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PP1:Tautomycetin Complex Reveals a Path toward the Development of PP1-Specific Inhibitors

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Abstract

Selective inhibitors for each serine/threonine phosphatase (PPP) are essential to investigate the biological actions of PPPs and to guide drug development. Biologically diverse organisms (e.g., cyanobacteria, dinoflagellates, beetles) produce structurally distinct toxins that are catalytic inhibitors of PPPs. However, most toxins exhibit little selectivity, typically inhibiting multiple family members with similar potencies. Thus, the use of these toxins as chemical tools to study the relationship between individual PPPs and their biological substrates, and how disruptions in these relationships contributes to human disease, is severely limited. Here, we show that tautomycetin (TTN) is highly selective for a single PPP, protein phosphatase 1 (PP1/PPP1C). Our structure of the PP1:TTN complex reveals that PP1 selectivity is defined by a covalent bond between TTN and a PP1-specific cysteine residue, Cys127. Together, these data provide key molecular insights needed for the development of novel probes targeting single PPPs, especially PP1.

The PPP-family of serine/threonine protein phosphatases (PP1/PPP1C, PP2A/PPP2CA, PP2B/calcineurin/PPP3C, PP4/PPP4C, PP5/PPP5C, PP6/PPP6C and PP7/PPPEF1) catalyze the dephosphorylation of thousands of proteins that play diverse roles in biology.¹ However, we have a limited understanding of the relationship between individual PPPs and their

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Notes

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Atomic coordinates and structure factors for the PP1:TTN complex have been deposited in the Protein Data Bank (PDB 6ALZ).

Supporting Information

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Methods (protein production,¹⁹ enzyme assays,⁶ crystallization, structure determination^{20,21}) (PDF)

biological substrate(s). Furthermore, how the disruption of PPPs substrate relationships contributes to human disease remains largely an open question. This is because the active sites of PPPs are highly conserved,^{2,3} and to date the development of potent inhibitors that are selective for a single PPP, which would allow their individual functions to be readily determined, have failed.

Natural toxins produced by organisms as diverse as cyanobacteria, *Streptomyces* and beetles have proven useful to distinguish the actions of a subset of PPP-family phosphatases (i.e., PP1, PP2A, PP4, PP5 and PP6) from other cellular phosphatases.⁴ These toxins include cyclic peptide-based inhibitors (i.e., microcystin-LR) and linear inhibitors (i.e., okadaic acid, fostriecin and tautomycin).⁵ Despite the sequence conservation of the PPP active sites² (Figure S1), a few natural toxins exhibit specificity toward a subset of PPPs. For example, fostriecin is selective for PP2A (PP2A vs PP1/PP2B/PP5; selectivity $>10^4$); however, fostriecin inhibits PP2A to nearly the same extent as PP4, limiting its usefulness for cellular assays.⁶

Tautomycin (TTN; Figure 1a) is a complex linear polyketide that has antitumor and immunosuppressive activities.⁷ TTN is the only compound that demonstrates increased potency against PP1 versus PP2A.⁸ However, the molecular basis for this PP1 selectivity has remained elusive for over 1 decade. We performed a series of in vitro dephosphorylation assays to define the inhibitory activity of TTN against PPP family members (Figure 1b). The first screen tested the effect of TTN on the PPP-catalyzed hydrolysis of an established substrate at a single concentration (DiFMUP; 100 μ M). Ten-point dose-response studies were then conducted with PPPs demonstrating $>50\%$ inhibition in order to assess the relative inhibition strength. This revealed inhibitory activities for PP4, PP5 and PP6 and showed that TTN is a potent and highly selective inhibitor of PP1. To test inhibition at pM enzyme concentrations,⁹ we used a [³²P]-phosphohistone radiolabeled assay, which confirmed that TTN potently and selectively inhibits PP1 (IC₅₀, 38 pM) as the IC₅₀ values for TTN-inhibition of other family members are considerably higher (Table 1; PP2A, ~139-fold; PP5, ~313-fold; $>10^3$ for the other PPPs tested). TTN has also been reported to inhibit the tyrosine phosphatase SHP2.¹⁰ However, the strength of inhibition for SHP2 is much lower (IC₅₀ of TTN for SHP2 is 2900 nM,¹⁰ $>10^4$ -fold higher than that for PP1; Table 1). Together, these data show that TTN is the most selective inhibitor of PP1 reported to date.

To determine the molecular basis for the PP1 specificity, we determined the structure of the PP1:TTN complex to 2.3 Å (Figure 2a,b; Table S1). Electron density for the bound TTN molecule was readily visible in the initial electron density maps (Figure 3a). TTN binds the active site with the TTN diene/alkene moiety extending into the PP1-substrate binding groove, known as the hydrophobic groove (Figure 2a).¹¹ Although produced by *Streptomyces* sp. as an anhydride, the diacid tautomeric center of TTN is observed at the active site of PP1 (Figures 1a, 2a, *right*; in solution, TTN exists as a mixture of the anhydride and diacid forms¹²). The TTN diacid carboxyl groups bridge the active site via salt bridge and hydrogen bonding interactions (Figure 2b). The C6' _{TTN} carboxyl forms a bidentate salt bridge with the guanidinium group of Arg96_{PP1} and a hydrogen bond with the hydroxyl of Tyr272_{PP1}, whereas the C7' _{TTN} carboxyl forms a bidentate salt bridge with Arg221_{PP1}. In addition, the C3' _{TTN} hydroxyl forms a hydrogen bond with the backbone

amide nitrogen of Val250_{PP1}, whereas the C12_{TTN} and C14_{TTN} hydroxyls form hydrogen bonds with Arg221_{PP1}. These interactions position the C7'_{TTN} carboxyl over the active site, with the oxygens forming hydrogen bonds with the two active site Mn²⁺-coordinated waters. The remainder of TTN binds the PP1 hydrophobic groove (Cys127, Ile130, Val192, Trp206 and Val223) via polar and hydrophobic contacts. An overlay of free PP1 (PDBid 4MOV¹³) with the PP1:TTN complex (RMSD = 0.26 Å) shows that TTN does not induce a structural change in PP1 (Figure S2).

To understand the selectivity of TTN for PP1, we compared the structure of PP1:TTN with that of PP1:tautomycin (TTM).¹⁴ TTM is structurally similar to TTN, sharing the 2,3-disubstituted maleic anhydride moiety but differing at the opposite end, in which the diene/alkene group of TTN is replaced by a spiroketal group in TTM (Figure S3a). In spite of their similarities, only TTN is selective for PP1 (TTN is >100-fold selective for PP1 versus any other PPP tested, this work; TTM inhibits PP1, PP2A, PP4 and PP5 to a similar extent⁴). A comparison of the PP1:TTN and PP1:TTM complexes shows that the diacid of both inhibitors binds identically to PP1 (Figures S3b–e). In contrast, the interaction of the TTN diene/alkene group with PP1 differs significantly from that of the TTM spiroketal group. Both moieties bind in a pocket near the bottom of the PP1 hydrophobic binding groove. However, only TTN forms a covalent bond with residue Cys127_{PP1} (Figures 3a, S3b–e). This is because TTM does not have a diene/alkene group capable of selective conjugate addition. We confirmed the presence of a covalent bond between PP1 and TTN using ESI mass spectrometry (Figure 3b). The observed 624.9 Da increase in the MW of the PP1:TTN complex compared to PP1 corresponds to the expected mass of the diacid form of TTN (624.8 Da; a time-course analysis showed that adduct formation occurs in less than 10 min). The formation of the covalent bond between TTN and Cys127_{PP1} is likely achieved via a mechanism in which the S- of Cys127_{PP1} functions as a nucleophilic donor and the diene/alkene group of TTN functions as an α,β -unsaturated acceptor. Consistent with this, a cysteine reactivity prediction algorithm¹⁵ identifies Cys127 as the most reactive cysteine in PP1 (predicted pK_a, 5.96), as the deprotonation of the Cys127_{PP1} thiol is predicted to be stabilized by Ser129_{PP1} and Asp194_{PP1} (Figure S4). Together, these data show that only the diacid tautomer of TTN binds to PP1 and that TTN and PP1 form a covalent bond upon binding.

Our structure, coupled with TTN inhibition assays and an analysis of the sequence conservation within this family, explains both the general ability of TTN to inhibit sensitive PPPs and the high selectivity of TTN for PP1 (IC₅₀: PP1 ≪ PP2A ~ PP5 ~ PP4 ~ PP6 ≪ PP2B). The residues needed for PPP-catalytic activity are 100% conserved among all PPP family members, including those that make key interactions with the TTN diacid (Arg96_{PP1}, Arg221_{PP1}, Tyr272_{PP1}; Figures 3c, 4a). This conservation explains why TTN functions as a general inhibitor of most members of this family. In contrast, residues that line the PP1 hydrophobic groove are divergent (Figure 4a). Most significantly, Cys127_{PP1}, the residue that forms a covalent bond with TTN (Figure 4b), is not conserved. A cysteine is only present in PP2B (Cys153_{PP2B}). Thus, the other PPPs (PP2A/PP4/PP5/PP6) are unable to form a covalent bond with TTN, which explains why TTN is selective for PP1.

To confirm that Cys127_{PP1} is critical for selectivity, we measured the IC₅₀ of TTN for PP1 and the PP1 Cys127Ser mutant (Figure S5; C127S_{PP1}). The data show that the IC₅₀ of TTN for C127S_{PP1} increases nearly 10-fold compared to WT; in contrast, no difference in IC₅₀ is observed for another PP1 inhibitor (Microcystin-LR) that does not form an adduct with Cys127_{PP1}. Further, Lineweaver–Burk kinetic analysis shows that TTN exhibits competitive inhibition of C127S_{PP1}, but exhibits noncompetitive inhibition of PP1, indicative of irreversible enzyme inhibition¹⁶ and consistent with Cys127_{PP1} forming a covalent bond with TTN (Figure S6).

TTN does not effectively inhibit PP2B because residues that line the hydrophobic group are not conserved between PP1 and PP2B (Figure 4a). In particular, a superposition of the PP1:TTN complex with PP2B (PDBid 4F0Z¹⁷) reveals that (1) the Val253_{PP2B} side chain (Asp220_{PP1}) clashes with the C18_{TTN} methyl group and (2) the bulky side chain of His155_{PP2B} (Ser129_{PP1}) blocks access of the TTN diene/alkene group to Cys153_{PP2B} (Figures 4c,d). These differences suggest that PP2B is unable to bind TTN in a manner conducive for covalent bond formation. We confirmed this using ESI-MS (Figure S7). Together, our structural and inhibition assay data show that the selectivity of TTN for PP1 manifests from key residue differences in the hydrophobic groove of the PPPs, especially the lack of conservation of Cys127_{PP1} (Figure 4b). As a consequence, TTN is able to bind and form a stable, covalent bond with only PP1, resulting in a 100-fold increased potency of TTN for PP1 than any other PPP.

TTN is not the first natural PPP inhibitor shown to form a covalent adduct with a cysteine in a PPP. Fostriecin forms a covalent bond with Cys269 upon PP2A binding;¹⁸ similarly, Microcystin-LR forms a covalent bond with Cys273 upon PP1 binding (Figure S8).¹¹ However, these cysteines are also present in other PPPs (Cys269_{PP2A} is conserved in PP4 and PP6; Cys273_{PP1} is conserved in PP2A, PP4, PP5 and PP6), rendering these toxins nonselective. Another concern with fostriecin is that the unsaturated C3 of the lactone, which functions as the electrophile, can oxidize during storage. This greatly reduces its selectivity. In contrast, TTN is selective for PP1 because the residue it forms a covalent adduct with, Cys127_{PP1}, is not present in any other PPP capable of binding TTN (PP2A, PP4, PP5, PP6 or PP7). In addition, TTN demonstrates excellent storage stability (>5 years in DMF or DMSO at –20 °C) and is cell membrane permeable. Together, these observations demonstrate that targeting noncatalytic Cys residues that are both (1) near the active site and (2) unique to a particular family member (i.e., Cys127_{PP1}, Cys256_{PP2B}, Cys404_{PP5}) is a promising strategy for the further development of highly selective PPP-family inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Tautomycetin is a PP1-specific inhibitor. (a) Chemical structure of TTN, highlighting the diacid (green) and diene/alkene (purple) groups. (b) Inhibitory effect of TTN on the activities of purified PP1 (yellow squares), PP2A (blue diamonds), PP4 (orange circles), PP5 (green triangles), PP6 (magenta stars) and PP2B (red inverted triangles). Inhibition assays were conducted using purified enzymes with 100 μ M DiFMUP as a substrate (see Methods). Each point is the mean $\pm$ SD ($n = 4-8$). (c) PP1 (■), PP2A (●) and PP5 (▲) were further tested using [^{32} P]-labeled phosphohistone (specific activity: 7.4×10^6 cpm/nmol incorporated phosphate; 25 pM PP1, 30 pM PP5, ~50 pM PP2A)⁴. Each point is the mean $\pm$ SD ($n = 4$). IC₅₀ values are reported in Table 1.

Figure 2.

Tautomycetin binds the PP1 hydrophobic groove and occludes the active site. (a) The PP1-TTN complex (PP1, gray; TTN, yellow). PP1 residues that contact TTN are in lavender. The hydrophobic groove and active site are indicated. *Right*, active site with TTN shown as sticks. (b) Stereo image of the interactions between PP1 (lavender) and TTN (yellow). Ionic and hydrogen bonding interactions indicated by black dashed lines. PP1 Mn^{2+} ions, magenta spheres; two active site coordinated waters, blue spheres. TTN carbon numbering as in Figure 1a.

Figure 3.

TTN forms a covalent bond with Cys127_{PP1}. (a) Simulated annealing composite omit map (blue mesh, 1 σ , TTN omitted) of TTN illustrating the covalent bond with Cys127_{PP1} (orange arrow). TTN is colored by atomic B-factor. (b) ESI-MS of PP1 (top) and PP1-TTN (bottom). PP1 expected MW, 34 124.19 Da. (c) PP1-TTN with PP1 color coded according to PPP family sequence conservation. TTN shown as yellow sticks.

Figure 4.

TTN is selective for PP1 due to sequence variability in the hydrophobic groove. (a) Overlay of PP1-TTN, PP2A, PP2B and PP5, with key TTN interacting residues (and their PPP homologues) shown as sticks. (b) Sequence alignment of the Cys127_{PP1} loop, colored as in panel a. (c) Model of PP2B bound to TTN (superposition of PP2B with PP1-TTN), highlighting that the replacement of Ser129_{PP1} by the bulky His155_{PP2B} hinders access to Cys153_{PP2B}. (d) PP1-TTN complex illustrating the location of Ser129_{PP1} and Asp197_{PP1}, which stabilizes the Cys127_{PP1} thiolate ion.

Table 1

Inhibitory Activity of TTN against PPP- and PTP-Family Phosphatases

Phosphatase	IC ₅₀ (nM) ^a	Fold change (relative to PP1)
Ser/Thr		
PP1	0.038 ± 0.009 ^b	–
PP2A	5.3 ± 0.26 ^b	139 ^b
PP5	11.9 ± 0.05 ^b	313 ^b
PP4	44.4 ± 4.2	1168
PP6	101 ± 7.0	2657
PP2B	17500 ± 3200	>10 ⁵
Tyrosine		
SHP2	2900 ± 200	>10 ⁴
SHP1	14600 ± 100	>10 ⁵
Lyp	20000 ± 2000	>10 ⁵
PTP1B	41200 ± 4400	>10 ⁵
PTP α	>50000	>10 ⁵
HePTP	>50000	>10 ⁵
CD45	>50000	>10 ⁵
VHR	>50000	>10 ⁵
CDC14A	>50000	>10 ⁵

^aIC₅₀ values calculated from a 10-point dose–response concentration curve by a 4-parameter logistic fit of the data, using 4–8 replicates.

^bIC₅₀ values calculated from assays using [³²P]-labeled phosphohistone as a substrate. IC₅₀ values for tyrosine phosphatases derived from [10]. Data represent the mean ± SD.