FINAL REPORT

THE ROLES OF NOVEL INHIBITOR MOLECULES AND PROTEIN–PROTEIN INTERACTIONS IN THE REGULATION OF PROTEIN PHOSPHATASE-1

Introduction and major aims of the study

Reversible phosphorylation of proteins at serine (Ser)/threonine (Thr) residues is an important way to regulate cellular processes [1]. The phosphorylation status of cellular proteins depends on the balance of the activities of protein kinases and phosphatases. Initially, protein kinases were assumed as the most important regulatory targets of these processes. However, protein phosphatases have also been shown to be strictly regulated enzymes and they were even declared as the 'winners' of this regulatory competition with the kinases [2].

Protein phosphatase-1 (PP1), -2A (PP2A), and -2B (PP2B, or calcineurin (CN)), are three major phospho-Ser/Thr-specific enzymes [3]. They are responsible for the dephosphorylation of more than 90% of all cellular proteins at phospho-Ser/Thr residues. The present proposal mainly focused on the regulation of PP1, but possible roles of PP2A and PP2B/CN were also addressed.

The major hypotheses for this proposal, based on the literature and the preliminary results were: *(i)* PP1 inhibitory polyphenol molecules might serve as 'lead' compounds for the development of novel PP1-specific inhibitors and conjugation of these molecules with peptides might result in inhibitors that would be able to interact with the substrate-binding grooves; *(ii)* the PP1-type myosin phosphatase (PP1M) plays an important role in neurotransmitter release and neurotransmission by interacting with and dephosphorylating a number of key regulatory proteins at both pre- and postsynaptic locations.

In light of these hypotheses, the aims of the proposal were as follows:

I. To develop novel PP1-specific inhibitors for the determination of the cellular functions of PP1.

II. To reveal the functions and roles of myosin phosphatase/PP1M and ROK at pre- and/or postsynaptic locations in the regulation of exocytosis and neurotransmitter release in synaptosomes and PC12 cells.

III. To investigate the physiological relevance of protein phosphorylation and protein-protein interactions in neurotransmission: morphological and electrophysiological studies of neurons.

The project has been executed according to the major lines of the original aims, therefore we state that it conforms to the original research plan. However, regarding the detailed research questions during the course of study these have been modified at some points and we have also pursued a few novel developments related to the planned studies. The results are presented in the following section in a manner that if publications (***indicated with bold italics***) have appeared on the topics the presentation is more focused. In contrast, more experimental data are provided with regards of that studies for which manuscripts submission is only in the preparation phase.

RESULTS AND DISCUSSION

I. To develop novel PP1-specific inhibitors for the determination of the cellular functions of PP1

Biochemical and cellular characterization of known phosphatase inhibitors

One of the major goals of this research project has been to assay known phosphatase inhibitors and develop novel PP1-specific inhibitors for the determination of the cellular functions of PP1. We have done experiments to establish model cellular systems to investigate these novel inhibitors. To this end we used well-known classical phosphatase inhibitors, calyculin-A (CLA) and tautomycin (TM), and characterized their type specific inhibitory effects in leukemic cells (***Dedinszki et al. CELLULAR SIGNALLING 27: pp. 363-372. 2015***) [4] and HaCaT cells (***Dedinszki et al. BIOCHIMICA ET***

BIOPHYSICA ACTA-MOLECULAR BASIS OF DISEASE 1852: pp. 22-33. 2015) [5]. Phosphatase activity assays in the presence of inhibitor-2, a specific inhibitor of protein phosphatase-1 (PP1), was developed to assess the cellular inhibitory effects of CLA and TM. It was found that CLA (50 nM) was a protein phosphatase-2A (PP2A), while TM (1 μM) was a PP1 specific inhibitor in cells. These assays helped to prove that PP1 and PP2A decrease the chemosensitivity of leukemic cells to chemotherapeutic drugs. It appears that the molecular background to these events include specific dephosphorylation of the retinoblastoma protein (Rb) by PP1, while PP2A might have an indirect influence on this process via mediating the phosphorylation level of PP1 inhibitory proteins such as kinase enhanced phosphatase inhibitor (KEPI) and the myosin phosphatase target subunit-1 (MYPT1). *These data imply the importance of PP1 inhibitory proteins in controlling the phosphorylation state of key proteins and regulating drug sensitivity and apoptosis in leukemic cells* [4].

The role of PP1 was investigated in the response of HaCaT cells and mouse skin to UV radiation. PP1c-silencing decreased the phosphatase activity and suppressed the viability of HaCaT cells. Exposure to UVA induced HaCaT cell death and resulted in 30 % decrease of phosphatase activity. PP1c-silencing and UVA irradiation altered the gene expression profile of HaCaT cells and the expression of 19 genes was regulated by the combined treatments with many of these genes being involved in malignant transformation. Inhibition of PP1 by TM increased pigmentation of mouse skin, which was even more apparent when TM was followed by UVA irradiation. *Our data identify PP1 as a regulator of the normal homeostasis of keratinocytes and the UV response* [5].

To gain a better insight of the mechanism of the action of phosphatase inhibitory molecules the interaction of okadaic acid (OA), tautomycin (TM), microcystin-LR (MC-LR), cantharidin (CA), epigallocatechin-gallate (EGCG) and cyclosporin A (CsA) with liposome covered surfaces prepared from the lipid extracts of bovine brain, heart and liver was investigated by surface plasmon resonance (SPR) based binding technique (***Bécsi, et al. CHEM. PHYS. LIPIDS 183C 68-76. 2014***) [6]. Distinct lipid composition specificities were reflected in different saturation values of inhibitor binding in a decreasing order of liver > heart >> brain lipids. Assaying the effect of OA, TM, MC-LR, CA and EGCG on the activity of protein phosphatases in neuroblastoma B50, cardiomyoblast H9C2 and hepatocarcinoma HepG2 cells implied that the *cell type specific association of phosphatase inhibitors with membrane lipids may influence their inhibitory potencies exerted on intact cells* [6].

The interaction of protein phosphatases with cellular proteins is an important aspect of the regulation of their enzyme activity and specificity. Microcystin-LR (MC-LR), a potent inhibitor of PP1 and PP2A, was biotinylated, immobilized to streptavidin-coated sensorchip surface and used in surface plasmon resonance (SPR) binding experiments to isolate phosphatase binding proteins from HaCaT cell lysate (***Bécsi et al. J. PHOTOCHEM. PHOTOBIOL. B 138C 240-248. 2014***) [7]. Biotin-MC-LR captured PP1c, PP2Ac and many of their regulatory proteins including MYPT1, MYPT family TIMAP, inhibitor-2 as well as PP2A A- and B-subunits from normal and UVA-irradiated HaCaT cell lysates as revealed by dot blot analysis of the recovered proteins. *Our results imply that biotin-MC-LR is a suitable capture molecule in SPR for isolation of protein phosphatase interacting proteins from cell lysates in sufficient amounts for immunological detection* [7].

In collaboration with other groups we compared the inhibition of mammalian protein phosphatase 1 (PP1) and Candida (*C. albicans*) PPZ1 using microcystin-LR (MC-LR) and inhibitor-2 (I2). We applied phosphorylated 20 kDa myosin light chain (P-MLC20) as substrate for both PP1 and PPZ1, for the latter it was identified as an excellent novel substrate (***Chen et al. mBIO 7(4):e00872-16. doi:10.1128/mBio.00872-16.***) [8]. We showed that PPZ1 was active with P-MLC20 and also was as susceptible to inhibition by MC-LR and I2 as PP1, its most similar human homolog. Despite its 66% sequence identity of PPZ1 to PP1, the catalytic domain of PPZ1 contains novel structural elements that are not present in PP1. These structural differences may imply possibilities for the development of novel drugs that specifically target *C. albicans* without influencing human phosphatases. In another PPZ1 related project we established a P-MLC20 based activity assay to prove the regulatory and inhibitory features of two C. albicans gene product (CaHal3 and CaCab3) on the catalytic activity of Candida PPZ1 (***Petrényi et al. PLoS ONE 11(8): e0160965***) [9].

We also determined changes of PP1 and PP2A activities upon treatment of *Vicia faba* with MC-LR and showed that mitotic spindle anomalies induced by MC-LR paralleled with decrease in the catalytic activity of these two types of phosphatase (***Garda et al. JOURNAL OF PLANT PHYSIOLOGY 199, 1–11. 2016***) [10].

Recognition of novel phosphatase inhibitor proteins

In collaboration research we identified the ankyrin domain of myosin 16 (My16Ank) as a PP1c binding protein (***Kengyel et al. EUR BIOPHYS J 44:207–218. 2015***) [11]. Binding of different isoforms of PP1c (PP1cα and PP1cδ) to My16Ank was proved by surface plasmon resonance (SPR) based binding studies and in phosphatase activity assays using phosphorylated myosin light chain as substrate. *My16Ank inhibited both skeletal muscle and recombinant PP1c. Further studies are necessary to uncover the physiological significance of this interaction and phosphatase inhibition.*

Relating to the role of protein phosphatases in leukemic cells we tested the presence of phosphorylated CPI-17 (C-kinase phosphorylated inhibitor of 17 kDa), a PP1 inhibitory phosphoprotein in these cells and found relatively low level of phospho-CPI-17. However, several other proteins in higher molecular mass range (65-75 kDa) cross-reacted with the anti-phospho-CPI17 antibody (**Fig. 1**).

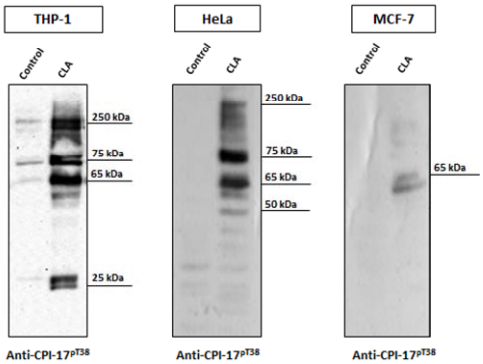


Figure 1. Presence of CPI-17 related phosphoproteins in different cancerous cell lines. Phosphorylation of CPI-17-like proteins was tested in the absence and the presence of 50 nM CLA. Several proteins in higher molecular mass range were detected, that cross-reacted with the anti-phospho-CPI-17 antibody and their phosphorylation increased markedly upon CLA treatment. Our results show that the amount of CPI-17-like proteins varies between the different cell lines.

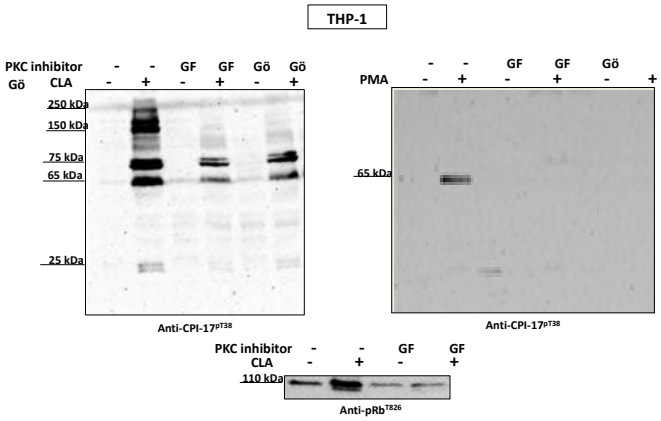


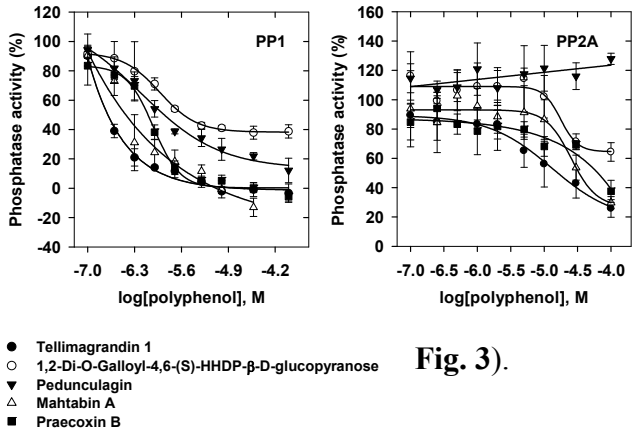
Figure 2. Effect of PKC inhibitors on the phosphorylation of CPI-17-like proteins and retinoblastoma protein in THP-1 cells treated with CLA or PMA. Cells were pretreated with PKC inhibitors (5 μM GF109203x or 0.2 μM Gö6976) for 30 minutes, then treated with 50 nM CLA or 100 nM PMA (an activator of PKC) for 30 minutes. PKC inhibitors in combination with CLA decreased the phosphorylation of CPI-17-like proteins. PMA alone increased the phosphorylation of the 65 kDa protein only, and this effect was completely eliminated in the presence of PKC inhibitors. Combined treatment with GF and CLA decreased significantly the stimulatory effect of CLA on pRb^{T826} phosphorylation.

The phosphorylation of these proteins increased markedly upon treatment with CLA, while inhibition of protein kinase C (PKC) decreased this CLA-induced phosphorylation, suggesting the roles of PP2A and PKC in these phosphorylation events. As LIMK2 showed significant sequence similarity to CPI-17, therefore the 75 kDa protein may be this enzyme. Accordingly, the phosphatase activity of PP1 was inhibited by unphosphorylated Flag-LIMK2 slightly, while the phosphorylated Flag-LIMK2 decreased the activity of PP1 markedly in a concentration dependent manner. The cellular influence of LIMK is still, however, doubtful since among the investigated cell lines the 75 kDa LIMK including the CPI17-like phosphorylation sequence present only in HeLa cells, while similar phosphorylated band is still also identified in THP-1 cells where this isoform is absent. Nevertheless, our results suggest that LIMK2 may be a novel inhibitor of PP1 and it may mediate the phosphorylation level of proteins important in the regulation of tumorigenesis. It is also apparent that PKC inhibitors suppress the phosphorylation of the 65 and 75 kDa proteins in THP-1 cells only partially (**Fig. 2**) and PKC activation by PMA treatment increased the phosphorylation level of the 65 kDa protein, but not the 75 kDa. *The goal of our future*

studies to identify these phosphatase inhibitor candidate proteins and characterize their cellular roles in more details.

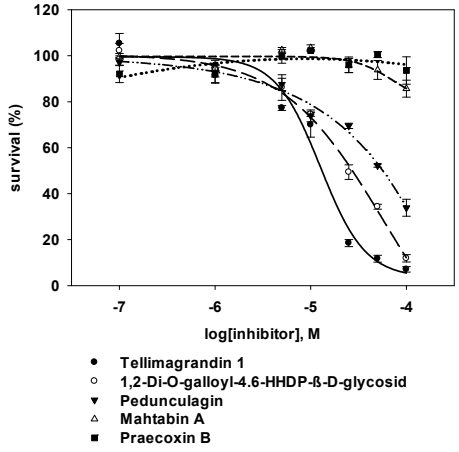
Identification of novel phosphatase inhibitory molecules

Our recent studies have shown that polyphenolic molecules, such as penta-O-galloyl-β-D-glucose (PGG) or epigallocatechin-3-gallate (EGCG) and its derivatives are more potent inhibitors of PP1c than that of PP2Ac. We have also pointed out that these compounds exert their inhibitory effects by interacting with part of the hydrophobic groove in PP1c [12]. We studied other polyphenolic molecules in order to find more effective and selective PP1 inhibitor. We assayed the inhibitory potency and the cell death inducing effects of gallic acid coupled glucose derivatives (tellimagrandin 1, praecoxin B, mahtabin A, pedunculagin, 1,2-Di-O-Galloyl-4,6-HHDP-β-Dglucose). Tellimagrandin 1 (IC50=0.1-0.22 μM) proved to be the most potent inhibitor of PP1, while the inhibitory potency of other derivatives (praecoxin B, mahtabin A, pedunculagin or 1,2-Di-O-Galloyl-4,6-HHDP-β-D-glucoside) varied (0.33-4 μM). Inhibition of PP2Ac occurred at ~100-fold higher IC50 values for each derivative than that of determined for PP1c suggesting a reasonable selectivity of these compounds toward PP1c (**see Fig. 3**).



IC50/LD50 (μM)	PP1c native	PP2Ac native
tellimagrandin 1	0.197 ± 0.023	19.52 ± 8.6
Praecoxin B	0.788 ± 0.111	63.86 ± 5.516
Mahtabin A	0.413 ± 0.186	33.77 ± 8.44
Pedunculagin	2.473 ± 0.216	> 100
1.2-Di-O-galloyl-4.6-(S)-HHDP-β-D-glucopyranose	1.41 ± 0.296	> 100

Figure 3. The effect of polyphenols on the activity of PP1c and PP2Ac. PP1c or PP2Ac was incubated inthe absence or the presence polyphenols (0.1–100 μM) for 10 minutes at 30 °C. The dephosphorylation reaction was started by the addition of 1 μM phosphorylated (^{32}P-labelled) 20 kDa myosin light chain (^{32}P-MLC20) and further incubated for 10 minutes. Phosphatase activity was determined as the released $^{32}P_i$ and was taken as 100% in the absence of polyphenols.



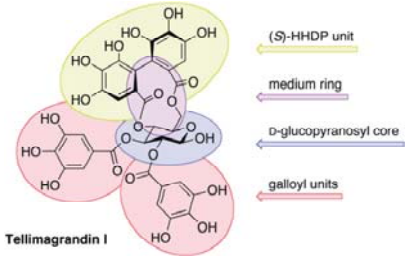


Figure 4. The effect of polyphenols on the viability of HeLa cells.

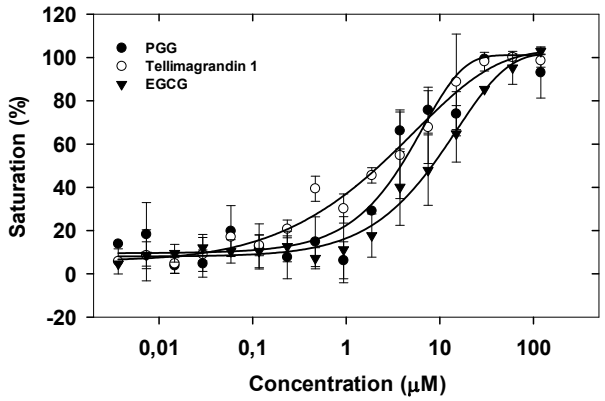


Figure 5. Comparision of the binding affinity of tellimagrandin 1, PGG and EGCG to PP1c determined by microscale thermophoresis. K_D = 1.78 ± 0.59 μM; PGG: K_D = 3.08 ± 0.35 μM; EGCG: 8 2 ± 4.3 μM.

It is interesting that there is no direct relation between the phosphatase inhibitory and cell death inducing effect of the polyphenols. For instances, Mahtabin A is a good phosphatase inhibitor, however, it hardly influences the viability of HeLa cells (**Fig. 4**). Binding affinities to PP1c of previously identified phosphatase inhibitors such as PGG and EGCG as well as tellimagrandin 1 were determined by

microscale thermophoresis (MST) method and compared *suggesting effective binding of tellimagrandin 1 to PP1as an inhibitor* (**Fig. 5**). The interaction of PGG with PP1c was further verified by surface plasmon resonance (SPR), isothermal titration calorimetry (ITC) and NMR-saturation difference (STD) based binding studies (**Fig. 6**).

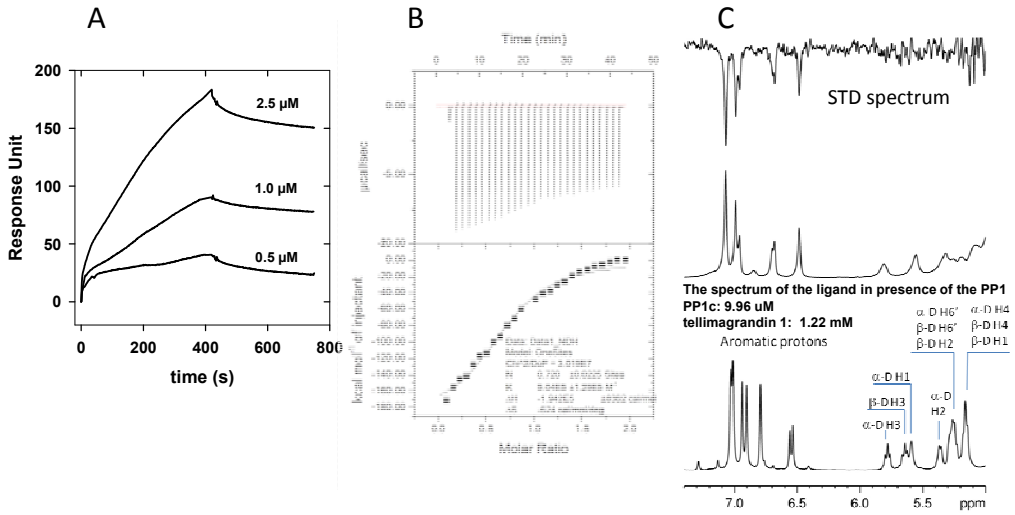


Figure 6. Interaction of tellimagrandin 1 with PP1c as revealed by SPR, ITC and NMR-STD studies. A: SPR binding studies were carried out on sensorchip with immobilized PP1c injecting 0.5, 1 and 2.5 µM tellimagrandin 1 over the surface. B:ITC was carried out with 1 µM PP1c titrated with 20 µM of tellimagrandin 1. C: NMR spectrum of tellimagrandin 1 in the absence and presence of PP1c.

The dissociation constants for PP1-tellimagrandin 1 interaction were K_D=0.31 µM and K_D=1.1 µM determined by SPR and ITC, respectively. From the NMR-STD spectrum it could be realized that based on the STD signals (for the aromatic protons) *there is hydrophobic interaction between PP1c and tellimagrandin 1 in which the substrate binding hydrophobic groove of PP1c and the aromatic rings of the molecule could be involved. Tellimagrandin 1 has a free glycosidic hydroxyl, implying that its modification with gallic acid (like in PGG) on this hydroxyl is unnecessary for the inhibitory function. Thus, direct modification of the glycosidic hydroxyl in tellimagrandin 1 with bifunctional reactant(s) would result in a molecule which is conjugated with other phosphatase inhibitory molecules such as with peptides for example shown below (**Figs 7-8**).*

MYPT1 peptide:
NH2-RQSRRSTQGVTL-COOH
MYPT1-phosphopeptide :
NH_2-RQSRRS-Thr(PO_3H_2)-QGVTL-COOH

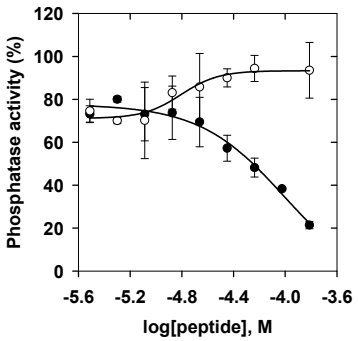


Figure 7. Effect of MYPT1 inhibitory peptide on the activity of PP1c: nonphospho- (○) and phospho- (●) peptide.

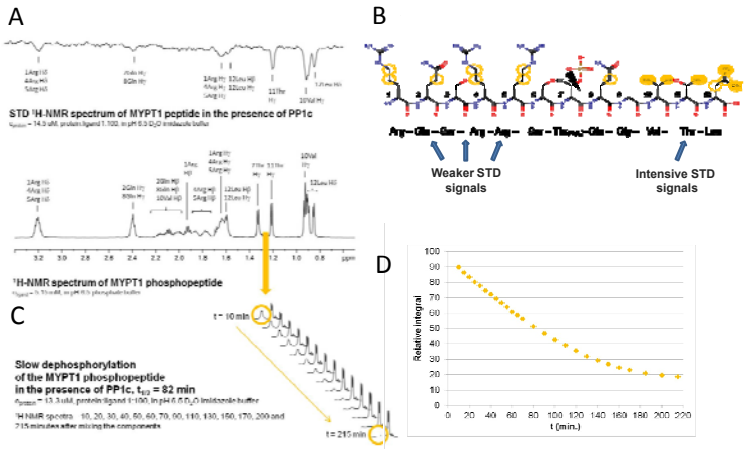


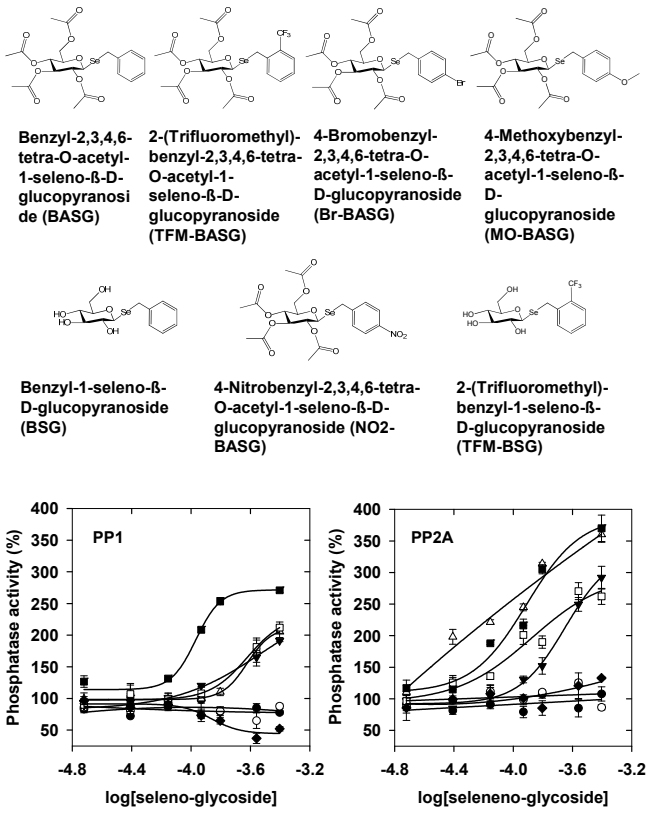
Figure 8. Interaction of MYPT1 phosphopeptide with PP1c as revealed by NMR-STD studies. A: NMR spectrum for the peptide and its STD spectrum in the presence of PP1c. B: Identification of amino acids with STD signals. C, D: Determination of the half-life of phosphopeptide during NMR studies.

In

search for such peptides that could be conjugated with tellimagrandin 1 we assayed the effect of non-phosphorylated and phosphorylated forms of an inhibitory peptide (see **Fig. 7**) derived from the MYPT1 regulatory subunit of myosin phosphatase. It is apparent that only the phosphorylated peptide is inhibitory and the IC_{50}=52 μM for the inhibition. This inhibitory peptide includes both hydrophobic and charged (basic) regions, therefore its interaction with PP1c may occur via both the hydrophobic and acidic grooves. These binding features were checked by NMR-STD studies recording the ^{1}H-NMR spectrum of the peptide in the absence and in the presence of PP1c (**Fig. 8**). Strong STD signals for the hydrophobic amino acids and somewhat weaker for the basic residues were identified. Another important observation was that the phosphopeptide was dephosphorylated by PP1c during the NMR assay with a half-life of 72 min. *These results suggest that both the nonphospho- and phosphopeptide interact with PP1c, however, only the phosphopeptide is inhibitory implying the essential role of phosphate in the inhibition. The results also imply that the PP1 inhibitory proteins/peptides actually act as an inhibitor being "a bad substrate" for the enzyme.*

Taken together the result with the polyphenols and the MYPT1 inhibitory peptide the original research plan was thought over with respect to the conjugation of PP1 inhibitors with peptides. Although the peptide and tellimagrandin 1 did not compete for inhibition or were not synergistic in inhibition either (their inhibitory effect was additive, not documented) the requirement of phosphorylation of the peptide and the slow dephosphorylation of the peptide by PP1c complicated the plans for conjugation. First, the conjugation reaction might result in many products due to the hydroxyls present on the aromatic rings beside the glycosidic one. Secondly, the conjugation would require a peptide with no hydrolysable phosphate for effective and maintained inhibition, but obtaining the necessary phosphate derivative is quite expensive. For the above reasons, the synthesis of phosphatase inhibitors by conjugation of polyphenols with peptide was not hardly pursued according to the original plans because of the indefinite outcome. *The results of the studies with polyphenols and MYPT1 peptide are prepared for publication and the manuscript(s) is planned to be submitted in the near future.*

Novel developments: identification of phosphatase activators



In the course of studies in search of glucose containing phosphatase inhibitors (as PGG and tellimagrandin) we assayed aromatic selenoglycoside derivatives for influencing the activity of PP1 and PP2A. It led to an interesting discovery, namely, that most of the assayed selenoglycosides proved to be activators of PP1c and PP2Ac (**Fig. 9**). It is well documented that inhibition of protein phosphatases by protein inhibitors/regulatory subunits favors cancer cell survival [13, 14] while activation of these enzymes induces cell death [15]. Therefore, selenoglycosides arised as model compounds for "drug candidate" phosphatase activators. Selenoglycoside derivatives assayed activated both PP1c and PP2Ac when the glucose hydroxyls were acetylated, but they were without effects in the non-acetylated forms.

Figure 9. The effect of the benzyl-seleno-glycosides on the activity of PP1c an PP2Ac. BSG (●); TFM-BSG (○); BASG (▼); TFM-BASG (Δ); Br-BASG(■); MO-BASG(□); NO_2-BASG(♦).

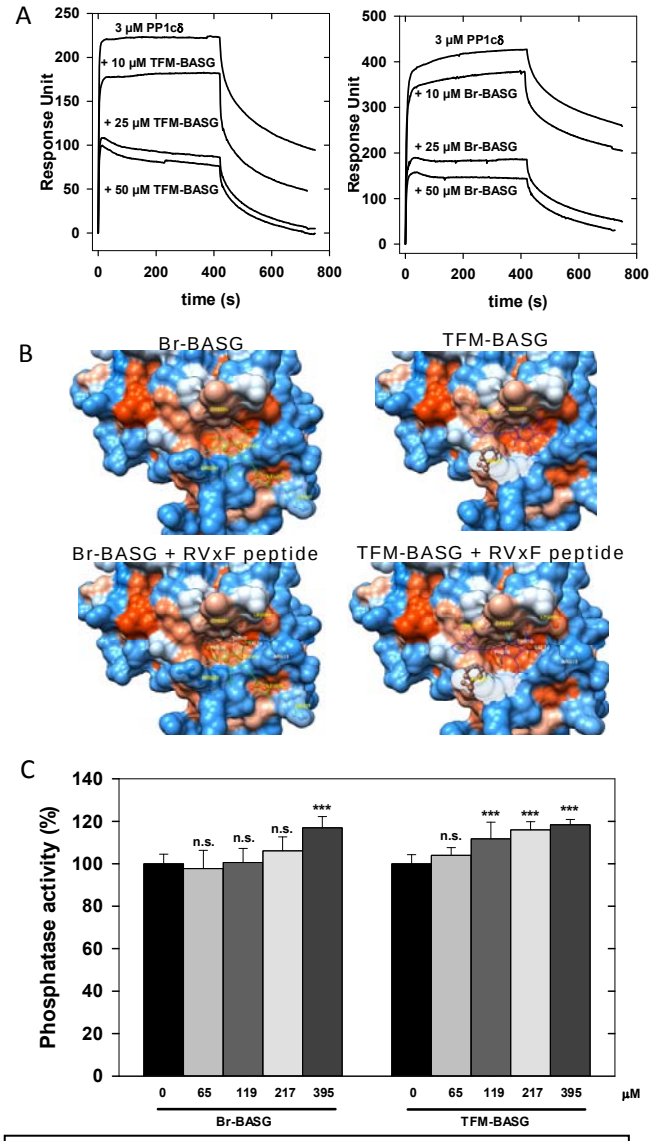


Figure 10. Interaction of selenoglycosides with PP1c and ther effect on the phosphatase activity of HeLa cells. A: Selenoglycosides prevent binding of PP1c on a GST-MYPT1 surface in SPR binding experiments. B: Docking of selenoglycosides on the RVxF motif binding surface of PP1c. C: Effects of selenoglycosides on the phosphatase activity of HeLa cells.

Among the selenoglycosides 2-trifluoromethylbenzyl- 2,3,4,6-tetra-O-acetyl-1-seleno-β-Dglucopyranoside (TFM-BASG) and 4-bromobenzyl-2,3,4,6-tetra-O-acetyl-1-seleno-β-D-glucopyranoside (Br-BASG) proved to be the most effective activators (EC50~80-120 μM). The interaction of Br-BASG and TFM-BASG with PP1c was confirmed by microscale thermophoresis and K_d values of 44.1 μM and 2.5 μM were determined. Both Br-BASG and TFM-BASG prevented binding of PP1c to MYPT1 in surface plasmon resonance based binding experiments (**Fig. 10**), and they also attenuated inhibition of PP1c by inhibitor-2 (not documented). Molecular modelling studies docked Br-BASG and TFM-BASG on the binding surface of PP1c (**Fig. 10B, upper part**) for the RVxF binding motifs of regulatory/inhibitory subunits (including MYPT1) and identified partial overlap in the binding of selenoglycosides and an RVxF binding peptide (**Fig. 10B, lower part**).

In addition, Br-BASG and TFM-BASG also caused a slight, but significant increase in the phosphatase activity of HeLa cells (**Fig. 10C**). These data suggest that selenoglycosides are able to activate PP1 and PP2A holoenzymes, however, the slight activation indicates that the stimulation of PP1c and PP2Ac are presumably hindered when they are associated with regulatory subunits. Another reason for the slight activation could be the low membrane permeability of the selenoglycosides.

Our results suggest that hydrophobic selenoglycosides stimulate PP1c activity by interacting with the surface on PP1c which is essential for binding the PP1c-binding motifs (RVxF) in interactor proteins. PP2Ac might also include a similar binding site for selenoglycosides. Gaining further insight into the structural background of PP1c-selenoglycoside interaction may provide useful tools for planning and synthesizing effective phosphatase activator molecules. Selenoglysosides have limited aqueous solubility and they are toxic. Our furthe plan is to screen molecular library to identify similar families of inhibitors with higher effectiveness.

This part of the research project is in the phase of manuscript preparation and submission.

II. To reveal the functions and roles of myosin phosphatase/PP1M and ROK at pre- and/or postsynaptic locations in the regulation of exocytosis and neurotransmitter release in synaptosomes and PC12 cells

Myosin phosphatase/PP1M and ROK in the regulation of exocytosis and neurotransmitter release in synaptosomes and PC12 cells

We investigated the effect of RhoA-activated kinase (ROK) and MP/PP1 on the phosphorylation of one potential neuronal substrate, the synaptosomal-associated protein of 25 kDa (SNAP-25). SNAP-25 is a

member of the SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) complex, along with synaptobrevin and syntaxin, and the primary role of SNAP25 is to mediate vesicle fusion. We showed that MYPT1 interacts with SNAP-25, as revealed by immunoprecipitation and surface plasmon resonance based binding studies (**Horváth et al. *PLoS ONE* 12 (5), e0177046.**) [16]. Mass spectrometry analysis and in vitro phosphorylation/dephosphorylation assays demonstrated that ROK phosphorylates, while MP dephosphorylates SNAP-25 at Thr138. Silencing MYPT1 in B50 neuroblastoma cells increased phosphorylation of SNAP-25 at Thr138. Inhibition of PP1 with tautomycetin increased, whereas inhibition of ROK by H1152, decreased the phosphorylation of SNAP-25 at Thr138 in B50 cells, in cortical synaptosomes, and in brain slices. In response to the transduction of the MP inhibitor, kinase enhanced PP1 inhibitor (KEPI), into synaptosomes, an increase in phosphorylation of SNAP-25 and a decrease in the extent of neurotransmitter release were detected. The interaction between SNAP-25 and syntaxin increased with decreasing phosphorylation of SNAP-25 at Thr138 upon inhibition of ROK. *Our data suggest that ROK/MP play a crucial role in vesicle trafficking, fusion, and neurotransmitter release by oppositely regulating the phosphorylation of SNAP-25 at Thr138.*

In accordance with the original aims we also tested the effects of the newly identified phosphatase inhibitory polyphenols on the exocytosis of synaptosomes.

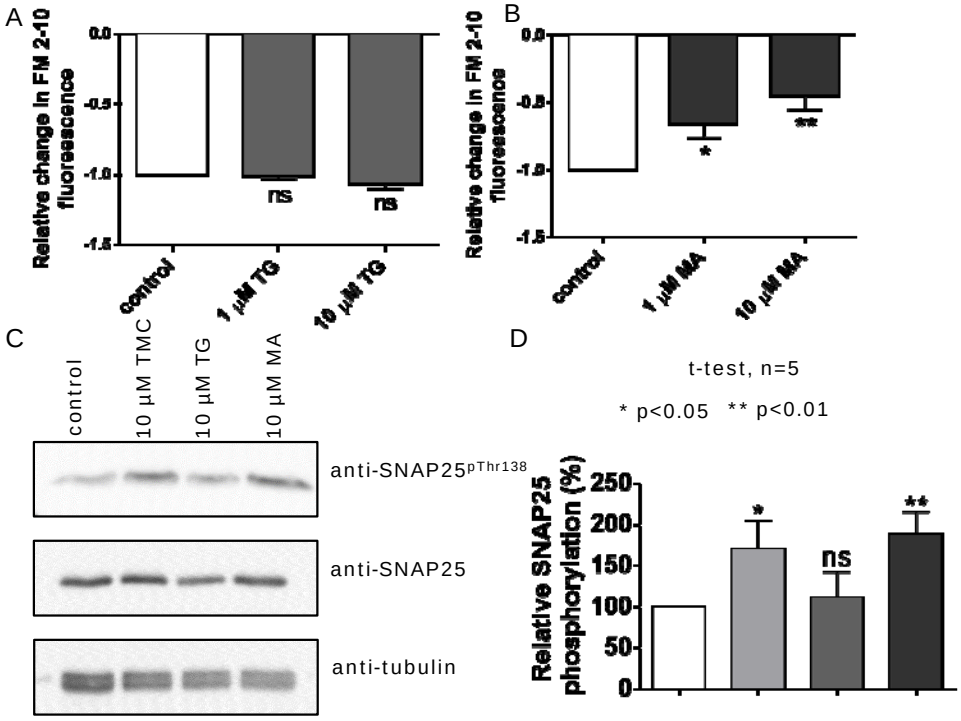


Figure 11. Effect of tellimagrandin 1 (TG) and mahtabin A (MA) on the exocytosis of cortical synaptosomes (A and B), and on the phosphorylation of SNAP-25 at Thr138 (C and D).

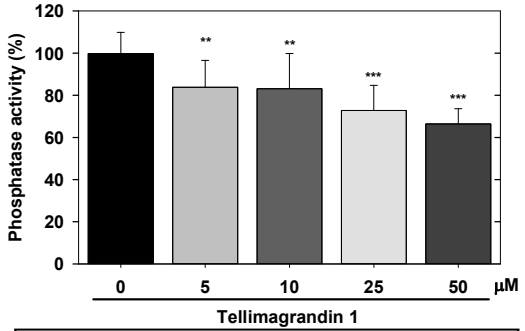


Figure 12. The phosphatase activity of THP-1 cells after 1 hour treatment with tellimagrandin 1.

It is interesting that tellimagrandin 1 (TG), which proved to be the most potent polyphenolic inhibitor was without effect on synaptosome exocytosis, while mahtabin A (MA) decreased the extent of exocytosis in a concentration dependent manner. In addition, MA increased the phosphorylation level at Thr138 in SNAP-25, similarly to tautomycin (TM) as revealed in our previous studies [16], while TG had no influence. It was previously shown [16] that increased phosphorylation of SNAP-25^{Thr138} is coupled with decreased exocytosis of synaptosomes.

The difference in the action of TG and MA is not understood yet. TG induced cell death of HeLa cells, while MA was without effect (see **Fig. 4** in part I). To complicate to get straightforward conclusion TG was found to inhibit phosphatase activity in THP-1 cells (**Fig. 12**) and according to our preliminary results it was concomitant with increased phosphorylation level of JNK and induction of cell death (not shown).

Neurotransmitter release from PC12 cells will be discussed in the following section (part III).

Myosin phosphatase/PP1M and ROK in the regulation of other cellular processes

We also investigated the role of myosin phosphatase/PP1M and ROK in cellular processes distinct from the neuronal ones. We identified the protein arginine methyltransferase 5 (PRMT5) enzyme of the methylosome complex as a MYPT1-binding protein uncovering the nuclear MYPT1-interactome of hepatocellular carcinoma cells (***Sipos et al.. SCIENTIFIC REPORTS 7, 40590. 2017***) [17]. It is shown that PRMT5 is regulated by phosphorylation at Thr80 by RhoA-associated protein kinase and MP. Silencing of MYPT1 increased the level of the PRMT5-specific symmetric dimethylation on arginine residues of histone 2 A/4, a repressing gene expression mark, and it resulted in a global change in the expression of genes affecting cellular processes like growth, proliferation and cell death, also affecting the expression of the retinoblastoma protein and c-Myc. The phosphorylation of the MP inhibitory $MYPT1^{Thr850}$ and the regulatory $PRMT5^{Thr80}$ residues as well as the symmetric dimethylation of H2A/4 were elevated in human hepatocellular carcinoma and in other types of cancers. These changes correlated positively with the grade and state of the tumors. *Our results suggest the tumor suppressor role of MP/PP1M via inhibition of PRMT5 thereby regulating gene expression through histone arginine dimethylation.*

III. To investigate the physiological relevance of protein phosphorylation and protein-protein interactions in neurotransmission: morphological and electrophysiological studies of neurons.

Protein-protein interaction and its role in neuronal and other cellular processes

The major goals of the study were to investigate the role of protein-protein interaction in neurotransmission and in other cellular processes focusing mainly PP1M and its MYPT1 subunit, PP2A, PP2B, nitric oxide synthases (eNOS, nNOS) and CaM-kinase II.

We found before that CsA induced increase in intracellular Ca^{2+} in endothelial cells influencing plasma membrane Ca^{2+}-channel(s) [18] and we planned to investigate this phenomenon also on brain slices. However, from the experimental data we obtained on cyclosporine A stimulation on brain slices suggested only slight effect on Ca^{2+} transients (results are not shown), and even these influences were exerted on a mixed population of cells (i.e. besides the neurons on astrocytes, too). Experiments on this research topic were discontinued.

As a model system and a prelude to the neuronal studies we investigated the interaction of endothelial NOS (eNOS) and myosin phosphatase (MP)/PP1M, especially focusing for the role of eNOS-MYPT1 interaction and the dephosphorylation of eNOS by MP/PP1M (**Bátori, R. et al. SCIENTIFIC REPORTS. 7, 44698; 2017.**) [19]. Inhibitory phosphorylation of endothelial nitric oxide (NO) synthase (eNOS) at Thr497 ($eNOS^{pThr497}$) by protein kinase C or RhoA-activated kinase is a major regulatory determinant of eNOS activity. The signaling mechanisms involved in the dephosphorylation of $eNOS^{pThr497}$ have not been clarified yet. We showed that the MP holoenzyme consisting of PP1c and MP target subunit-1 (MYPT1) was an $eNOS^{pThr497}$ phosphatase. In support of this finding were: (*i*) eNOS and MYPT1 interacted in various endothelial cells (ECs) and in in vitro binding assays (*ii*) MYPT1 targeted and stimulated PP1c toward $eNOS^{pThr497}$ substrate (*iii*) phosphorylation of MYPT1 at Thr696 ($MYPT1^{pThr696}$) controlled the activity of MP on $eNOS^{pThr497}$. Phosphatase inhibition by CLA (at a PP2A inhibitory concentration) suppressed both NO production and transendothelial resistance (TER) of ECs suggesting an indirect role of PP2A via dephosphorylation of $MYPT1^{pThr696}$ in these physiological events.

The characterization of eNOS/myosin phosphatase/PP2A axis in ECs served as a model to apply similar studies on the possible interaction of neuronal NOS (nNOS) with MYPT1 as well as

phosphorylation of inhibitory (nNOS^{Ser852}) and activatory (nNOS^{Ser1417}) phosphorylation sites in nNOS corresponding to the sites eNOS^{Thr497} and eNOS^{Ser1179} in eNOS, respectively.

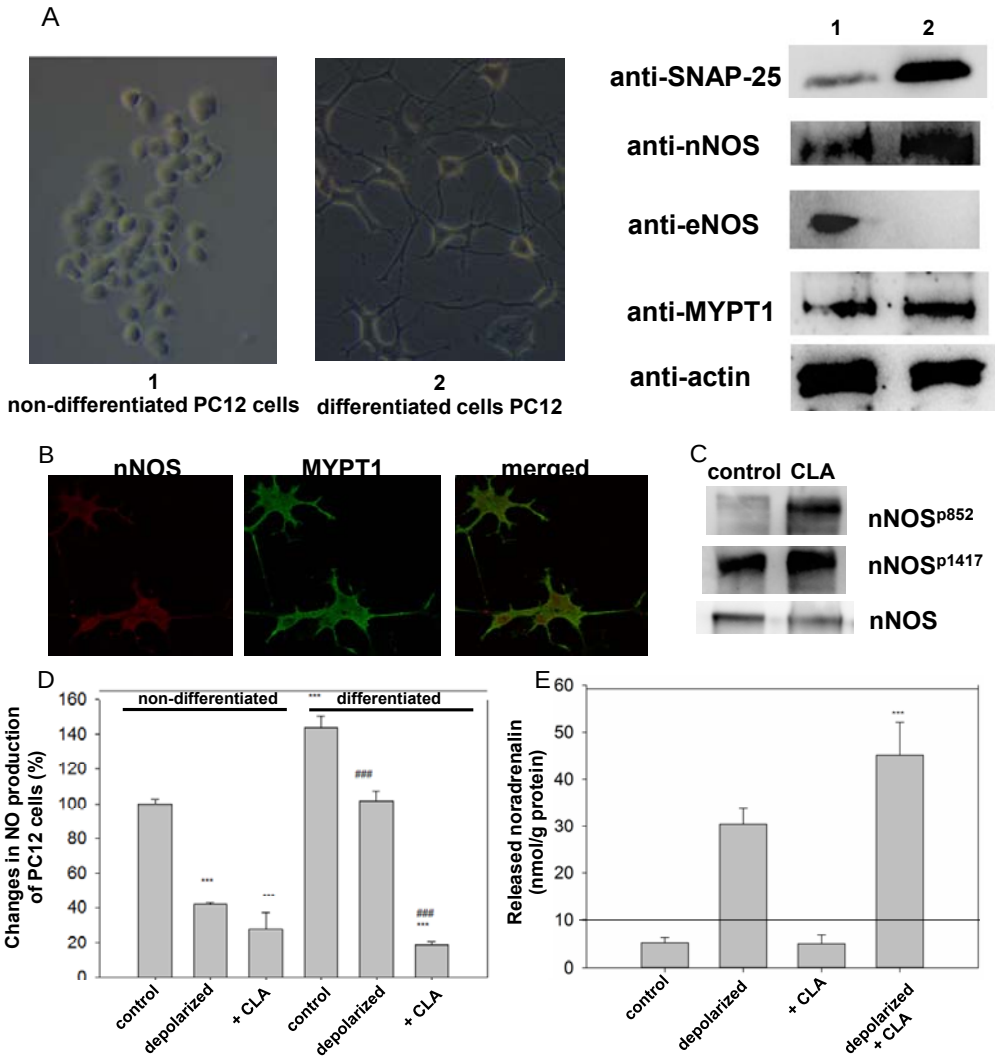


Figure 13. Preliminary results with PC12 cells for differentiation (A), interaction of nNOS and MYPT1 (B), and the effect of phosphatase inhibitor CLA on nNOS phosphorylation (C), NO production (D) and noradrenalin release (E).

First, we investigated how the expression of key proteins changed during PC12 cell differentiation (**Fig. 13A**). An important difference between non-differentiated and differentiated cells was that while non-differentiated cells contained both eNOS and nNOS, in the differentiated ones only nNOS was identified. SNAP-25, a key component of vesicle fusion during exocytosis, was significantly higher in differentiated than in non-differentiated cells, while MYPT1 was present in both cells at comparable amounts. In addition, in differentiated cells partial colocalization of MYPT1 and nNOS was observed (**Fig. 13B**) by confocal microscopy suggesting that nNOS might interact with MYPT1 in similar manner as it was identified in case of eNOS. It is also apparent that the inhibitory sites in nNOS (eNOS^{Ser852}) became phosphorylated upon CLA treatment, while nNOS^{pSer1417} was only slightly influenced (**Fig. 13C**). In accordance with the increased inhibitory phosphorylation CLA suppressed NO production in both cells (**Fig. 13D**), while it has a positive effect on noradrenalin release (**Fig. 13E**). Of course, no farfetched conclusions could be drawn from these preliminary experiments with respects to the effect of MYPT1 and nNOS phosphorylation on noradrenalin release in PC12 cells. However, the establishment of the key experimental setups and the initial results are promising for future experimentation to gather more knowledge on the role of these proteins in neurotransmitter release.

Novel developments: identification of signaling pathway for phosphatase activation

Phosphatase inhibition suppresses both NO production and transendothelial resistance (TER) of endothelial cells (ECs) implying that phosphatase activation may have opposite effects. We have identified epigallocatechin-3-gallate (EGCG) as a physiological activator of protein phosphatases in endothelial cells (**Bátori, R. et al. SCIENTIFIC REPORTS. 7, 44698; 2017.**) [19]. EGCG signals ECs via the 67 kDa laminin-receptor (67LR) resulting in protein kinase A (PKA) dependent activation of protein phosphatase-2A (PP2A). PP2A dephosphorylates MYPT1^{pThr696} (a MP inhibitory phosphorylation site) and thereby stimulates MP activity inducing dephosphorylation of eNOS^{pThr497} and the 20 kDa myosin II light chains. Phosphatase activation by EGCG is not observed in ECs when the catalytic subunit of PKA is silenced. *Our data suggest that an interplay of MP and PP2A is involved in the physiological regulation of EC functions implying that an EGCG dependent activation of these phosphatases in a PKA dependent manner leads to enhanced NO production and EC barrier improvement.*

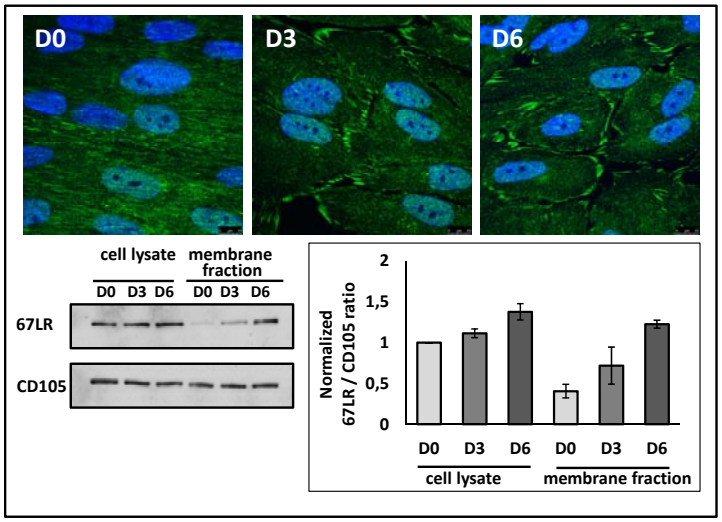


Figure 14. The localization of 67LR in MSCs on day 0., 3. and 6. of the adipogenic differentiation. Confocal microscope images show that 67LR translocated to the cell membrane during the adipogenic differentiation ofMSCs, which was confirmed by Western blot experiments on subcellular fractions.

The effect of EGCG was also investigated on the adipogenic differentiation of chorion-derived human mesenchymal stem cells (MSCs). The adipogenesis is stimulated by the enhancer binding proteins (C/EBPs) and the peroxisome proliferator-activated receptors (PPARs). The activity of these proteins is largely dependent on their phosphorylation state. PP2A is able to dephosphorylate C/EBPβ thereby can regulate the adipogenic process. We have proved that EGCG suppresses the lipid deposition and decreases the expression of the major transcriptional factors during the differentiation. 67LR is localized in the cytoplasm and the nucleus in undifferentiated MSCs, but it translocates to the cell membrane during adipogenic differentiation (**Fig. 14**). Phosphatase activity assays confirm that the PP2A activity increases while the phosphorylation level of C/EBPβ decreases in the presence of EGCG following the membrane localization of 67LR, but these changes are not observed in the presence of a PKA inhibitor. *These data suggest that EGCG influences the adipogenic differentiation of MSCs via binding to 67LR inducing PP2A activation and changes in the phosphorylation level and expression of key adipogenic transcription factors. It also directs attention to the importance of receptor translocation in these signaling events.*

PhD and students' scientific activities within this research project

On this research project 4 PhD students worked (*Bálint Bécsi, Dóra Dedenszki, Róbert Bátori* and *Zoltán Kónya*) under the supervision of the Principal Investigator (**Ferenc Erdődi**) and during the research period *Bálint Bécsi* (2014) and *Dóra Dedinszki* (2015) obtained PhD degree. *Róbert Bátori* and *Zoltán Kónya* have finished necessary exams and they are in the phase of writing their thesis (thesis defenses are planned at the beginning of 2018). With the supervision of **Beáta Lontay** (her students are *Adrienn Sipos, Daniel Horváth* and *István Tamás*) and **Andrea Kiss** (her student is *Emese Tóth*), participating Senior Researchers, the indicated PhD students have also worked on this project and *Adrienn Sipos* (2017) received PhD degree. *Andrea Reszegi*, a molecular biology MSc student, completed students' scientific research within the project (advisors are **Ferenc Erdődi** and **Zoltán Kónya**) and had presentation on the local conference in 2016 (recognized with a special prize) and wrote a student scientific paper which was recognized as her diploma thesis defense and fulfillment to her final examination.

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