

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

**Regulation of protein phosphatase-1 and -2A by natural polyphenols, synthetic peptides
and selenoglycosides**

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Regulation of Protein Phosphatase-1 and -2A by natural polyphenols, synthetic peptides and selenoglycosides

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At 10:00 am, 16th of December 2015.

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Introduction

The structure and the functions of at least one third of the cellular proteins are regulated by reversible phosphorylation. The phosphorylation state of proteins is determined by the delicate balance of the activity of protein kinases and protein phosphatases. The kinases drawn more attention at the beginning of the protein phosphorylation era, because their inhibitors are more type specific and almost all types of enzyme, except for a few, regulate only a couple of proteins which provides a more direct approach to identify the substrates and the functions of these kinases. Later the attention turned towards protein phosphatases when it was recognized that phosphatases are also under strict regulation and important players in the mediation of the phosphorylation level of proteins. Numerous substrates can be dephosphorylated by the same type of phosphatase catalytic subunit with the aid of several regulatory proteins. The identification of substrates and functions of phosphatases are not directly possible with the application of inhibitors acting on the catalytic subunit. Protein phosphatase-1 (PP1) and 2A (PP2A) belong to the most important phosphoserine/threonine (P-Ser/Thr) specific phosphatases due to their broad range of substrate specificity and functions and our major aim was to study the regulation of these enzymes.

The structure, regulations and functions of PP1c

The catalytic subunit of PP1 (PP1c) is responsible for the catalytic activity. Its catalytic center localizes at the bifurcation point of a Y shaped substrate binding groove and coordinates Mn^{2+} and a Fe^{2+} ions. The arms of Y include the hydrophobic, acidic and the C-terminal grooves, which orient the phosphoproteins to the right position on the surface of the enzyme to enhance dephosphorylation efficiency. Another important structural component of the enzyme is the $\beta 12$ - $\beta 13$ loop at the catalytic center. The structure of the loop can influence the interaction of the enzyme with proteins and different types of small molecules at the catalytic cleft. The genes encoding PP1c results in expression of 5 isoforms: PP1c α_1 and α_2 , PP1c β/δ , PP1c γ_1 and PP1c γ_2 . These isoforms are involved in the formation of holoenzyme complexes. The interactions are driven by the binding of short interacting motifs to PP1c. The interacting motifs of the regulatory proteins differ slightly. One of these is the RVxF motif which's presence and structure is a key feature in the affinity of regulatory proteins to PP1c and it also facilitates the interaction of secondary binding sites to PP1c. The competition of regulatory proteins for PP1c is highly probable, because PP1c has limited surface area for specific interaction due to the small size, thus the binding of modulators is restricted to the same well characterized surfaces of the catalytic subunit. Proteins with inhibitor functions are also

known (I1, I2, DARPP32, NIPP-1, CPI-17). Their function is to inhibit the activity of holoenzymes or free PP1c, and to enhance the exchange of regulatory proteins in holoenzyme complexes. The posttranslational modification of the proteins is also an important way to regulate holoenzyme functions.

PP1 fulfill important biological functions in many cellular processes like glycogen metabolism, lipid metabolism, synaptic plasticity, cell cycle progression, smooth muscle contraction, and insulin signaling as well as cytoskeleton rearrangement.

Subunit interactions in the myosin phosphatase holoenzyme

One of the most well characterized holoenzymes of the PP1 is the myosin phosphatase (MP). In MP the PP1c β/δ isoform interacts with the myosin phosphatase target subunit 1 (MYPT1). The substrate specificity and the subcellular localization of MP are determined by MYPT1. It contains an RVxF interaction motif (KVKF) and the binding of this sequence is a prerequisite for the interaction of secondary MYPT1 binding regions. The phosphorylation at Thr696 and Thr853 residues in MYPT1 can exert inhibition of PP1c in MP holoenzyme.

The surrounding amino acid sequences at the Thr696 and Thr853 in MYPT1 are similar to the sequence of 20 kDa molecular mass myosin regulatory light chain (MLC20) around the Ser19 phosphorylation site. This suggests that, they may act like a substrate analogue dephosphorylated at a much slower rate compared to the substrate proteins, thereby competing with the substrate and causing inhibition.

The structure, regulation and function of PP2A

The catalytic center of the protein phosphatase-2A catalytic subunit (PP2Ac) contains Mn²⁺ ions. The amino acid sequence of the PP2Ac is 50 % identical to PP1c. The identical sequences are involved in the formation of the globular protein structure of catalytic subunits. One of the key features in the non-identical sequences is the structure of β 12- β 13 loop. A scaffold protein (PR/65A (A), α and β isoforms) is associating with the PP2Ac (C) creating the base structure (PP2A-AC) of the enzyme. An additional B regulatory protein associate with the AC dimer regulating PP2A substrate specificity and cellular localization. Four families are known for the B subunits (PR55/B, PR56/61/B', PR48/59/72/130/B'', PR93/110/B''').

Inhibitor proteins are also known for PP2A (I₁^{PP2A} (PHAP-I) and the I₂^{PP2A} (SET)). These proteins have features in common with the inhibitors regulating PP1 with regards of specific inhibition of PP2Ac. The postsynthetic modifications of PP2A proteins are also important to modulate the function of PP2A just like in case of PP1.

The phosphorylation of Thr, tyrosine (Tyr) and Ser residues of B subunits regulate the activity of the holoenzyme. At the same time the methylation of PP2Ac alters the affinity of B, B', B'', B''' subunits to the holoenzyme by changing the binding strength between the proteins. PP2A enzymes are involved in the regulation of several processes like cell proliferation, cell death and motility, cytoskeletal rearrangement, and in certain steps of cell cycle.

The inhibition of PP1 and PP2A by toxins and tannins

Several natural toxins can exert inhibition on the activity of PP1 and PP2A with different specificity, thus they are used to differentiate between the activities of these enzymes. These compounds bind to the substrate binding groove(s) and some of them interact with the catalytic center blocking the binding of substrate proteins to the enzyme. Okadaic acid (OA), a polyether compound, was the first identified toxin inhibitor of phosphoprotein phosphatases (PPP). *In vitro* experiments showed different effectiveness for inhibition of PP1 (IC_{50} = 15 nM) compared to PP2A (IC_{50} = 0.1 nM). The catalytic center, the β 12- β 13 loop and the hydrophobic substrate-binding groove of the enzymes are involved in the interaction of OA with the enzyme. Calyculin A (CLA), another polyether, is a more potent inhibitor of PP1 and PP2A compared to OA. Its binding sites partially overlap to that of OA, but in addition it also occupies the acidic groove and it is less selective for PP1 and PP2A compared to OA. The cyclic peptide microcystin exert similar inhibitory efficacy on the activity of both PP1 and PP2A (IC_{50} = 0.2 nM). Fostriecin, a polyketide, binds to PP2A and PP4 by forming a covalent linkage with a cysteine residue of these enzymes Fostriecin is more selective towards PP2A compared to PP1 (PP1, IC_{50} = 4 μ M; PP2A, IC_{50} = 40 nM).

Plant tannins and catechins, an example for the latter is epigallocatechin-3-gallate (EGCG), inhibits the activity of PP1c (IC_{50} = 1.35 μ M) and PP2Ac (IC_{50} = 15 μ M) with partial selectivity towards PP1c. *In vitro* studies proved that EGCG interacts with PP1c occupying part of the hydrophobic substrate binding groove. The hydrogen bonds formed between EGCG and PP1c stabilize this interaction. Penta-O-galloyl- β -D-glucose (PGG) is the most abundant gallotannin in plants. The inhibition it exerts on protein phosphatases (PP1c, IC_{50} = 0.4 μ M; PP2Ac, IC_{50} = 6.6 μ M) is similar to EGCG. NMR-STD experiments proved also that in PGG, the hydrophobic aromatic rings are involved in the interaction with PP1c and this is presumably further stabilized by hydrogen bonding.

The activation of PP1 and PP2A

The PP1c activators known so far are peptides derived from regulator proteins. These peptides inhibit the interaction of regulatory and inhibitory proteins with PP1c acting as deinhibitors. For example, the peptide with the RVxF sequence motif derived from skeletal muscle G-subunit of PP1G holoenzyme is capable to inhibit the association of DARPP32 with PP1c thus freeing the catalytic subunit which can exert increased activity towards certain substrates. Interestingly, the N-acetyl-Arg-Adda (derived from MCY-LR, a very potent inhibitor) or the D-eritro-C₁₈-Ceramide were also identified as direct activator compounds.

The activators of PP2A often have a hydrophobic core structure and/or functional groups which can form hydrogen bonds. Ceramides are carrying both features and they can act as activators or deinhibitors depending on the molecular structures. The D-eritro-C₆-ceramide releases SET (I2^{PP2A}) protein from the PP2Ac-PR65-SET complex and restores the activity of PP2Ac from inhibition. Other activators (e.g. FTY720) share the same mechanism of action.

The D-eritro-C₁₈-ceramide, phenothiazines and perphenazines are direct activators which can stimulate the activity of heterotrimeric forms of PP2A holoenzymes. An alternative way to enhance PP2A activity is the inhibition of the expression of CIP2A inhibitor protein. This can be accomplished by chemotherapeutic compounds such as bortezomib and erlotinib. Celastrol, derived from plants, can induce the ubiquitination and degradation of CIP2A. These actions can also increase the activity of PP2A.

Aims

Several cellular processes can be modulated by the activation or inhibition of PP1 and PP2A enzymes since more than 90 % of the P-Ser/Thr residues in cellular proteins are dephosphorylated by these two enzymes. Uncovering the precise cellular functions of these enzymes is not without limitations. Inhibitory toxins were applied for these purposes, but they are not selective enough under cellular conditions and they are cytotoxic, too. Previous results established that inhibition of PP1 and PP2A often contribute to tumorigenic transformation and could help survival of cancer cells. In contrast, activation of PP1 and PP2A is considered to contribute to tumor suppression, primarily by the involvement of phosphatase activation in the induction of cell death of cancer cells. Thus, the exploration and pharmacological utilization of type specific phosphatase inhibitors and activators have become the central focus of several recent studies. Our research group identified gallotannins and catechins as partially selective inhibitors of PP1. Our aim was to study the effects of the structurally similar naturally occurring ellagitannins on the activity of PP1 and PP2A and on the viability of cell cultures. Phosphorylated MYPT1 was also shown earlier to inhibit the activity of PP1c. However, peptides including the inhibitory phosphorylation sites of MYPT1 are yet to be analyzed on the activity of PP1c. We hypothesized that other glucose derivatives like selenoglycosides could influence the activity of PP1 and PP2A, Surprisingly, these compounds proved to exert activation on both enzymes which was a novel finding.

Overall, our experiments aimed to answer the following questions:

1. Effects of ellagitannins on the activity of PP1 and PP2A: assay of type specificity, interaction analysis and their influence on the viability of cells.
2. Examination of the effect of phospho-MYPT1 peptide on PP1c activity and exploration of its inhibitory mechanism.
3. Characterization of the activation of protein phosphatases by selenoglycosides: description of structure-activatory effect relationships.

To answer these questions a wide range of methods of biochemistry as well as cell and molecular biology were applied.

Materials and Methods

The preparation of phosphatases and phosphatase activity measurements

Native PP1c and PP2Ac were isolated from rabbit skeletal muscle while recombinant rPP1 α and rPP1 δ were expressed and purified from bacterial expression systems. The activity of protein phosphatases was measured with 1 μ M ^{32} P-MLC20 substrate at 30°C. The purified proteins or HeLa cell lysate were preincubated in the presence or absence of ellagitannins, selenoglycosides or MYPT1 peptides and the reaction was started by the addition of the substrate. The radioactivity of the released ^{32} P_i of the samples were measured in a scintillation counter.

Cell culturing, treatments and viability assays

HeLa cells were cultured according to the guidelines of provider. After serum starvation cells were incubated in the presence or absence of compound dissolved in serum free culture medium and placed in an incubator (30°C, 80 % humidity) for 24 hours. The treating media was exchanged to serum free media and the viability was assayed with Alamar blue assay.

Surface Plasmon Resonance

The interaction of selenoglycosides or 2,3-di-O-galloyl-4,6-O-(S)-hexahydroxydiphenoyl- β -D-glucose (tellimagrandin I) with rPP1 δ was assayed by surface plasmon resonance (SPR) based binding technique using Biacore 3000 instrument. The effect of selenoglycosides on the interaction of PP1c with MYPT1 was studied by injecting PP1c in the absence or presence of selenoglycosides over a GST-MYPT1 surface. Anti-GST antibody was covalently immobilized to the CM5 sensor surface by amine coupling kit and GST or GST-MYPT1 proteins were immobilized on these surfaces. 3 μ M rPP1 δ was injected over the surfaces in presence or absence of 10-50 μ M selenoglycosides (association phase), then surfaces were washed (dissociation phase) with buffer. The GST and GST MYPT1 surfaces were treated identically to determine nonspecific binding, which was subtracted from the data obtained on the GST-MYPT1 surfaces. As a result, rPP1 δ binding specific signal was obtained as resonance signal as a function of time. The tellimagrandin I-rPP1 δ interaction was studied by experiments in which rPP1 δ was immobilized to CM5 sensor chip and tellimagrandin I (0-2.5 μ M) was injected over the control and rPP1 δ surfaces. The association and dissociation phases lasted for 7 minutes and 6 minutes, respectively. The kinetic and the dissociation/association parameters

for the interactions were derived by the BiaEvaluation 3.1 software (Biacore AB, Uppsala, Sweden) from the sensorgrams with the assumption of 1 : 1 stoichiometric ratio.

Isotherm titration calorimetry (ITC)

rPP1 α protein was dialyzed in assay buffer, then diluted to 5-10 μ M and loaded in the sample cell of MicroCal ITC200 instrument. The reference cell was loaded with the assay buffer. The syringe was filled with tellimagrandin I (60-100 μ M in assay buffer) and the titration was carried out by 18 step injections of 2 μ l volumes to the sample cell with 5 μ cal/sec compensation power and constant stirring at 25°C. The titration data were analyzed by Microcal ITC-Origin software assuming 1 : 1 ligand-protein interaction.

Microscale thermophoresis (MST)

Microscale thermophoresis experiments were carried out by Monolith NT.115 and Monolith NT.LabelFree instruments (NanoTemper Technologies GmbH, Munich, Germany). Experiments on Monolith NT.LabelFree instrument were carried out with non-labeled rPP1 α diluted to 250 nM. In case of the Monolith NT.115 rPP1 α was labeled with NT-647 dye by NHS labeling kit (NanoTemper Technologies GmbH, Munich, Germany) and applied for the experiments diluted to 170 nM in assay buffer. Two-fold dilution series (16 samples) were prepared with the ligands and mixed with labeled or non-labeled rPP1 α to generate a concentration series of ligands in the range of 7.32 nM to 120 μ M for polyphenols or the range of 61 nM to 1 mM for P-MYPT1⁶⁹⁰⁻⁷⁰¹ peptide. The sample solutions were loaded in glass capillaries for the measurements. The intrinsic (NT.LabelFree) or extrinsic (NT.115) fluorescence signal change of rPP1 α upon interaction with the ligands was measured. The obtained data were analyzed by MO AffinityAnalysis software (NanoTemper Technologies GmbH, Munich, Germany).

NMR saturation transfer difference (STD)

NMR measurements were carried out on Bruker Avance II 500 spectrophotometer. The ¹H-NMR and the saturation transfer difference (STD) spectrums of tellimagrandin I dissolved in D₂O phosphate buffer were recorded at 293 K in the presence or absence of 10-13 μ M rPP1c.

Docking simulations

The X-ray structure of PP1c interacting with an RVxF motif containing peptide was used to the docking procedures. The surfaces involved in the binding of RVxF within the peptide were the

reference for the simulations. A grid box with the size of 25 x 25 x 25 Å was set and the simulations were carried out by Autodock Vina software. The docking structures representing the highest affinities were chosen for further analysis. The hydrophobic surfaces involved in the interface of PP1c-selenoglycoside interaction were identified and analyzed with MGM Tools Visualization and analysis of structures were performed with UCSF Chimera software (Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (NIGMS P41-GM103311)).

The preparation and treatment of synaptosomes

Synaptosomes were extracted from C3H mouse cerebral cortex. The cortex tissues were collected in homogenization buffer and lysed with potter at 4°C. Synaptosomes were obtained after differential and gradient centrifugations. The synaptosomes were further diluted in Krebs buffer containing 1.2 mM CaCl₂, then incubated for 45 minutes in the presence or absence of tellimagrandin I, 1,4,5-tri-O-galloyl-2,3-O-(R)-hexahydroxydiphenyl-β-D-glucose (mahtabin A, 1, 10, 100 μM) or tautomycin (TMC, 10 μM) at 30°C. After the treatments the samples were used for exocytosis assay or were lysed in RIPA buffer and analyzed for the phosphorylation level of SNAP-25 by Western blot.

Synaptosomes were loaded with FM 2-10 dye and exocytosis was induced by the addition of 30 mM KCl. The fluorescence signal change of FM 2-10 dye incorporated in the synaptosomes was recorded by Fluoroskan FL. The fluorescent intensity of FM 2-10 styryl dye correlates negatively with the extent of synaptosomal exocytosis.

The protein concentrations for Western blot analysis were measured by BCA assay kit. The synaptosome proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked and incubated with primary-antibody solutions (diluted as recommended by the manufacturers) for 16 hours at 4°C. After washing the membranes were incubated with peroxidase conjugated secondary-antibody and the antigen-antibody interaction was detected with chemiluminescence reagent by Chemidock Touch instrument. The data were analyzed by Fiji (Image J) software.

Results and conclusions

Ellagitannins regulate the activity of PP1 and PP2A

Ellagitannins exert inhibitor effect on PP1c and PP2Ac with a higher preference towards PP1c. The catechin EGCG and the gallotanin PGG were proved to act on PP1c and PP2Ac with similar manners but the selectivity of these compounds for PP1c was less pronounced compared to ellagitannins. The differences in the structure of the compounds may explain the observed differences in selectivity. The structure of the studied ellagitannins differed from PGG in the presence of covalently coupled galloyl units designated as hexahydroxydiphenol groups, and in the number and positions of unesterified hydroxyl groups of β -D-glucose: Tellimagrandin I, 2,3; 4,6-di-(S)-hexahydroxydiphenyl- β -D-glucose (pedunculagin) and 4,5-di-O-galloyl-2,3-O-(S)-hexahydroxydiphenyl- β -D-glucose (praecoxin B) contains free glycosidic hydroxyl group, while 1,2-di-O-galloyl-4,6-O-(S)-hexahydroxydiphenyl- β -D-glucose (GHG) contains free alcoholic hydroxyl at position 3 of β -D-glucose.

Tellimagrandin I has a more rigid structure compared to PGG due to the presence of the 4,6-hexahydroxydiphenol group and it has a more pronounced inhibitory effect on the activity of PP1c. Mahtabin A has no free glycosidic hydroxyl group and it includes 2,3-hexahydroxydiphenol unit exerting weaker inhibition on PP1c than tellimagrandin I. If we analyze the inhibition-structure relationship comparing the more effective tellimagrandin I to praecoxin B it could be concluded that the position of hexahydroxydiphenol group has also influence on the inhibitory activity. Tellimagrandin I and GHG both include 4,6-hexahydroxydiphenol group a common structural element, but the glycosidic hydroxyl group of GHG is esterified with gallic acid and it has a free hydroxyl group at the position 3 of the β -D-glucose. As GHG proved to be a less effective inhibitor compared to tellimagrandin I or even to praecoxin B, the conclusion could be drawn that esterification at position 3 of the glucose can positively influence inhibition. Pedunculagin is the most rigid studied ellagitannin due to the presence of two hexahydroxydiphenol groups. The increased rigidity is coupled with reduced inhibitory potential compared to the rest of the compounds.

The inhibition exerted by ellagitannins on PP1c is similar to PGG, but their effect on PP2Ac is less pronounced. The difference is reflected in the selectivity ratios calculated from IC_{50} values (PP1c/PP2Ac= 1: 80/97 for ellagitannins and 1: 17 for PGG). Molecular docking simulations based on X-ray structures of PP1c and PP2Ac and the 3D model of EGCG proved that the binding positions of EGCG in PP1c or PP2Ac is different but involves the hydrophobic

substrate-binding groove in both cases. PP2Ac contains a salt bridge at the lower region of the hydrophobic groove which may restrict the proper binding of EGCG. Ellagitannins, with their larger size compared to EGCG, are also presumed to bind to the hydrophobic groove as revealed by NMR-STD experiments, therefore their interactions may also be influenced by the above structural features of PP2Ac.

The hydrophobicity of the aromatic rings of ellagitannins was proved to be an important feature in the interaction with PP1c by NMR-STD experiments. Earlier experiments also confirmed the hydrogen bonds established by the hydroxyl groups of such rings can stabilize the interaction between PP1c and EGCG for example. Although we did not confirm the role of these hydroxyl groups in the interaction of ellagitannins with PP1c, but some literature data suggested that interaction between these phenolic compounds and proteins are also stabilized by hydrogen bonds and this could not be excluded in case of PP1c-ellagittannin interactions either.

The interaction of tellimagrandin I with PP1c was assessed by several binding methods. The hydrophobic manner of the interaction was verified by NMR-STD experiments. The kinetic and association/dissociation parameters were characterized by ITC, SPR and MST methods. Depending on the experimental settings the K_D value was between 0.23 and 1.78 μM , which is in good agreement with the determined IC_{50} values. Although one binding site is proven for interaction of PGG or EGCG with PP1c, SPR studies suggested higher level of bindings to the PP1c surfaces which would be expected from the 1:1 stoichiometric ratio. This may be due to the phenomenon called „stacking” when the binding compounds of interests interact with each other on the surface of the target protein. From the SPR data we gained it is obvious that “stacking” could not be excluded in case of ellagitannins either, which might occur during ITC or MST measurements too, influencing the actual K_D values determined.

The viability and phosphatase activity of HeLa cells were reduced by tellimagrandin I. On contrary, mahtabin A was without effect in cell cultures. The reason which accounts for this controversial behavior of the two ellagitannins might be differences in the membrane permeability of these compounds. In addition, the phosphorylation level of SNAP-25^{Thr138} was increased while the extent of KCl inducible exocytosis was decreased in synaptosomes by treatments with mahtabin A, while tellimagrandin I was without effect. There is no experimental data available presently to explain these defferent behavior of tellimagrandin I and mahtabin A on cells and membrane surrounded ex vivo preparation like synaptosomes.

In conclusion, ellagitannins were found to be a novel family of PP1 and PP2A inhibitors with a greater preference towards PP1c. Tellimagrandin I-PP1c interaction was shown to be a medium stability complex by quantitative binding analysis. This interaction involves the hydrophobic substrate binding groove in PP1c and the aromatic rings and phenolic hydroxyls in the ellagitannins.

The effects MYPT1⁶⁹⁰⁻⁷⁰¹ and P-MYPT1⁶⁹⁰⁻⁷⁰¹ peptides on PP1

The effect of the phosphorylated inhibitory peptide (P-MYPT1⁶⁹⁰⁻⁷⁰¹) derived from MYPT1 was assessed on the activity of PP1c. P-MYPT1⁶⁹⁰⁻⁷⁰¹ caused inhibition of the activity of PP1c ($IC_{50} = 32 \mu M$), although by the relatively high IC_{50} value this peptide cannot be considered as a promising phosphatase inhibitor.

The NMR-STD analysis of the PP1c-P-MYPT1⁶⁹⁰⁻⁷⁰¹ complex led to two new findings. First, the hypothesis of P-MYPT1⁶⁹⁰⁻⁷⁰¹ peptide interacting with PP1c by hydrophobic and basic amino acid side chains was strengthened. This is in good agreement with that the peptide forms interaction with the hydrophobic and the acidic grooves in PP1c. Second, the NMR experiments confirmed that P-MYPT1⁶⁹⁰⁻⁷⁰¹ was dephosphorylated by PP1c during the NMR-STD experiment at this high concentrations of both the enzyme (10-14 μM) and the peptide (1.3 mM) in the solution, although the half-life of the dephosphorylation turned out to be quite high ($t_{1/2} = 82$ min). The MYPT1⁶⁹⁰⁻⁷⁰¹-PP1c interaction was also confirmed by NMR-STD measurements, but not with MST, which suggest a weak interaction between the dephosphorylated peptide and PP1c. However, the dephosphorylated peptide has no inhibitor effect on PP1c, which imply that phosphorylation is a prerequisite for the inhibition.

The high half-life of the dephosphorylation of P-MYPT1⁶⁹⁰⁻⁷⁰¹ suggest that the peptide is a “bad substrate” for PP1c. Due to the similar affinity of the inhibitory peptide and the substrate to PP1c the slowly dephosphorylating P-MYPT1⁶⁹⁰⁻⁷⁰¹ would compete with the substrate for dephosphorylation causing inhibition which mechanism was recently referred to as “unfair” competition. Our current molecular docking simulations suggest that the P-MYPT1⁶⁹⁰⁻⁷⁰¹ peptide really is a substrate analogue and the position of the P-Thr is unfavorable for dephosphorylation. Moreover, the coordination of the metal ions in PP1c changes upon interaction with the peptide and this alteration may also contribute to the slower dephosphorylation.

In conclusion, the short peptides derived from regulatory proteins are not effective inhibitors of PP1c, nevertheless further analysis of their effects and the structural background to the inhibitory mechanism may help to create more potent peptide inhibitors.

The regulation of PP1 and PP2A by selenoglycosides

Acetylated aromatic selenoglycosides activated both PP1c and PP2Ac. Since only the acetylated selenoglycosides proved to be effective activators the hydrophobicity of the compounds is considered to play a major role in the activation. Our results suggest that selenoglycosides interact with hydrophobic surfaces of the phosphatases distinct from the hydrophobic substrate binding groove, but it proved to be the surface involved in the binding of the RVxF motif of regulatory and inhibitory proteins. Molecular docking simulation also provided data suggesting that the binding surface of selenoglycosides and the hydrophobic amino acid residues (Val and Phe) in the RVxF motif overlap only partially. SPR-binding data suggested that the selenoglycosides and the RVxF motif containing peptides/proteins mutually weakened each others' binding, however did not completely ceased that. Selenoglycosides lowered the extent of binding of PP1c to MYPT1, but the formation of MYPT1-PP1c complex still be seen even at the highest applied selenoglycoside concentrations. We suppose the formation of a PP1c-selenoglycoside-MYPT1 complex in which the partners can still associate with each other but with a decreased affinity. This is confirmed by the results that selenoglycoside treatments increase the phosphatase activity of PP1 and PP2A holoenzymes in HeLa cells to a low, but significant extent.

In summary, we uncovered a novel family of PP1 and PP2A activators, however, they are still could not be considered as pharmacologically relevant candidates. Selenoglycosides have less pronounced intracellular effects, which is coupled with cytotoxicity even at lower concentration for both the acetylated and the nonacetylated compounds. The facts that non-acetylated selenoglycosides has also cytotoxic effects, although less pronounced than the acetylated ones, suggest that phosphatase activation may be only partially implied in the induction of cell death. Despite all these controversies, our present results imply that selenoglycosides can serve as structural template to design and synthesize phosphatase activators, or to screen molecular libraries for structurally related compounds with improved features of possible physiological relevance. The importance of the application of such phosphatase activators is related to the facts that protein phosphatases are considered as tumor suppressors as their activation can induce cancer cell death. Our data imply also that PP2Ac possesses at least two separate hydrophobic sites for binding of activator compounds, distinct from that of the PP1c binding surface.

Summary

Protein phosphatases and kinases are key regulatory enzymes of various cellular processes. Protein phosphatase- 1 (PP1) and -2A (PP2A) are important representatives of the phosphatase enzymes and they are believed to dephosphorylate more than 90 % of phosphoserine/threonine (P-Ser/Thr) proteins regulating a wide range of cellular processes. Our aims were to identify novel inhibitors and activators of PP1 and PP2A and to uncover the molecular mechanisms behind these inhibitory/activatory effects.

We identified ellagitannins as inhibitors of PP1 and PP2A with partial selectivity against PP1 compared to PP2A. The interaction of tellimagrandin I, the most effective inhibitor, with PP1 catalytic subunit (PP1c) was characterized by NMR saturation transfer difference (NMR-STD) spectroscopy. Quantitative features of this interaction ($K_D = 0.23\text{--}1.78\ \mu\text{M}$) were determined by surface plasmon resonance (SPR), isotherm titration calorimetry (ITC) and microscale thermophoresis (MST) based binding methods. Tellimagrandin I inhibited the phosphatase activity of HeLa cells and decreased cell viability, while mahtabin A increased the phosphorylation level of neuronal proteins and decreased the extent of exocytosis in cortical synaptosomes. These data suggest that the phosphatase inhibitory features of ellagitannins might also be involved in mediation of distinct cellular events.

A phosphopeptide (P-MYPT1⁶⁹⁰⁻⁷⁰¹), synthesized based on the phosphatase inhibitory sequence of myosin phosphatase regulatory subunit (MYPT1), inhibited ($IC_{50} = 32\ \mu\text{M}$) the activity of PP1c. This inhibition was due to the slow dephosphorylation of the P-Thr in the peptide by which an “unfair” competition with the P-Ser containing physiological substrate could occur. It is assumed that the distinct positioning of the P-Thr and P-Ser at the catalytic centre of PP1c might be responsible for the above phenomena.

Acetylated selenoglycosides were identified as activators ($EC_{50} = 80\text{--}300\ \mu\text{M}$) of both PP1 and PP2A. Competition assays with kinetic and SPR interaction analyses proved that the binding site for selenoglycosides in PP1c partially overlaps with the binding surface of RVxF motif, a sequence essential to the binding of regulatory proteins to PP1c. Selenoglycosides stimulated the phosphatase activity in HeLa cells to a small, but significant extent as well as decreased cell viability, which at least in part, may be due to phosphatase activation.

In summary, we identified ellagitannins and selenoglycosides as novel families of phosphatase inhibitor and activator molecules. These compounds might serve as templates for the development of new phosphatase mediator molecules of pharmacological significance.

List of publications



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Registry number: DEENK/300/2019.PL
Subject: PhD Publikációs Lista

Candidate: Zoltán Kónya
Neptun ID: MN0FM4
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Kónya, Z.**, Bécsi, B., Kiss, A., Horváth, D., Hadháziné Raics, M., Kövér, K. E., Lontay, B., Erdődi, F.: Inhibition of protein phosphatase-1 and -2A by ellagitannins: structure-inhibitory potency relationships and influences on cellular systems.
J. Enzym. Inhib. Med. Chem. 34 (1), 500-509, 2019.
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2. **Kónya, Z.**, Bécsi, B., Kiss, A., Tamás, I., Lontay, B., Szilágyi, L., Kövér, K. E., Erdődi, F.: Aralkyl selenoglycosides and related selenosugars in acetylated form activate protein phosphatase-1 and -2A.
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List of other publications

3. Szabó, K., **Kónya, Z.**, Erdődi, F., Farkas, I., Dombrádi, V.: Dissection of the regulatory role for the N-terminal domain in *Candida albicans* protein phosphatase Z1.
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