

Miklós Bodanszky Award Lecture: Advances in the selective targeting of protein phosphatase-1 and phosphatase-2A with peptides

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Protein phosphatase-1 and phosphatase-2A are two ubiquitously expressed enzymes known to catalyze the majority of dephosphorylation reactions on serine and threonine inside cells. They play roles in most cellular processes and are tightly regulated by regulatory subunits in holoenzymes. Their misregulation and malfunction contribute to disease development and progression, such as in cancer, diabetes, viral infections, and neurological as well as heart diseases. Therefore, targeting these phosphatases for therapeutic use would be highly desirable; however, their complex regulation and high conservation of the active site have been major hurdles for selectively targeting them in the past. In the last decade, new approaches have been developed to overcome these hurdles and have strongly revived the field. I will focus here on peptide-based approaches, which contributed to showing that these phosphatases can be targeted selectively and aided in rethinking the design of selective phosphatase modulators. Finally, I will give a perspective on www.depod.org, the human dephosphorylation database, and how it can aid phosphatase modulator design. © 2017 The Authors. Journal of Peptide Science published by European Peptide Society and John Wiley & Sons Ltd.

Keywords: protein phosphatases; allosteric modulation; active site inhibition; peptidomimetics; macrocyclic peptides; microcystin; peptide-based drug design; phosphatase database DEPOD

Introduction: Protein Phosphorylation, Protein Phosphatase-1 (PP1) and Phosphatase-2A (PP2A)

Protein serine/threonine kinases and phosphatases are important enzymes governing the process of phosphorylation by adding and removing, respectively, a phosphate group on the hydroxyl groups of serine and threonine amino acids in proteins. Phosphorylation is the most common reversible chemical modification in eukaryotes [1], regulating, for example, the interactions of proteins, their physicochemical properties, their activity, and their stability and degradation [2]. The balance between phosphorylation and dephosphorylation is fundamental in many aspects of cell physiology, and disruption of this balance contributes to diseases [3,4].

Among the different types of serine/threonine-specific phosphatases, the phosphoprotein phosphatases (PPPs), which are part of family 5 (Figure 1(a)) [5], are known to dephosphorylate the majority of phospho-residues on serine and threonine. Their catalytic active sites are highly conserved, which has hampered selective inhibitor design in the past. Within the PPPs, phosphatase-2A (PP2A) and phosphatase-1 (PP1) carry out the majority of dephosphorylation reactions, counteracting more than 100 kinases [5]. While on first sight it would seem that this fact speaks for PP1 and PP2A being non-specific, the opposite is actually the case when considering how these enzymes are presented and regulated in cells. They are always bound to regulatory proteins in the form of holoenzymes, altogether accounting for an estimated number of more than 250 PP1 and PP2A holoenzymes [7,8].

The catalytic subunit of PP1 (PP1c), which exists in the three isoforms PP1 α , β , and γ (γ 1 and γ 2), interacts with more than 200

structurally largely unrelated proteins that determine when and where the phosphatase acts [8]. These PP1 interacting proteins (PIPs) function, for example, as substrate-targeting and/or location-targeting subunits, substrate-specifiers, or inhibitors

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Abbreviations: Adda, – (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Bpa, – benzoylphenylalanine; DARPP32, – dopamine and cyclic adenosine monophosphate-regulated phosphoprotein of molecular weight 32 kDa; DEPOD, – dephosphorylation database; DNA, – deoxyribonucleic acid; G_M, – muscle form of the PP1 regulatory G subunit; G_m, – PP1 targeting inhibitor peptide derived from G_M; HeLa, – Henrietta Lax; HIV, – human immunodeficiency virus; I-2, – Inhibitor-2; MC, – microcystin; NIPP1, – nuclear inhibitor of PP1; PDP, – PP1 disrupting peptide; pH3, – phosphorylated histone 3; pl-1, – phosphorylated inhibitor-1; PIP, – PP1 interacting protein; PP, – protein phosphatase; PP (number), – catalytic subunit of a PP; PPP, – phosphoprotein phosphatases; Repo-Man, – Recruits PP1 onto mitotic chromatin at anaphase protein; RVxF, – single-letter amino acid code, x = any amino acid excluding proline.

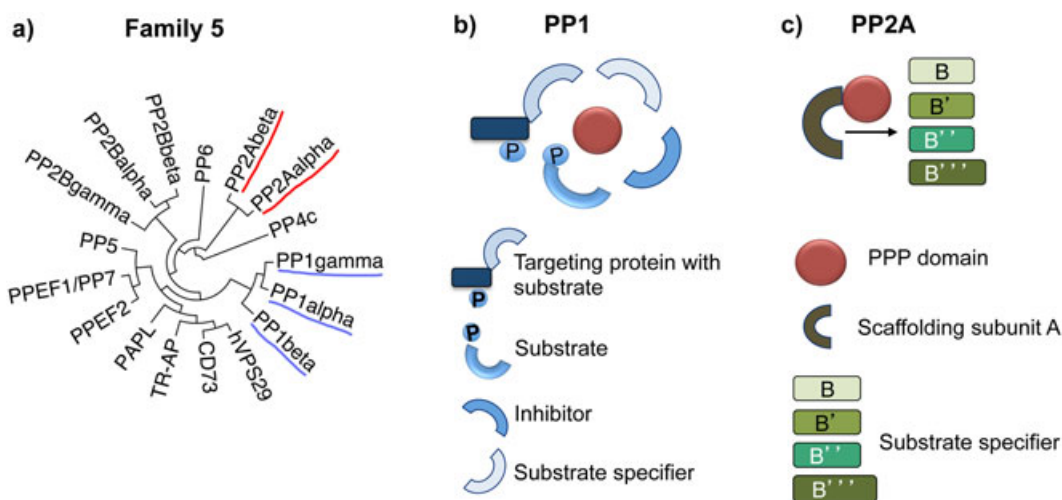


Figure 1. Schematic representation of (a) family 5 phosphatases including the PPPs PP1, PP2A, PP2B, PP4, PP5, PP6, and PP7 (adapted from [5], PP1 and PP2A isoforms underlined), (b) PP1, and (c) PP2A holoenzymes (adapted from [6]).

Author biography



Maja Köhn studied Chemistry at the University of Kiel (Germany). She finished her PhD under the supervision of Prof. H. Waldmann at the Max-Planck-Institute of Molecular Physiology and the Technical University in Dortmund (Germany) in 2005. Afterwards, she joined Prof. G. Verdine's group at Harvard University (USA) supported by a postdoctoral fellowship of the German Academic Exchange

Service. In November 2007, she started as a group leader at the European Molecular Biology Laboratory in Heidelberg (Germany). In 2010, she was awarded an Emmy Noether Research group funded by the German Research Foundation, and in 2014, she became a European Research Council Investigator (starting grant). In 2016, she became full Professor for Integrative Signaling Studies at the Faculty of Biology and the Centre for Biological Signaling Studies (BIOSS) at the University of Freiburg (Germany). She was awarded with the Friedrich Weygand Prize of the Max Bergmann Circle in 2008, the Friedmund Neumann prize of the Schering Foundation in 2014, the 2015 Tetrahedron Young Investigator Award in Bioorganic and Medicinal Chemistry, and the Miklós Bodanszky Award of the European Peptide Society in 2016. She is a member of the editorial advisory boards of Bioorganic and Medicinal Chemistry and ACS Chemical Biology. Her research interests are to control and investigate protein phosphatases using interdisciplinary approaches comprising synthetic chemistry, biochemistry, and molecular as well as cell biology.

structural subunit A. Regulatory subunit B proteins direct the enzyme to different locations and confer substrate specificity, whereas the subunit A helps to form the holoenzyme complex (Figure 1(c)) [7]. These three subunits assemble in a combinatorial fashion to make over 90 PP2A holoenzymes [7]. Whereas most of the complexes are trimeric, PP2A can also occur as a dimer with just one A and one C subunit [7]. These PP2A holoenzymes regulate, for example, cell proliferation and death, mobility, signaling pathways, mitosis, and DNA damage repair [7,16]. In disease, PP2A holoenzymes play multiple roles in Alzheimer's [17,18] and cancer [16,19,20]: PP2A is known to be a tumor suppressor [16]. In this regard, it is the A and B subunits that are genetically altered in different types of cancers leading to functional impairment of PP2A, and increased expression of PP2A inhibitory proteins often correlates with cancer aggressiveness and poor prognosis [7]. On the other hand, inhibition of PP2A has also been shown to lead to cancer cell death. This is attributed to apoptosis being a consequence of improper cell cycle progression, for which proper PP2A function is crucial. Furthermore, PP2A plays a role in DNA damage repair, and co-treatment of a PP2A inhibitor with a DNA damaging agent or a DNA repair inhibitor was shown to sensitize cancer cells to chemotherapy-induced cell death [7].

Considering the diverse roles of PP1 and PP2A in health and disease, it is critical to know the identity and understand the roles of the particular holoenzymes in cellular processes, and to not view them only as PP1 and PP2A catalytic subunits alone.

Modulation of PP1 or PP2A Activity with Chemical Compounds

(Figure 1(b)) [8]. PP1 holoenzymes function in numerous cellular processes, including cell cycle progression, protein synthesis, muscle contraction, transcription, calcium signaling, and splicing [8–10]. Given their diverse functions, it is not surprising that PP1 holoenzymes also play many roles in a number of diverse pathologies such as heart failure [11], memory loss [12], viral infections [13], type-2 diabetes [14], and cancer [15].

PP2A holoenzymes are usually trimeric complexes that consist of the catalytic subunit C (PP2Ac), a regulatory B subunit, and the

Traditionally, inhibiting enzymatic activity has been accomplished by using compounds that target the active site of the enzyme. However, the conserved nature of the active site pocket in PPPs has made it very difficult to achieve selectivity for PP1 or PP2A over the other, and the other PPPs have often been neglected when determining selectivity issues [4]. For example, various molecules such as microcystins (MCs), calyculin A, and cantharidin are not specific and are often problematic in their use due to low solubility and cell permeability. Fostriecin is selective toward PP2A over PP1

and PP5, but it is unstable [4]. While this continues to be a challenge, new developments now offer the prospect of reaching selective, potent, cell-active chemical modulators of PP1 and PP2A holoenzyme activity. This has largely been achieved by targeting the holoenzymes instead of the active site of the proteins. Small molecules such as Sephin1, which interferes with PP1 activity [21], and FTY720193 as well as its analog AAL(S), which were shown to activate PP2A [22,23], have been groundbreaking for the development of PPP modulators. For a recent overview on these, the reader is referred to reference [4].

In essence, targeting holoenzymes means targeting protein-protein interactions, either on the catalytic subunit or the regulatory protein side, which comes with its own challenges such as the need to disrupt large interaction areas or targeting intrinsically disordered proteins, which are the majority of the interaction partners of PP1 [8]. Often peptides are the compounds of choice to achieve this, but they also frequently have inherent properties that are not ideal for chemical modulator development, such as low cell permeability, low stability, or low solubility [24,25]. Nevertheless, many examples indicate that these challenges can be overcome to design cell-active chemical modulators [24,25].

In a proof-of-principle study, A. Garcia and his group developed a peptide-based approach to target PP1 and PP2A with peptides containing a binding-region and a cell-penetrating region (Figure 2) [26]. To identify the binding sequences that target PP1 and PP2A, they screened cellulose-bound peptides derived from known interacting proteins. The binding sequences were fused to a cell-penetrating peptide sequence discovered in the same work. The fusion peptides containing either a binding sequence for PP1 or PP2A were shown to co-precipitate PP1 and PP2A, respectively, and to induce apoptosis. While the modes of action or the selectivity were not investigated at the time, this study demonstrated that cell-penetrating peptides targeting these phosphatases at sites other than the catalytic pocket have an effect in cells.

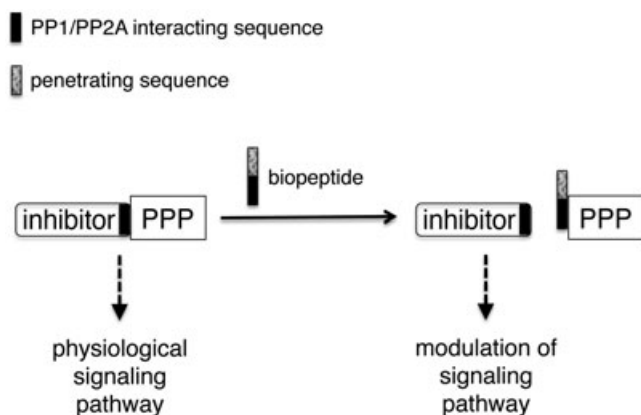


Figure 2. Depiction of the principle of fusing a cell-penetrating sequence to a PPP-binding sequence to modulate PPP activity in cells (adapted from [26]).

Targeting the PP1-Regulatory Protein Interaction with Peptides to Disrupt PP1 Holoenzymes

PP1 contains several binding sites for PIPs, but the major one that is thought to bind to most PIPs is the so-called RVxF-binding site

(single-letter amino acid code, x = any amino acid excluding proline). It is a hydrophobic channel on PP1 to which PIPs containing an RVxF-type motif bind. This annotation is not strictly defined, as several sequence definitions for this motif have been proposed [27–29]. Both peptides and small molecules have been developed to target the RVxF-binding site on PP1 [8].

The first examples of peptides targeting this site were used to study the binding modes of endogenous PIPs *in vitro*. These were peptides derived from the natural sequences of DARPP32 (dopamine and cyclic adenosine monophosphate-regulated phosphoprotein of molecular weight 32 kDa) [30] and inhibitor 1 [31]. A peptide based on spinophilin from residues 438 to 461 disrupted the PP1-spinophilin holoenzyme in cell lysates at a 100 μ M concentration [32]. While inhibitor 1 and DARPP32 peptides inhibited PP1 catalytic activity, the spinophilin sequence-based peptide did not, but it did disrupt holoenzyme formation. This shows that peptides targeting the RVxF-binding site can be used to modulate PP1 activity in different ways depending on the peptide sequence beside the RVxF-motif. A first example of applying PP1-modulating peptides in cells was a peptide that consisted of residues 63 to 75 of the muscle form of the PP1 regulatory G subunit, G_M [33,34]. This peptide contained the RVxF-motif and disrupted PP1 holoenzymes but not its catalytic activity and was termed PP1 targeting inhibitor peptide G_M [34]. For cellular uptake, it needed to be dialyzed into cells through a patch pipette [34]. Further 4 to 13-mer peptides based on the sequence of G_M were shown to activate PP1 and disrupt the complex between PP1 and DARPP32 using low micromolar concentrations *in vitro*, demonstrating that a tetrapeptide (Ac-RVVF-NMe) could be sufficiently potent, but neither their activity in cells nor their selectivity was tested [35]. Finally, expression of an 82-mer fragment of the central domain of nuclear inhibitor of PP1 (NIPPI) was reported to disrupt the interaction between HIV (human immunodeficiency virus)-1 transactivator of transcription protein and PP1, inhibiting HIV-1 replication in cells [13]. No other tested PIPs were affected in this context, which could be due to the relatively low affinity of transactivator of transcription protein to PP1 that was blocked, whereas endogenous holoenzymes that have a higher affinity were not disrupted [8,13,36].

Building on these findings, in collaboration with Mathieu Bollen, KU Leuven, Belgium, we set out to develop peptides that would target the RVxF-binding site on PP1, modulate PP1 activity selectively in cells, and be cell penetrating as well as proteolytically stable, and we called these PP1-disrupting peptides (PDPs) [24]. The design was based on data from the Bollen lab, which identified the 20-mer peptide NIPPI1 (191–210) as being able to compete for PP1c inhibition with full-length NIPPI1, as well as with the endogenous protein inhibitors phospho-inhibitor-1 (pI-1) and inhibitor-2 (I-2) [37]. The first peptide following this design was termed PDP0. Alanine scans based on PDP0 were conducted with different peptides, where either single or multiple amino acids were replaced by alanine. These were screened by measuring the decrease of inhibition of PP1c by I-2 through the resulting increase in PP1c phosphatase activity on the dephosphorylation of 32 P-labeled phosphorylated glycogen phosphorylase *a* (starting at 30% and releasing to 100% phosphatase activity) (Figure 3(a)). Truncation of PDP0 led to a reduction in potency, revealing that the N-terminal polybasic sequence and also the C-terminal isoleucines ($\Theta\Theta$ -motif [38]) were important for binding. This approach led to an optimized sequence, PDP1, which showed nanomolar potency in the assay (Figure 3(b)). Further attempts

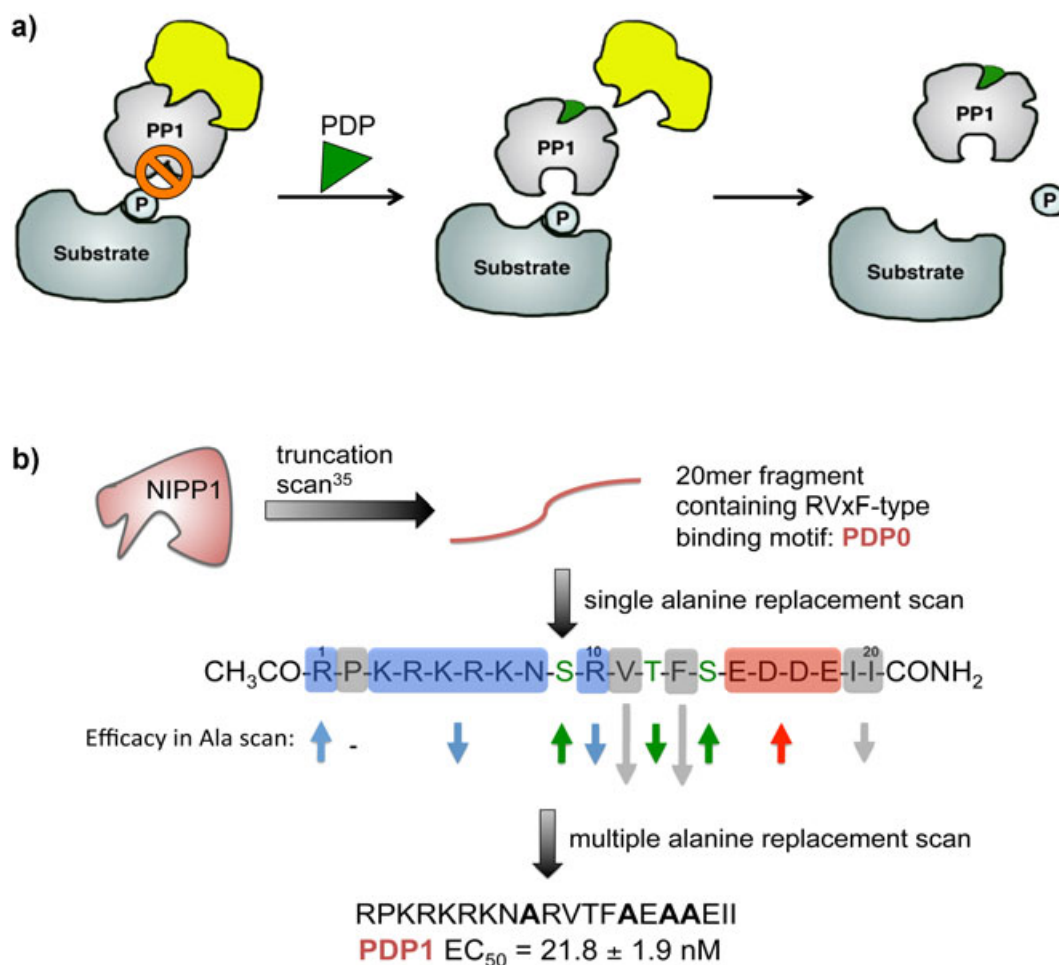


Figure 3. Mode of action of PDPs (a) and their design (b). (a) PDPs disrupt PP1 holoenzymes by binding to the RVxF-binding site on PP1c and competing with endogenous PIPs. The released catalytic subunit can dephosphorylate nearby substrates. For testing the potency of the peptides, the release of the inhibitory protein I-2 (yellow) from PP1c (gray) was measured by the resulting increase in PP1c phosphatase activity on the dephosphorylation of [³¹P]-labeled phosphorylated glycogen phosphorylase *a* (blue substrate). (B) Based on a 20-mer fragment of NIPP1 and PDP0, single and multiple alanine replacement scans led to the design of PDP1.

to enhance the potency and stability by cyclizing the peptides were not successful, which suggests that the reported conformational flexibility of the RVxF-motif and the surrounding sequence [39] is beneficial for binding.

While cells did not take up PDP1, extending the polybasic sequence by sequential addition of lysine and arginine residues (RRK) to the *N*-terminus provided the cell penetrating PDP2. Subsequently, we showed that PDPs do not bind to PP2A and that they disrupt PP1 holoenzymes, the extent of which is dependent on the affinity of the PP1–PIP interaction. The crystal structure of the PP1–PDP2 complex was provided as proof of direct interaction *in vitro*. Furthermore, we designed PDP3 based on PDP2, which carried the photo-crosslinkable amino acid 4-benzoylphenylalanine (*Bpa*) instead of alanine adjacent to the phenylalanine of the RVxF-motif. This sequence was crosslinked to PP1c in cell lysates, indicating direct interaction under these conditions. Finally, we showed that PDP3, but not PDP2, induces PP1 activity in cells by studying phosphorylated histone 3 (pH3) dephosphorylation by PP1 in mitosis. PDP2 is less stable in cells than PDP3 and is therefore likely degraded before it can act in the rather long time frame of this process. During mitosis, the PIP Repo-Man guides PP1 to its substrate pH3. Knockdown of Repo-Man prevents the guidance, and PP1 is not able to

dephosphorylate pH3. PDP3 rescued this phenotype, suggesting that it binds to PP1 and releases the PDP3-bound PP1 catalytic subunit that can then dephosphorylate nearby substrates [24]. The scope of substrates dephosphorylated by this PP1–PDP3 complex is at this point unclear, as PP1 could act promiscuously or specifically depending on the substrates, their (local) availability, and regulation. This is currently a very difficult question to address given that PP1 is assumed to dephosphorylate the majority of proteins on serine and threonine in eukaryotic cells [39], that comparably only few substrates are known, and that it is unclear which substrates are dephosphorylated redundantly by different PPPs [5].

We used the PDPs to study the acute effect of PP1 activity on calcium signaling [40]. Treatment of HeLa cells with PDP2 and PDP3 triggered calcium oscillations in a concentration-dependent manner within seconds, visualized by the ratiometric Fura-2 dye. Calcium spikes triggered by PDP3 displayed a higher frequency than the ones for PDP2. While washing out of PDP2 abolished the calcium signals, the cells treated with PDP3 followed by washing continued calcium spiking. Both observations are in agreement with the lower stability of PDP2 [24], which is still able to trigger calcium signals but to a lower extent and reversibly, revealing the intriguing possibilities of fine-tuning the cellular

response toward peptides by varying their proteolytic stability. We went on to show that the released calcium originated from the endoplasmic reticulum and that the inositol 1,4,5-trisphosphate receptor was involved in the release [40]. Finally, we demonstrated that the first calcium spike was triggered by PP1, revealing that elevated PP1 activity leads to upregulation of calcium signaling and that PDPs did not activate PP2A and PP2B activity, confirming their selectivity and demonstrating the applicability of these chemical tools to address questions concerning PP1 biology [40].

Subsequent to our work, 4 to 11-mer peptides based on the N-terminal domain of inhibitor 1 that disrupt the interaction between PP1 and pI-1 as well as PP1 and I-2 were reported [41]. The most active peptides were a nonapeptide (SPRKIQFTV) and a hexapeptide (RKIQFT), containing a variation (RKIQF) of the RVxF-motif, both with low micromolar potencies *in vitro* in an assay measuring the decrease of inhibition of PP1c by pI-1 (from 50 to 100% PP1 activity). In order to check the activity of the peptides inside cells, a polyarginine sequence was attached for cellular uptake. Treatment of cardio myocytes with 10 μ M of the polyArg-nonapeptide led to the reduction of isoprenaline-induced phosphorylation levels of phospholamban on Ser16 but not of cardiac troponin inhibitor on Ser23/24. Selectivity over other phosphatases was not tested [41].

Taken together, when targeting the RVxF-binding site on PP1 with peptides, different sets of PP1 holoenzymes can be disrupted depending on the potency of the compounds, the affinity of the PP1-PIP interaction, and the cellular context. The sequence adjacent to the RVxF-motif can be varied to either inhibit PP1, which means blocking the active site in addition to the RVxF-site, or to

release the free catalytic subunit, leading to dephosphorylation of nearby substrates.

Efficient Synthesis of a Complex Macrocyclic Peptide Renders an Unselective Active Site Inhibitor Selective

Many PPP inhibitors are natural toxins, and they are usually not selective toward a certain PPP [4]. With the exception of norcantharidin and similar compounds, these natural toxins have complex chemical structures and are challenging to synthesize, which has hampered their structure-activity relationship studies in the past [42]. Peptide natural toxins that inhibit PPPs include the MCs, which are cyclic heptapeptides with the typical structure depicted in Figure 4 [43]. Around 100 different MCs, so-called congeners, are known, and they mostly differ in the amino acid composition in positions 2 and 4 [44]. MCs indiscriminately target PPP active sites, which are highly conserved, and are highly potent inhibitors with potencies in the picomolar to nanomolar range. The so-called 'Adda' side chain at position 5 was reported to be required for binding (Figure 4) [42]. We reasoned that given that the general structure of the MCs covers a larger area in and around the PPP active sites, small changes in the molecules might give insights into determinants for selectivity between PP1 and PP2A [43]. We therefore set out to synthesize several MC derivatives and to study their structure-activity relationship toward PP1 and PP2A. However, since MCs require multi-step syntheses that result in low yields, we first designed an efficient solid/solution-phase synthetic approach and simplified the

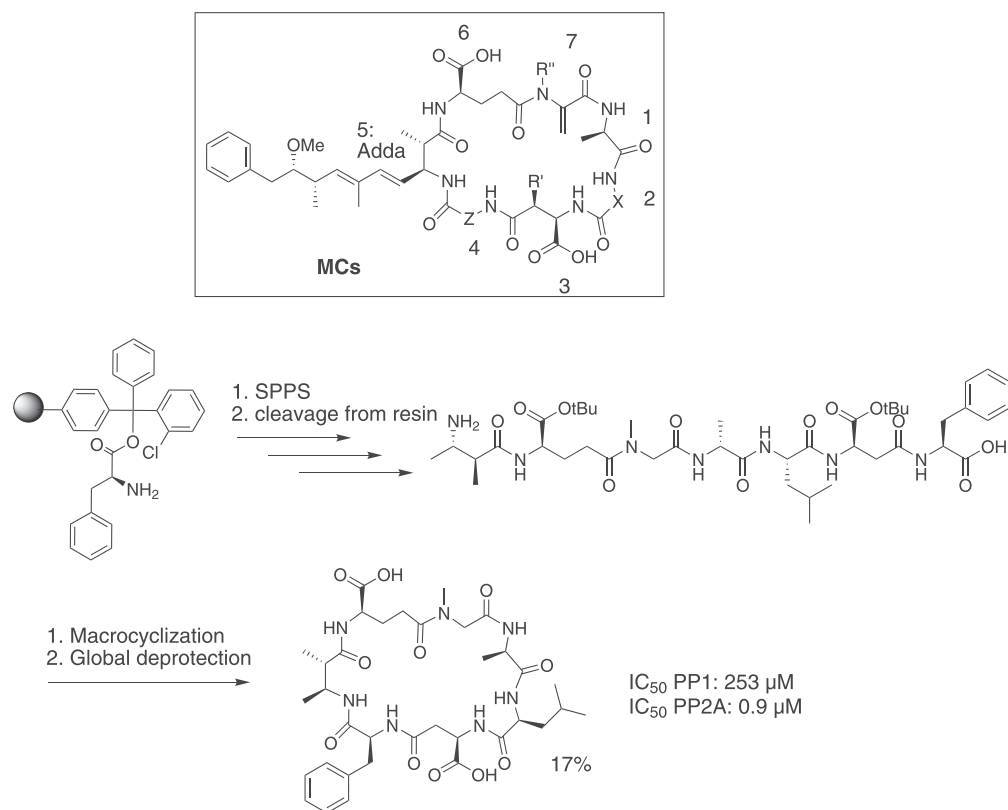


Figure 4. The general structure of MCs and their synthetic procedure, exemplified by the most selective derivative, are shown. The potency toward PP1 and PP2A of this derivative is included. Adda = (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid [43].

molecules. Besides other minor changes, we replaced the long lipophilic tail in the beta position of the Adda amino acid at position 5 with shorter tails and synthesized derivatives with or without the α -methyl group, as this residue had not been investigated for its role in binding before. Our synthetic approach included the assembly of the linear peptide on resin, cleavage, and then macrocyclization in solution followed by global deprotection (Figure 4), leading to good overall yields of the final compounds and enabling the synthesis of 11 MC analogs. We discovered that the α -methyl group on the backbone of the amino acid in position 5 is highly beneficial for binding, likely replacing a water molecule in a small hydrophobic pocket. Most surprisingly and against previous observations stating that the lipophilic side chain in Adda is required for binding, we obtained sub-micromolar inhibitors for PP2A that included the α -methyl group but not the lipophilic tail in position 5. Finally, we discovered inhibitors that were more than 250-fold selective toward PP2A over PP1 (Figure 4), which is an unprecedented value, obtained through chemical modification of a natural toxin. We showed that this selectivity is largely based on a span of residues in PP1 and PP2A that is not completely conserved: Y(265)CYRC (269) in PP2A aligns with Y(272)CGEF(276) in PP1. While R268 in PP2A can form a hydrogen bond with a carbonyl group on the MC analog, E275 in PP1 cannot. In addition, C269 in PP2A is less bulky than F276 in PP1, helping to accommodate the sterics of MC in the binding pocket [43]. Interestingly, this stretch is conserved between PP2A, and PP4 and PP6, which are the closest homologs to PP2A; thus, the MC analogs could also favor these PPPs. However, these residues are not conserved in PP5 (YCDQM), adding another opportunity to achieve selectivity for this phosphatase. PP3 and PP7 are not strongly inhibited by MCs due to unique regions in their catalytic domains [4]. Together with studies on non-peptidic toxins and small molecules [4,42,45], these results demonstrate that selective inhibitors targeting the active site of PPPs can be achieved.

depod.org: A Database Aiding Phosphatase Modulator Design

When we began our studies, data on phosphatases and their substrates and interacting proteins used to be scattered around in the literature. There was no comprehensive database to aid phosphatase studies, so we began to develop the human DEPhosphorylation Database (DEPOD; www.depod.org). In collaboration with Janet Thornton, European Bioinformatics Institute, UK, we built the database to include all human phosphatases, their substrates (protein and non-protein), links to the corresponding kinases in other databases, links to small molecule inhibitors (ChEMBL database), and information on the involvement of phosphatases and substrates in cellular pathways [5]. We furthermore added interacting proteins (other than substrates) to the database [46]. It is searchable for all internal data (phosphatases, substrates, pathways, dephosphorylation sites, and sequences) (Figure 5) and offers an interactive network to visualize phosphatase–substrate interactions. We analyzed the data in several ways; for example, we did a structural evolutionary analysis that led to the re-classification of phosphatases showing previously unrecognized relationships, and we looked for substrate specificity on the protein domain and sequence levels [5].

DEPOD offers valuable information for the design of chemical modulators of phosphatases. It gives information on substrates and pathways that would be affected upon chemical modulation of a certain phosphatase, and it provides information on direct interactors that could be used to design peptidic modulators of phosphatases, as outlined above. In the case of substrate-based inhibitors, one can directly analyze which other phosphatases might be affected by the inhibitor in case they recognize the same substrate that was the basis for the compound. Together with other databases (for an overview see reference [4]), DEPOD is a valuable resource for phosphatase research and modulator design.

The screenshot displays the DEPOD web interface. On the left is a navigation menu with sections: DEPOD Home, About DEPOD, User Manual; Human Phosphatases, Protein Substrates, Non-Protein Substrates, Pathway Mapping, Dephosphorylation Network; Download, Deposit Data; Feedback, Acknowledgement, Contact; and Links. Below the menu are social media icons and a 'Statistics' box listing: 226 human active and 11 inactive phosphatases in total; 194 phosphatases have substrate data; 305 protein substrates; 89 non-protein substrates; 1114 dephosphorylation interactions; 213 KEGG pathways; 206 NCI Nature PID pathways; and 560 Reactome pathways. The main content area features a header with a 3D molecular model and the DEPOD logo. Below the header is a search bar with tabs for 'Search Phosphatases', 'Search Substrates', 'Search Dephosphorylation Sites', 'Search Pathways', and 'Sequence search (BLAST)'. An example search string is provided: 'Example: PTPN1 (gene name), PTP1B (protein name), P18031 (UniProt AC), ENSG00000196396 (Ensembl gene ID), 5770 (Entrez gene ID)'. At the bottom, there are icons for 'HUMAN PHOSPHATASE', 'PROTEIN SUBSTRATES', 'NON-PROTEIN SUBSTRATES', 'PATHWAY MAPPING', 'DEPHOSPHORYLATION NETWORK', 'DOWNLOAD', and 'DEPOSIT DATA'. A footer contains a citation: 'Please cite the following reference for this resource: Duan, G., Li, X., Köhn, M. (2015). The human DEPhosphorylation database DEPOD: a 2015 update. Nucleic Acids Research, 43(Database issue):D531-5.' and a row of links: 'About DEPOD | User Manual | Feedback | Acknowledgement | Contact'.

Figure 5. Web interface of www.depod.org.

Perspectives

The complex regulation of PP1 and PP2A is not only a challenge but also offers the possibility to take advantage of the several ways that PP1 and PP2A interact with their regulatory proteins and substrates in order to interfere with their roles in certain cellular pathways and, by extension, various diseases. This disruption can be achieved by targeting either the catalytic subunits or the holoenzymes. Exciting new structural insights into the interactions of different PPPs with their regulatory proteins will strongly support modulator design [38]. Peptides play a special role in these strategies: they allow the targeting of larger surfaces and were shown to impart selectivity to modulators targeting these phosphatases. Addressing specific holoenzymes or their cellular localization and distinguishing between the different PPPs still remain major challenges. Given the recent advances in utilizing peptides as the basis for modulator design and the insights obtained so far from employing these strategies, peptides will undoubtedly aid in overcoming these challenges and continue to play an important role in the progression of our understanding of phosphatase biology.

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References

- Khoury GA, Baliban RC, Floudas CA. Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. *Sci. Rep.* 2011; **1**: srep00090.
- Nishi H, Hashimoto K, Panchenko A. Phosphorylation in Protein-Protein Binding: Effect on Stability and Function. *Structure* 2011; **19**: 1807–1815.
- Fontanillo M, Köhn M. Phosphatases: Their roles in cancer and their chemical modulators. *Adv. Exp. Med. Biol.* 2016; **917**: 209–240.
- Fahs S, Lujan P, Köhn M. Approaches to study phosphatases. *ACS Chem. Biol.* 2016; **11**: 2944–2961.
- Li X, Wilmanns M, Thornton J, Köhn M. Elucidating human phosphatase-substrate networks. *Sci. Signal.* 2013; **6**: rs10.
- DeMunter S, Köhn M, Bollen M. Challenges and opportunities in the development of protein phosphatase-directed therapeutics. *ACS Chem. Biol.* 2013; **8**: 36–45.
- Lambrecht C, Haesen D, Sents W, Ivanova E, Janssens V. Structure, regulation and pharmacological modulation of PP2A phosphatases. *Methods Mol. Biol.* 2013; **1053**: 283–305.
- Chatterjee J, Köhn M. Targeting the untargetable: recent advances in the selective chemical modulation of protein phosphatase-1 activity. *Curr. Opin. Chem. Biol.* 2013; **17**: 361–368.
- Cohen PT. Protein phosphatase 1-targeted in many directions. *J. Cell Sci.* 2002; **115**: 241–256.
- Ceulemans H, Bollen M. Functional diversity of protein phosphatase-1, a cellular economizer and reset button. *Physiol. Rev.* 2004; **84**: 1–39.
- Nicolaou P, Hajjar RJ, Kranias EG. Role of protein phosphatase-1 inhibitor-1 in cardiac physiology and pathophysiology. *J. Mol. Cell Cardiol.* 2009; **47**: 365–371.
- Genoux D, Haditsch U, Knobloch M, Michalon A, Storm D, Mansuy IM. Protein phosphatase 1 is a molecular constraint on learning and memory. *Nature* 2002; **418**: 970–975.
- Ammosova T, Yedavalli VR, Niu X, Jerebtsova M, Van Eynde A, Beullens M, Bollen M, Jeang KT, Nekhai S. Expression of a protein phosphatase 1 inhibitor, cdNIPP1, increases CDK9 threonine 186 phosphorylation and inhibits HIV-1 transcription. *J. Biol. Chem.* 2011; **286**: 3798–3804.
- Xia J, Scherer SW, Cohen PT, Majer M, Xi T, Norman RA, Knowler WC, Bogardus C, Prochazka M. A common variant in PPP1R3 associated with insulin resistance and type 2 diabetes. *Diabetes* 1998; **47**: 1519–1524.
- Nuytten M, Beke L, Van Eynde A, Ceulemans H, Beullens M, Van Hummelen P, Fuks F, Bollen M. The transcriptional repressor NIPP1 is an essential player in EZH2-mediated gene silencing. *Oncogene* 2008; **27**: 1449–1460.
- Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol. Med.* 2008; **14**: 152–160.
- Sontag E, Luangpirom A, Hladik C, Mudrak I, Ogris E, Speciale S, White CL, 3rd. Altered expression levels of the protein phosphatase 2A AB α C enzyme are associated with Alzheimer disease pathology. *J. Neuropathol. Exp. Neurol.* 2004; **63**: 287–301.
- Vogelsberg-Ragaglia V, Schuck T, Trojanowski JQ, Lee VM. PP2A mRNA expression is quantitatively decreased in Alzheimer's disease hippocampus. *Exp. Neurol.* 2001; **168**: 402–412.
- Mumby M. PP2A: unveiling a reluctant tumor suppressor. *Cell* 2007; **130**: 21–24.
- Sangodkar J, Farrington CC, McClinch K, Galsky MD, Kastrinsky DB, Narla G. All roads lead to PP2A: exploiting the therapeutic potential of this phosphatase. *FEBS Journal* 2016; **283**: 1004–1024.
- Das I, Krzyzosiak A, Schneider K, Wrabetz L, D'Antonio M, Barry N, Sigurdardottir A, Bertolotti A. Preventing proteostasis diseases by selective inhibition of a phosphatase regulatory subunit. *Science* 2015; **348**: 239–242.
- Yang Y, Huang Q, Lu Y, Li X, Huang S. Reactivating PP2A by FTY720 as a Novel therapy for AML with CKIT tyrosine kinase domain mutation. *J. Cell. Biochem.* 2012; **113**: 1314–1322.
- Toop H, Dun M, Ross B, Flanagan H, Verrills N, Morris J. Development of novel PP2A activators for use in the treatment of acute myeloid leukaemia. *Org. Biomol. Chem.* 2016; **14**: 4605–4616.
- Chatterjee J, Beullens M, Sukackaite R, Qian J, Lesage B, Hart DJ, Bollen M, Köhn M. Development of a peptide that selectively activates protein phosphatase-1 in living cells. *Angew. Chem. Int. Ed.* 2012; **51**: 10054–10059.
- Fosgerau K, Hoffmann T. Peptide therapeutics: current status and future directions. *Drug Discov. Today* 2015; **20**: 122–128.
- Guergnon J, Dessauge F, Dominguez V, Viallet J, Bonnefoy S, Yuste VJ, Mercereau-Puijalon O, Cayla X, Rebollo A, Susin SA, Bost PE, Garcia A. Use of penetrating peptides interacting with PP1/PP2A proteins as a general approach for a drug phosphatase technology. *Mol. Pharmacol.* 2006; **69**: 1115–1124.
- Wakula P, Beullens M, Ceulemans H, Stalmans W, Bollen M. Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. *J. Biol. Chem.* 2003; **278**: 18817–18823.
- Hendrickx A, Beullens M, Ceulemans H, Den Abt T, Van Eynde A, Nicolaescu E, Lesage B, Bollen M. Docking Motif-Guided Mapping of the Interactome of Protein Phosphatase-1. *Chem. Biol.* 2009; **16**: 365–371.
- Meiselbach H, Sticht H, Enz R. Structural analysis of the protein phosphatase 1 docking motif: Molecular description of binding specificities identifies interacting proteins. *Chem. Biol.* 2006; **13**: 49–59.
- Kwon YG, Huang HB, Desdouts F, Girault JA, Greengard P, Nairn AC. Characterization of the interaction between DARPP-32 and protein

- phosphatase 1 (PP-1): DARPP-32 peptides antagonize the interaction of PP-1 with binding proteins. *Proc. Natl. Acad. Sci. U. S. A.* 1997; **94**: 3536–3541.
- 31 Endo S, Zhou X, Connor J, Wang B, Shenolikar S. Multiple structural elements define the specificity of recombinant human inhibitor-1 as a protein phosphatase-1 inhibitor. *Biochemistry* 1996; **35**: 5220–5228.
- 32 Yan Z, Hsieh-Wilson L, Feng J, Tomizawa K, Allen PB, Fienberg AA, Nairn AC, Greengard P. Protein phosphatase 1 modulation of neostriatal AMPA channels: regulation by DARPP-32 and spinophilin. *Nat. Neurosci.* 1999; **2**: 13–17.
- 33 Johnson DF, Moorhead G, Caudwell FB, Cohen P, Chen YH, Chen MX, Cohen PT. Identification of protein-phosphatase-1-binding domains on the glycogen and myofibrillar targeting subunits. *Eur. J. Biochem.* 1996; **239**: 317–325.
- 34 Westphal RS, Tavalin SJ, Lin JW, Alto NM, Fraser ID, Langeberg LK, Sheng M, Scott JD. Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex. *Science* 1999; **285**: 93–96.
- 35 Tappan E, Chamberlin AR. Activation of protein phosphatase 1 by a small molecule designed to bind to the enzyme's regulatory site. *Chem. Biol.* 2008; **15**: 167–174.
- 36 Ammosova T, Jerebtsova M, Beullens M, Lesage B, Jackson A, Kashanchi F, Southerland W, Gordeuk VR, Bollen M, Nekhai S. Nuclear targeting of protein phosphatase-1 by HIV-1 Tat protein. *J. Biol. Chem.* 2005; **280**: 36364–36371.
- 37 Beullens M, Van Eynde A, Vulsteke V, Connor J, Shenolikar S, Stalmans W, Bollen M. Molecular Determinants of Nuclear Protein Phosphatase-1 Regulation by NIPP-1. *J. Biol. Chem.* 1999; **274**: 14053–14061.
- 38 Peti W, Page R. Strategies to make protein serine/threonine (PP1, calcineurin) and tyrosine phosphatases (PTP1B) druggable: achieving specificity by targeting substrate and regulatory protein interaction sites. *Bioorg. Med. Chem.* 2015; **23**: 2781–2785.
- 39 Bollen M, Peti W, Ragusa MJ, Beullens M. The extended PP1 toolkit: designed to create specificity. *Trends Biochem. Sci.* 2010; **35**: 450–458.
- 40 Reither G, Chatterjee J, Beullens M, Bollen M, Schultz C, Köhn M. Chemical activators of protein phosphatase-1 induce calcium release inside intact cells. *Chem. Biol.* 2013; **20**: 1179–1186.
- 41 Sotoud H, Borgmeyer U, Schulze C, El-Armouche A, Eschenhagen T. Development of phosphatase inhibitor-1 peptides acting as indirect activators of phosphatase 1. *Naunyn. Schmiedeberg's Arch. Pharmacol.* 2015; **388**: 283–293.
- 42 Sakoff JA, McCluskey A. Protein Phosphatase Inhibition: Structure Based Design. Towards New Therapeutic Agents. *Curr. Pharm. Des.* 2004; **10**: 1139–1159.
- 43 Fontanillo M, Zemskov I, Häfner M, Uhrig U, Salvi F, Simon B, Wittmann V, Köhn M. Synthesis of highly selective submicromolar microcystin-based inhibitors of protein phosphatase (PP)2A over PP1. *Angew. Chem. Int. Ed.* 2016; **55**: 13985–13989.
- 44 Puddick J, Prinsep MR, Wood SAF, Kaufononga SA, Cary SC, Hamilton DP. High levels of structural diversity observed in microcystins from *Microcystis CAWBG11* and characterization of six new microcystin congeners. *Mar. Drugs* 2014; **12**: 5372–5395.
- 45 Swingle M, Volmar CH, Saldanha SA, Chase P, Eberhart C, Salter EA, D'Arcy B, Schroeder CE, Golden JE, Wierzbicki A, Hodder P, Honkanen RE. An Ultra-High-Throughput Screen for Catalytic Inhibitors of Serine/Threonine Protein Phosphatases Types 1 and 5 (PP1C and PP5C). *J. Biomol. Screen.* 2017; **22**: 21–31.
- 46 Duan G, Li X, Köhn M. The human DEPhosphorylation database DEPOD: a 2015 update. *Nucleic Acids Res.* 2015; **43**: Database issue D531–D535.