and Y272 (Figures 2(c) and (d)), an additional ligplot-derived drawing is shown in supporting information, Figure S2(b)). As observed in the PP1:OA crystal structure,¹⁶ tautomycin makes only one contact with the

PP1 β 12- β 13 loop: a critical hydrogen bond interaction with the hydroxyl-group of Y272. This illustrates why the mutation of Y272 results in an up to a 26000-fold increase of the IC_{50} of tautomycin for PP1.²⁸ In addition, it has also been previously reported that the C22 hydroxyl of tautomycin is required for its full inhibitory activity and that its removal severely weakens the PP1:tautomycin interaction.²⁶ Our PP1:tautomycin structure illustrates the molecular basis for this observation: a strong 2.3 Å intramolecular hydrogen bond is observed between the C22 hydroxyl and C6' acid moiety of tautomycin (Figure 2(d)). This hydrogen bond stabilizes the tautomycin diacid group, which is bound at the active site, forming a tight, roughly pseudo-cyclic structure. Interestingly, a similar intramolecular hydrogen bond was observed between the C24 hydroxyl and C1 acid of OA in the PP1:OA complex, highlighting that intramolecular toxin hydrogen-bonds are important for stabilizing the interaction of molecular toxins with PP1.¹⁶

The second functional fragment of tautomycin is the hydrophobic bicyclic ketal (spiroketal) moiety, which does not interact with the PP1 active site but instead binds the PP1 hydrophobic groove (Figures 2(c) and (d)). Hydrophobic interactions with PP1 residues W206, V223, I133, G222, S129 and C127 strongly anchor the spiroketal moiety of tautomycin in the PP1 hydrophobic groove. In addition, the side chain of PP1 residue W206 forms a water-mediated hydrogen bond with the C2 oxygen of tautomycin, further enhancing its interaction with PP1. While these interactions appear substantial, as demonstrated by Colby *et al.*,³⁰ removal of the C6-C14 spiroketal group does not abolish tautomycin inhibition of PP1. IC_{50} values for Colby's tautomycin derivative against PP1 are ~100 nM, only 500 times higher than the IC_{50} of 0.19 nM reported for full-length tautomycin. It is important to remember that mutating Y272 in the PP1 β 12- β 13 loop reduces the IC_{50} of tautomycin 26000-fold, highlighting the increased importance of the interaction of tautomycin at the PP1 active site versus the hydrophobic groove. Moreover, tautomycetin (Figure 1(c)), a recently identified structural analogue of tautomycin that lacks the spiroketal group, shows an even higher specificity for PP1 versus PP2A than tautomycin. The reported IC_{50} values of tautomycetin are 1.6 nM for PP1 and 62 nM for PP2A, a ~40-fold difference.³¹ This further emphasizes the importance of the interaction of these molecular toxins at the PP1 active site for PP1 inhibition and, especially, PPP selectivity. Lastly, our structure also illustrates critical differences from the PP1:OA interaction, an interaction that had previously been thought to be similar to the PP1:tautomycin interaction. Unlike tautomycin, OA requires its analogous spiroketal moiety for inhibition (C28-C38), as its removal ablates any PP1 or PP2A inhibitory activity.³² Taken together, these observations strongly suggest that tautomycin (and tautomycetin) inhibitory activity and selectivity for PP1 versus PP2A originates from the C18-C7' half of the molecule. The spiroketal group of tautomycin only contributes to its overall binding affinity thereby increasing its potency.³³

Using the PP1:tautomycin structure, it should be possible to detect structural differences that lead to the small preferred PP1 versus PP2A specificity of tautomycin (~1.5-4-fold). The catalytic domains of PP1 and PP2A are 49% identical in sequence. Superposition of PP1 with PP2A (PDBid: 2NPP)³⁴ illustrates that the active sites of these protein phosphatases are structurally identical. This suggests that interactions outside of the active site must be the basis for the differential binding of tautomycin. Nevertheless, extensive direct comparison of the PP1:tautomycin complex with PP2A does not lead to an obvious structural explanation for the small preferred PP1 versus PP2A specificity of tautomycin. It is critical to point out, and as is evident by the relatively broad range of literature reported IC_{50} values for the same PPP inhibitors (Table S1), that all literature reported IC_{50} values are influenced by quantity of inhibitor and enzyme used, mode of inhibition, buffers and reagents used, among other factors. Therefore, variations of the reported IC_{50} values from different laboratories are not surprising, and thus a structural basis for a small differences in IC_{50} values is likely not identifiable. This is further supported by the fact that while there are differences in the reported IC_{50} values of

nodularin-V and nodularin-R for PP1 (Table S1), structural differences between these two complexes are also not observed (Figure 3(a)).

Because of tautomycin's reported higher specificity towards PP1 than PP2A, there has been extensive interest in obtaining the 3-dimensional structure of this complex to facilitate the design of PP1 specific inhibitors. To meet this need, Colby *et al.* published a model of this complex in which tautomycin was docked into the PP1 active site (using the PP1 coordinates of PDBid 1JK7) and energy minimized.³⁰ Interestingly, comparison of the model with our experimentally determined structure illustrates that the proposed overall interaction pattern was largely correct, i.e. the diacid group binds the PP1 active site and the spiroketal moiety binds in the PP1 hydrophobic groove. However, there are also several key differences (Figure 3(b)). First, in the model, the diacid group enters the PP1 active site from the left. This removes all stabilizing hydrogen bonds within the active site and orients the diacid group $\sim 180^\circ$ away from its position in the crystal structure. Second, and as a result of the diacid group rotation, the C22 hydroxyl, which is involved in an intramolecular hydrogen bond in the crystal structure, is oriented away from the PP1 active site in the model. Therefore, the model did not explain the hydroxyl's previously reported importance for PP1:tautomycin binding. Third, the spiroketal group is rotated clockwise by $\sim 90^\circ$ such that atoms C1-C6 are pointed towards α -helix 4 instead of being buried within the PP1 hydrophobic groove. This rotation hinders the formation of hydrophobic contacts and also the W206-water mediated hydrogen bond observed in the crystal structure. Lastly, and critical for future studies, the orientation and positioning of PP1 active site residues changed during the energy minimization step, as compared to the experimentally determined structure. Due to the known rigidity of PP1 (based on the comparison of PP1 in all PP1 crystal structures determined to date; Figure S1) and, in particular, the extremely limited changes observed in the PP1 active site, it seems that the assumption that PP1 undergoes only limited conformational changes upon toxin binding will lead to better results for future "*in silico*" drug design of specific PP1 inhibitors.

This PP1:tautomycin crystal structure clearly demonstrates the importance of the interactions made between the tautomycin diacid and the PP1 active site residues. Moreover, the diacid core will serve as a new fragment for ongoing efforts to develop a lead compound for PP1 specific inhibitors. Based on the PP1:tautomycin structure, we expect the PP1:cantharidin complex to have an identical interaction with the active site, as cantharidin (Figure 1(d)) has an diacid group identical to that of tautomycin. Cantharidin is the smallest and most simple PP1 molecular toxin known and is used in traditional Chinese medicine for cancer treatments.³⁵ Indeed, numerous cantharidin analogs have already been synthesized by several academic groups with the goal of increasing binding affinity and/or PP1/PP2A selectivity. These compounds have been tested for PP1/PP2A inhibition and selectivity and also as therapeutics in cancer cell lines.^{5,36,37} To date, no significant improvements in binding potency or selectivity of these compounds as compared to unmodified cantharidin have been reported. In a related effort, Tatlock *et al.*, have shown that modified cantharidin-type molecules (endothall based) can also be used as PP2B inhibitors.³⁸ The inability of these efforts to produce more potent and selective cantharidin-based inhibitors clearly shows that a PP1:cantharidin structure is essential for these efforts to be successful.

Based on the expected similarities of the tautomycin/cantharidin PP1 active site interaction, we used the experimental PP1:tautomycin structure to create a model of the PP1:cantharidin complex (Figures 4(a) and 4(b)). The model illustrates, that modifications at the bridging-ether containing bicyclic skeleton of cantharidin result in significant steric clashes with PP1 active site residues. In addition, this region of PP1 leaves little room for large moieties. This is supported by the observation that the addition of large moieties at this site of cantharidin results in decreased PP1 binding. Furthermore, modification of the 7-O bridgehead atom itself would abolish hydrogen bonding interactions with PP1, destabilizing the PP1:cantharidin interaction.

Thus, it is no surprise that cantharidin analogs modified at 7-O always showed decreased binding affinities for PP1. Therefore, this PP1:cantharidin model is an important asset for the rational design of new inhibitors by medicinal chemists.

In summary, these PP1:inhibitor complexes provide multiple important insights for the generation of Ser/Thr protein phosphatase specific inhibitors. These inhibitors are of great need, as they will provide superior tools that will enable us to: 1) rapidly analyze essential biological pathways regulated by PPPs and 2) when sufficient selectivity for PP1 and/or PP1 holo-enzymes is achieved, lead to the development of a novel class of potent therapeutics for PP1-related diseases, including cancer.

PDB accession numbers

Structure factors and coordinates for the PP1 complexes have been deposited in the PDB with accession codes 3E7A for PP1:nodularin-R and 3E7B for PP1:tautomycin.

Table Caption

PP1 α DNA (human) encoding residues 7-300 (wt-PP1: 1-330) was cloned into an in-house vector (RP1B) in frame to an N-terminal His₆-tag and tobacco etch mosaic virus (TEV) protease cleavage site for cleavage of the purification tag.³⁹ The plasmid encoding PP1₇₋₃₀₀ is co-transformed with the pGRO7 plasmid encoding the chaperone GroEL/GroES (Takara) into the BL21 (DE3) *E. coli* (Invitrogen) cells.

To ensure the PP1 plasmid has been successfully transformed, several colonies are grown in rich LB medium (supplemented with Kanamycin and Chloramphenicol), at 37°C until OD₆₀₀ of ~0.6 is reached; one ml aliquots are removed and induced with 1 mM IPTG for three hours. Un-induced and induced samples are analyzed with SDS-PAGE to confirm PP1 expression.

An overnight starter culture of PP1 is grown at 37°C in LB medium supplemented with Kan, Cam and 1 mM MnCl₂. PP1 production is initiated by inoculating each liter of LB with 35 ml of starter culture. The cells are grown in LB medium supplemented with 1 mM MnCl₂ at 30°C to an OD₆₀₀ of ~0.5 when 2 g/L of arabinose is added to induce the expression of the GroEL/GroES chaperone. When OD₆₀₀ equals ~1, the temperature is lowered to 10°C (ice-bath) and PP1 expression is induced with 0.1 mM IPTG for ~20 hours. The cells are harvested by centrifugation, re-suspended in fresh LB medium (again supplemented with 1mM MnCl₂ and 200 µg/ml chloramphenicol to eliminate all ribosome activity) and agitated for two hours at 10°C. This final step allows for *in vivo* folding of PP1 and increases the yield of active, well-folded PP1. Harvested cells are frozen and stored at -80°C.

For purification, cell pellets are thawed at room temperature then resuspended in lysis buffer (50 mM Tris pH 8.0, 5 mM Imidazole, 700 mM NaCl, 1 mM MnCl₂, 0.1% Triton X-100 and complete EDTA-free protease inhibitor tablets) and lysed by high-pressure cell homogenization (Avestin C-3 Emulsiflex). Supernatants are clarified by centrifugation (30,000g/20 minutes/4°C) and purified *via* Ni-NTA IMAC (Qiagen). PP1 is eluted with 10 ml elution buffer (50 mM Tris pH 8.0, 250 mM Imidazole, 700 mM NaCl and 1 mM MnCl₂) into 40 ml of dilution buffer (50 mM Tris pH 8.0, 5 mM Imidazole, 700 mM NaCl and 1 mM MnCl₂) under constant stirring at 4°C. This dilution step is required to reduce the apo-PP1 concentration, which precipitates above ~5 µM. PP1 activity is confirmed using a standard pNPP assay (described below).

Following PP1 activity determination, nodularin-R or tautomycin (Alexis Chemicals) is added in a 1:1 molar ratio and incubated at 4°C for 15 minutes before the addition of the 27 kDa

catalytic domain of the Nuclear Inclusion a (NIa) protein encoded by the tobacco etch virus (TEV; his₆-tagged; produced in-house). The successfully cleaved PP1 is dialyzed against 50 mM Tris pH 8.0, 5 mM Imidazole, 700 mM NaCl and 1 mM MnCl₂ overnight at 4°C. Complete digestion is confirmed by SDS-PAGE. TEV and undigested PP1 are removed by a subsequent subtraction purification step (second Ni-NTA column).

The PP1:toxin containing flow through is prepared for ion exchange chromatography (Mono Q, GE Healthcare) by dialyzing overnight at 4°C against 20 mM Tris pH 8.0, 40 mM NaCl and 0.02% Tween-20. Precipitated protein is removed by filtration through 0.22µ filters. PP1 is eluted against a gradient of 20 mM Tris pH 8.0, 500 mM NaCl and 0.02% Tween-20. Fractions containing the PP1:nodularin-R or PP1:tautomycin complex are pooled and concentrated to 5-10 ml then applied to a Superdex 75 26/60 (GE Healthcare) size exclusion column in 50 mM Tris pH 7.0, 500 mM NaCl, 1 mM MnCl₂, and 0.02% NaN₃. For crystallization trials the PP1:nodularin complex was concentrated to ~4 mg/ml and the PP1:tautomycin complex to ~8 mg/ml as determined by a BCA assay (Pierce).

Phosphatase activity of recombinant PP1 was assessed using the commercially available pNPP assay (Sigma). Ni-NTA eluted PP1 was diluted to 1 µM in 50 mM Tris pH 8.0, 5 mM Imidazole, 700 mM NaCl, 1 mM MnCl₂. Michaelis-Menten kinetic analysis was conducted using final pNPP concentrations of 0.5, 1, 2, 4 and 8 mM in 96 well clear Corning Costar cell culture plates. pNPP tablets were dissolved in buffer solution containing 150 mM Bis-Tris pH 6.5 and 150 mM NaCl. Each 100 µL reaction well contained 20 µL buffer solution, 20 µL 1 µM PP1 and 60 µL pNPP solution. Reactions were incubated for five minutes at 30°C and stopped with the addition of 100 µL 1 M NaOH. The absorbance of p-nitrophenol was immediately measured at 405 nM and subtracted from the absorbance of the control well. Experiments were completed in triplicate and data analyzed with SigmaPlot (Systat).

Crystallization experiments were conducted at room temperature, under paraffin oil using 72 well Terasaki tissue culture plates (Micro-batch plate; Hampton Research). 0.5 µL of PP1:toxin complex was added to 0.5-1 µL precipitant and covered with paraffin oil (Hampton Research). PP1:nodularin-R crystallized in 20% PEG 3350 and 0.2 M NaI. PP1:tautomycin crystallized in 0.1M Tris pH 8.0, 30% PEG 6K, 1M LiCl. In both cases, crystals appeared within 24 hours and were harvested at day four by 'flash-cooling' to -180°C in cryoprotectant consisting of well buffer and 20% glycerol.

A 2.37 Å low resolution data set was collected in-house and an initial PP1:Nodularin-R model was determined by molecular replacement with Phaser⁴⁰ from the CCP4i⁴¹ suite using a the 2.1 Å crystal structure of PP1:Microcystin-LR (PDB code 1FJM¹⁸) as a starting model. A 1.63 Å data set was collected on a single PP1:nodularin-R crystal at the National Synchrotron Light Source at the Brookhaven National Laboratories (beamline X6A). Data were processed and scaled with HKL2000.⁴² The spacegroup is P2₁2₁2₁ with unit cell dimensions a=65.49 Å, b=77.18 Å and c=132.24 Å, with two molecules per asymmetric unit ($V_m=2.39 \text{ Å}^3/\text{Da}$) corresponding to a solvent content of 48%. The 1.63 Å, high resolution PP1:nodularin-R complex structure was determined using by molecular replacement with Phaser⁴⁰ from the CCP4i⁴¹ suite using the initial, low resolution crystal structure of PP1:Nodularin-R as a starting model (Z-score=71.05, final LLG=8662.94). A rigid body refinement ($R_{\text{cryst}}=27.6\%$, $R_{\text{free}}=29.5\%$) using REFMAC5⁴³ was performed using the 1.63 Å molecular replacement structure. Initial electron density maps were of excellent quality and facilitated manual building of the PP1 and nodularin molecules using Coot.⁴⁴ Unambiguous positive difference density showed the location of nodularin within the active site. REFMAC5⁴³ using TLS⁴⁵ refinement with riding hydrogens was used for refinement. The model was rebuilt into σ_A -weighted $3F_o-2F_c$, $2F_o-F_c$ and F_o-F_c electron density maps in Coot.⁴⁴ Water molecules were assigned automatically in ARP/wARP⁴⁶ at $>3\sigma$ F_o-F_c difference density peaks and verified by manual

inspection. The final model ($R_{\text{cryst}}=14.27\%$, $R_{\text{free}}=16.93\%$) is composed of 586 residues, four Mn^{+2} atoms, 692 water, two glycerol, two azide, four chloride, 14 iodine and two nodularin molecules. Residue 300 of monomer A and the side chains of residues 23, 26, 41, 98, 211, 214, 218, 230, 234, 260 and 275 were un-modeled due to lack of interpretable electron density residue as was residue 300 of monomer B and side chains of residues 23, 26, 77, 211, 214, 218, 230, 234, 260, 275 and 287. The quality of the model was analyzed using the programs MolProbity,⁴⁷ WHATIF⁴⁸ and PROCHECK.⁴⁹

Data was collected on a single crystal PP1:tautomycin crystal to 1.70 Å at Brookhaven National Laboratories beamline X6A. Data were processed and scaled with HKL2000⁴². The spacegroup is $P2_12_12_1$ with unit cell dimensions $a=61.76$ Å, $b=78.52$ Å and $c=130.76$ Å, with two molecules per asymmetric unit ($V_m=2.51$ Å³/Da) corresponding to a solvent content of 51%. The PP1:tautomycin complex structure was determined by molecular replacement with Phaser⁴⁰ from the CCP4i⁴¹ suite using the 1.63 Å crystal structure of PP1:nodularin (PDB code 3E7A) as a starting model ($Z\text{-score}=76.33$; final refined LLG=5372.45). An initial model was generated by performing a rigid body refinement ($R_{\text{cryst}}=31.56\%$, $R_{\text{free}}=30.99\%$) using REFMAC5.⁴³ Initial electron density maps were of excellent quality and facilitated manual construction of the PP1:tautomycin structure using Coot.⁴⁴ The model was rebuilt into σ_A -weighted $3F_o-2F_c$, $2F_o-F_c$ and F_o-F_c electron density maps in Coot.⁴⁴ Water molecules were assigned automatically in ARP/wARP⁴⁶ at $>3\sigma$ F_o-F_c difference density peaks and verified by manual inspection. Refinement was continued with REFMAC5⁴³ using TLS⁴⁵ refinement and with riding hydrogens. The tautomycin REFMAC library file was generated using PRODRG.⁵⁰ The final model ($R_{\text{cryst}}=15.3\%$, $R_{\text{free}}=17.5\%$) is composed of 588 residues, four Mn^{+2} atoms, one Cl^{-1} and one Na^{+1} atom, 487 waters, one glycerol, six azide and two tautomycin molecules. Residue 300 of monomer A and partial side chains of residues 18, 23, 98, 198, 211, 214, 218, 234, 260 and 275 were un-modeled due to lack of interpretable electron density as was residue 300 of monomer B and partial side chains of residues 23, 41, 98, 188, 197, 198, 211, 214, 218, 230, 234, 240, 260, 275 and 287. The quality of the model was analyzed using the programs MolProbity,⁴⁷ WHATIF⁴⁸ and PROCHECK.⁴⁹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

PP1	Protein Phosphatase-1
MCLR	Microcystin-LR
NODr	Nodularin-R
NODv	Nodularin-V
OA	Okadaic Acid
Adda	(2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6,-dienoic acid
Masp	D-erythro- β -methylaspartic acid
Mdhhb	2-(methylamino)-2-dehydrobutyric acid
rmsd	root mean square deviation
BME	β -mercaptoethanol

Figure 1. Structures of nodularin-R (a), tautomycin (b), tautomycetin (c) and cantharidin (d). Important interaction sites with PP1 identified in this study are highlighted in the figures. An asterisk marks the critical OH group in tautomycin. Details are given in the text.

Figure 2. Overview of nodularin-R and tautomycin binding to PP1. Stereo images of (a) 1.63 Å, simulated annealing omit map⁵¹ contoured at 3.0σ and (b) detailed active site interactions of nodularin-R. (c) 1.7 Å simulated annealing omit map⁵¹ contoured at 3.0σ map of tautomycin bound to the surface of PP1 and (d) detailed active site interactions of tautomycin. Dotted lines represent potential hydrogen bonding interactions. The tautomycin intramolecular hydrogen bond is shown as a solid black line. This figure was made with PyMol.⁵²

Figure 3.

Comparisons of the nodularin and tautomycin binding sites. (a) PP1:Nodularin-V (silver:blue) and PP1:Nodularin-R (green:yellow) PP1 active sites are overlaid. The location of a β-mercaptoethanol (BME) molecules of the PP1:Nodularin-V structure cause residues 194-199 to shift 4.0 Å away from the hydrophobic groove (movement depicted by black arrow) relative to those of PP1 bound to Nodularin-R. A second BME molecule, bound directly under the Adda residue of Nodularin-V, causes it to rotate 2.65 Å closer to α-helix 4. (b) Tautomycin from our PP1:tautomycin (green:yellow) crystal structure is overlaid with the hypothetical, molecular dynamics generated tautomycin model (purple) from Colby, *et al.*³⁰ The general binding mode of the hypothetical tautomycin model is correct. The diacid binds the active site but is oriented in the opposite direction relative to the crystal structure. The C22 hydroxyl (noted by arrows) is involved in an intra-molecular hydrogen bond in the crystal structure, which explains its biological importance;²⁶ this is not observed in the model. The spiroketal binds to the hydrophobic cleft; however, when compared to the crystal structure it's rotated

~90° away from α -helix 4 such that atoms C1-C6 are no longer buried within the cleft. Mn^{+2} atoms are silver spheres. All images in stereo. This figure was made with PyMol.⁵²

Figure 4. PP1:cantharidin model. (a) Hydrogen bond interaction network on cantharidin and the PP1 active site. PP1 residues Y272, R221 and R96 make critical stabilizing interactions. (b) Surface representation of PP1 of the PP1:cantharidin model. Four surface pockets that can potentially be used to tether cantharidin-based inhibitors onto PP1 are highlighted.

Table 1

Data processing, model and refinement statistics.

	PP1:nodularin-R (3E7A)	PP1:tautomycin (3E7B)
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions (Å)	a=65.4, b=77.3, c=132.4	a=65.8, b=78.5, c=130.8
Data Collection		
Resolution range (Å)	50-1.63 (1.69-1.63) ^a	50-1.70 (1.76-1.70)
Unique reflections	83995 (7804)	74731 (6917)
Completeness (%)	99.3 (93.5)	99.2 (93.3)
Redundancy	7.2 (4.1)	5.7 (4.4)
R _{sym} ^b (%)	7.2 (51.2)	4.6 (51.1)
Average I/σ (I)	18.6 (2.7)	22.7 (3.0)
Wavelength (Å)	1.000	1.000
Model and Refinement Statistics		
Resolution range (Å)	26.57-1.63	19.63-1.70
Reflections (total)	83884	74610
Reflections (test)	4185	3750
R _{cryst} ^c (%)	14.3	15.3
R _{free} ^c (%)	16.9	17.5
Protein atoms/toxin/water molecules	4708/113/692	4745/110/487
RMSD bonds (Å) / angles (°) / dihedrals (°)	0.010/1.37/6.61	0.012/1.52/6.55
<B> protein atoms / toxin / water molecules (Å ²)	15.5/21.4/33.5	25.2/32.6/39.3
Ramachandran Plot ^d	99.8/0.2	99.7/0.3

^aParentheses indicate the data statistics for the highest resolution shell used for each structure^bR_{sym}=100·Σ_i|I_i-<I_i>|/Σ_iI_i where I_i is the scaled intensity of the *i*th measurement, and <I_i> is the mean intensity for that reflection.^cR_{cryst}=Σ||F_O|-|F_C||/Σ|F_O|, where F_O and F_C are the observed and calculated structure factor amplitudes, respectively. R_{free} = as for R_{cryst}, but for 5 % of the total reflections chosen at random and omitted from refinement.^dNumber of residues in favored region/outlier region