The effects of chemically induced TrkA expression alteration in PC12 cells

Ph.D. thesis

Gergely Berta M.D.

Head of the doctoral school: Balázs Sümegi Ph.D., DSc
Head of the program: József Szeberényi M.D., Ph.D., DSc
Supervisor: György Sétáló, Jr. M.D. Ph.D.

University of Pécs
Medical School
Department of Medical Biology

2014.

Abstract

Our work's aim was to explore mechanisms that could explain how the chemical inhibition of heat-shock protein 90 reduces NGF (nerve growth factor) signaling in rat pheochromocytoma PC12 cells. Geldanamycin is an antibiotic originally discovered based on its ability to bind heat-shock protein 90. This interaction can lead to the disruption of heat-shock protein 90-containing multimolecular complexes. It can also induce the inhibition or even degradation of partner proteins dissociated from the 90 kDa chaperone and, eventually, can cause apoptosis, for instance, in PC12 cells. Before the onset of initial apoptotic events, however, a marked decrease in the activity of extracellular signal-regulated kinases ERK 1/2 (extracellular signal-regulated kinase 1 and 2) and protein kinase B/Akt can be observed together with reduced expression of the high affinity nerve growth factor receptor, tropomyosine-related kinase, TrkA (tropomyosin receptor kinase, frequently: tropomyosin-related kinase), in this cell type. The proteasome inhibitor MG-132 can effectively counteract the geldanamycin-induced reduction of TrkA expression and it can render TrkA and ERK1/2 phosphorylation by nerve growth factor again inducible. This latter effect was not observed in the case of protein kinase B/Akt. The altered intracellular distribution of TrkA that we have found in geldanamycin-treated and proteasome-inhibited PC12 cells may, at least partially explain why nerve growth factor remains without effect on protein kinase B/Akt in this case. The lack of protein kinase B/Akt stimulation by nerve growth factor in turn reveals why nerve growth factor treatment can't save PC12 cells from geldanamycin-induced programmed cell death. Our observations can help to better understand the mechanism of action of geldanamycin, the derivative compounds of which possess strong human therapeutical potential.

Introduction

Geldanamycin and the Hsp90

Geldanamycin is a member of the ansamycin antibiotic family originally discovered in Streptomyces bacteria. Heat-shock protein 90 (Hsp90), an abundantly expressed, homodimeric, ATP-dependent molecular chaperone was identified as a geldanamycin target by means of affinity chromatography. As part of multimolecular complexes Hsp90 maintains a number of proteins, including signaling kinases, in their functionally correct conformation.

The Hsp90 based complexes can bind a high variety of substrate proteins, the most widely known are the steroid receptors (which are released form the chaperone complex after the ligand binding and nuclear translocation of the hormone-receptor complex), and protein kinases participating in various signaling processes. Among these, based on cell type, the members of the ERK cascade are also found. Publications mention for example the Ras, Raf, and MEK proteins. The protein kinases are generally binding to the N-terminal binding site of the Hsp90 with their catalytic domain. The p23 and CDC37/p50 are essential members of the Hsp90 complexes formed this way.

Ansamycin antibiotics can efficiently compete with, and hence inhibit the ATP binding of 90 kDa chaperones. The resulting disruption of heat-shock protein and chaperone function can cause the dissociation of Hsp90-containing complexes, or it can alter their intracellular localization even compromising their stability. Binding of geldanamycin and its derivatives to Hsp90 can shift it from a refolding chaperone to one that promotes the degradation of client proteins inside Hsp90-containing complexes. Hsp90 is also capable of passive, ATP/ADP-independent chaperoning.

The Ras-ERK and protein kinase B/Akt signaling pathways in PC12 cells

The PC12 rat pheochromocytoma cell line is a popular model system to study nerve cell differentiation and survival *in vitro*. In serum-containing culturing medium PC12 cells do not require nerve growth factor (NGF) for survival, but upon addition of it they develop a sympathetic neuron-like phenotype. After binding to its ligand, TrkA trans-

/autophosphorylation and activation elicits prolonged activation of the ERK-cascade in PC12 cells, leading to differentiation.

Upon the binding of the ligand, the high affinity TrkA NGF receptor goes through dimerization and consequently creates phosphotyrosine binding sites on itself for signaling proteins possessing SH2 domains by its tyrosine protein kinase activity. As a result of this process multiple signaling ways are started, one of them is launched as adapter proteins (e.g. Shc and Grb2) activate a guanine nucleotide exchange factor (Sos) (**Figure 1.**). This protein turns on Ras, the G protein anchored to the inner surface of the cell membrane, the inactivation of which requires the operation of the GTPase activating protein or GAP. It helps Ras to hydrolyze its bound GTP back to GDP. Before this can happen, Ras, in its GTP bound, active state induces a chain of protein kinases phosphorylating each other, the ERK cascade, which belongs to the group of MAPK cascades. Its consecutive members are Raf (a MAPKKK), MEK (which belongs to the class of MAPKKs), and finally ERK (extracellular signal-regulated kinase). This latter phosphorylates target proteins in the cytoplasm, and is also translocated into the nucleus where it can activate transcription factors such as the Serum Response Factor and Ternary Complex Factor. As a result of this ERK enzymes induce gene expression changes promoting differentiation by influencing transcription. The prompt activation of early- followed by the induction of late response genes leads ultimately to phenotype changes like sympathetic neuronal differentiation.

TrkA has been shown to interact with Hsp90 in human neuroblastoma, and also in acute leukaemic cells. After the withdrawal of trophic factors the machinery of programmed cell death is activated in PC12 cells, whereas NGF stimulation of its cognate receptor tropomyosin-related kinase (TrkA) and its downstream indirect effector protein kinase B/Akt can inhibit apoptosis (**Figure 1.**). Along this pathway the activated TrkA stimulates the phosphatidylinositol 3-kinase enzyme, which catalyzes the production of a multi-phosphorylated lipid derivative, phosphatidylinositol trisphosphate (PIP_3), from phosphatidylinositol bisphosphate (PIP_2). This will lead to the phosphorylation and activation of PKB/Akt, which is one of the most important factors for promoting cell survival.

Depending on the examined cell type the result of geldanamycin treatment can be differentiation or programmed cell death. Apoptosis of PC12 cells, for example, can be induced by geldanamycin. Interestingly, this phenomenon could still be observed when NGF was present during geldanamycin treatment.

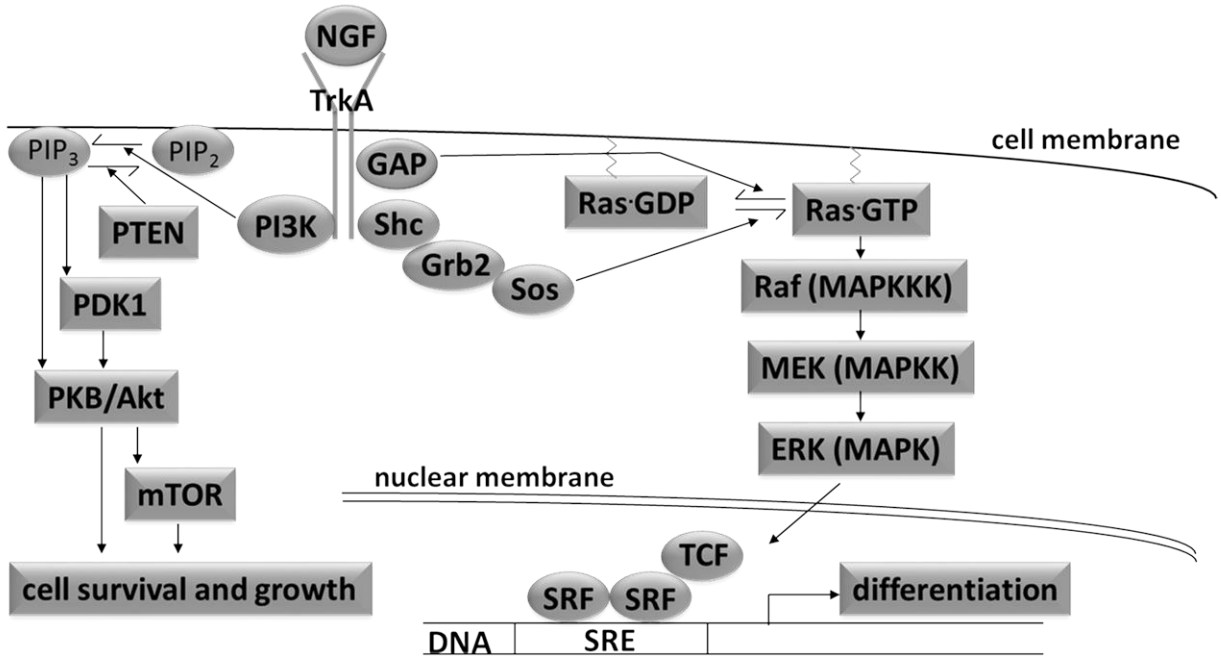

Figure 1.: General scheme of the ERK cascade and the PKB/Akt pathway. GAP: GTPase activating protein. Shc, Grb2: adapter proteins. Sos: a guanine nucleotide exchange factor. Ras: a monomeric G protein. Raf, MEK (MAPK/ERK kinase), ERK (extracellular signal-regulated kinase), PKB/Akt (protein kinase B), PDK1 (phosphoinositide-dependent kinase 1), mTOR (mammalian target of rapamycin): protein kinases. SRE: serum responsive element = an enhancer. SRF: serum responsive factor = a transcription factor. TCF: ternary complex factor = the cofactor of SRF. PI3K: phosphatidylinositol 3-kinase: a lipid kinase. PIP_3: phosphatidylinositol trisphosphate, a lipid-type second messenger molecule, generated from PIP_2 (phosphatidylinositol bisphosphate). PTEN: phosphatase and tensin homolog: a lipid phosphatase.

Objectives

In our work we have set out to identify mechanisms that could explain how geldanamycin treatment could interfere with NGF signaling via TrkA. In our PC12 cells treated with geldanamycin we aimed to study:

- the changes in the level of the high affinity NGF receptor TrkA, as an Hsp90 client protein, and the effects of these on the protein kinase B/Akt and ERK1/2 signaling pathways;
- the possible dissociation of Hsp90 complexes containing TrkA;
- the influence of the MG-132 proteasome inhibitor on the changes of TrkA concentration;
- the possibility of activating protein kinase B/Akt during treatments with geldanamycin combined with MG-132, so we could find an explanation for the apoptotic consequences of geldanamycin treatment;
- the difference of TrkA intracellular localization compared to untreated cells, which could explain the alteration of signaling processes;
- our work group further analyzed the inducing effect of the proteasome inhibitor on ERK1/2 phosphorylation.

Materials and methods

Reagents

All chemicals used were from Sigma-Aldrich (Budapest, Hungary) unless stated otherwise. NGF was purchased from Alomone Labs (Jerusalem, Israel).

Tissue culture

PC12 rat pheochromocytoma cells (kindly provided by G.M. Cooper) passage number 5 to 15) were plated onto plastic Petri dishes or Thermanox (Nalgene Nunc International, Rochester, NY, USA) coverslips (at 50% or 30% confluence, respectively) and maintained in DMEM complemented with 10% horse and 5% fetal bovine serum, for 24 hours to achieve sufficient adhesion. The cultures were kept at 37 °C in a humidified atmosphere containing 5% CO2. Next day the media were switched to 0.5 % horse serum-containing DMEM for 24 hours to silence growth factor-stimulated pathways before and during treatments.

Immunoprecipitation and Western blotting

After treatment the cultures were collected in ice-cold lysis buffer (50mM Tris-base, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM Na-orthovanadate, 5 μM $ZnCl_2$, 100 mM NaF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 1% Triton X-100) and frozen at -20 °C. Thawed samples were homogenized by vortexing for 20 seconds. The homogenate was centrifuged at 40,000 x g at 4 °C for 30 minutes and the protein concentration of the supernatant was determined (Lowry's method, Detergent Compatible Protein Assay Kit, Bio-Rad, Hercules, CA, USA). For caspase-3 activity measurements cells were lysed in Chaps cell extract buffer and processed as suggested by the antibody supplier (Cell Signaling Technology, Beverly, MA, USA).

For immunoprecipitation equal amounts of the samples (0.5 mg) were brought to 0.5 μg/μl by the addition of lysis buffer and immunoprecipitated with 1 μg of anti-TrkA antibody (R&D Systems, Minneapolis, MN, USA) under gentle rotation for 2 hours. Next, the precipitates were collected with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and washed according to the manufacturer's instructions. Conditions to minimize nonspecific Hsp90 contamination of the precipitates were adjusted as described in our previously published work.

Samples prepared from equal amounts of protein (see immunoprecipitates above or for Western blotting 50 μg in case of TrkA and 25 μg for the rest) were mixed with Laemmli buffer (the 4x stock contained 25 ml 1M Tris-HCl, pH 6.8, 40 ml glycerol, 8 g SDS, 10 ml 100 mM EDTA, 10 ml 100 mM EGTA and 1 ml 1% bromophenol blue brought up to 100 ml with distilled water) and denatured by boiling. Subsequently, they were loaded onto 10% (or 7,5 % for TrkA and 18% for cleaved caspase-3) SDS-containing polyacrylamide gels and separated based on molecular size. The gels were electro-blotted onto PVDF membranes (Hybond-P, GE Healthcare, United Kingdom).

Immunological detection of the protein of interest was carried out by first blocking the membrane in 3% nonfat dry milk in TBS-Tween (10mM Tris-base, 150mM NaCl, 0.2% Tween-20, pH 8.0), followed by addition of the primary antibody [Phospho-p44/42 MAP Kinase to detect phospho-ERK1/2, anti-Akt and phospho-Akt (Ser 473) beta-Actin and phospho-TrkA (Tyr490), cleaved caspase-3 (Asp175), (Cell Signaling Technology), ERK-1 (C-16), or ERK-2 (C-14), Trk (C-14), Hsp90 (Santa Cruz Biotechnology)] diluted in the blocking solution and incubated overnight. Antisera were diluted 1:1000. At this dilution rate methodical control samples prepared from antigen-deficient (nnr5) cells' lysates (for anti-TrkA and anti-phospho-TrkA), or by the omission of the primary antibodies (in all cases)

produced no specific immune signal expected at the right molecular weight range in pilot experiments. Excess antibody was removed by five washes of TBS-Tween. Membranes were incubated with a horseradish-peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Pierce, Thermo Fischer Scientific, Rockford, IL, USA) diluted 1:10,000 in blocking solution. Five washes in TBS-Tween were followed by detection of the enhanced chemiluminescent signal (Immobilon Western, Millipore Corporation, Billerica, MA, USA). Densitometry was carried out using the ImageJ software (National Institutes of Health, USA).

Evaluation of Western blots

Densitometric values were plotted on graphs as the mean ± S.D. for four or five independent experiments. Significance of differences was determined using ANOVA testing applying Bonferroni corrections for multiple samples. P values < 0.05 were considered to be significant.

DNA fragmentation analysis

Adherent and floating/dead cells alike were scraped into their culturing media and pelleted by centrifugation at 4 °C in 15 ml plastic tubes. After the removal of the supernatant the pellet was lysed in 0.5 ml ice-cold lysis buffer (5 mM Tris-HCl, 5 mM EDTA, 0.5 % Triton-X 100, pH 7.4) for 20 minutes on ice. The detergent-insoluble fraction was removed by centrifugation (at 40,000 x g for 30 minutes at 4 °C) and the lysate was deproteinized by two rounds of phenol and one round of chloroform extraction. DNA was precipitated by the addition of 1 ml absolute ethanol and 50 µl 3M Na-acetate to the 0.5 ml protein-free lysate and incubation at -20 °C overnight. The precipitated nucleic acid was collected by centrifugation (at 100,000 x g for 30 minutes at 4 °C). The pellet was washed in 0.5 ml of 70 % ethanol, then vacuum-dried. The dry material was solubilized in 20 µl of Tris-EDTA (pH 7.4) buffer and the contaminating RNA was digested with RNase A treatment. The remaining DNA fragments were resolved by electrophoresis in a 1.8% agarose gel and visualized for imaging by the addition of 100-fold diluted SYBR Gold nucleic acid dye (Molecular Probes, Eugene, OR, USA).

Fluorescence microscopy

Treatments were stopped by a quick rinse in 37 °C PBS (1.37 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4 \cdot 7H2O$, 0.14 mM KH2PO4, pH 7.4) followed by fixation in 4% paraformaldehyde in PBS, pH 7.4 for 1 hour at room temperature. Excess fixative was washed out in three changes of PBS, then three changes of TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). Blocking of nonspecific binding sites was carried out by incubation of the samples in 5% nonfat dry milk in TBS at 4 ˚C for 1 hour under gentle rocking. The primary antibodies

TrkA (R&D Systems) and Hsp90 (Santa Cruz Biotechnology) were diluted 1:500 in the blocking solution and incubated with the specimens overnight at 4 ˚C under gentle rocking. Five washes in TBS followed. The fluorescent signal was generated by the addition of a Cy3-conjugated donkey-anti-rabbit antibody (Jackson Immuno Research, Cambridgeshire, UK) diluted in the blocking solution 1:500. At this dilution rate the methodical control samples prepared from antigen-deficient (nnr5) cells (for anti-TrkA) or by the omission of the primary antibodies (for all samples) produced no immune signal using the applied microscope settings. Nuclei were counterstained with Hoechst 33342 (Calbiochem, La Jolla, CA, USA).

Confocal images were generated using an Olympus FV-1000 laser scanning confocal system. Single optical sections from areas of interest were taken using a 40X phase objective.

In the case of P-ERK1/2 immunocytochemistry the average intensity of pixels in the area of nuclei were determined using the ImageJ software (National Institutes of Health, USA), then normalized to background intensity and plotted on a graph. The significance of the differences was determined using ANOVA testing applying Bonferroni corrections for multiple samples. P values < 0.05 were considered to be significant.

Results

Pilot experiments repeatedly confirmed that dimethyl-sulfoxyde (DMSO), the vehicle of the compounds geldanamycin and MG-132, had no effect on the examined parameters of the experiments at the concentrations applied (0.025 v/v % for single treatments and 0.05 v/v % when geldanamycin and MG-132 were applied in combination).

1. The effect of geldanamycin on PC12 rat pheochromocytoma cells is time and dose dependent.

PC12 cells have responded to geldanamycin in a dose- and time-dependent manner. In keeping with recent results of Toyomura et al. (2012) if treated with the higher dose 1.78 μM (1 μg/ml) of the antibiotic the cells responded with apoptosis already after 16 hours as shown by the activation of caspase-3 on our Western-blot. One tenth of this dose (0.178 μM) had to be present in the culture for at least 24 hours to produce initial signs of caspase-3 cleavage but proved to be safe and was not followed by programmed cell death if used only for 16 hours. These results have also been confirmed by DNA fragmentation analysis.

2. Geldanamycin reduces the level of TrkA.

Next we examined the expression level of the TrkA NGF receptor under our above tested, non-apoptotic experimental settings with geldanamycin. The treatment unable to elicit apoptosis led to a markedly reduced expression of TrkA protein in PC12 cultures as seen on our Western-blots.

3. Inhibition of proteasome function partially restores TrkA protein in geldanamycin-treated PC12 cells.

When the specific proteasome inhibitor compound MG-132 was applied together with geldanamycin the effect of the latter to reduce the amount of TrkA was substantially weaker as shown by our Western blot results.

4. Dissociation of TrkA and Hsp90 upon geldanamycin treatment.

Having seen the reduced level of TrkA upon inhibiting Hsp90 by geldanamycin we decided to examine the intracellular association of the high affinity NGF receptor and the 90 kDa chaperone by means of co-immune precipitation. While TrkA and Hsp90 were found to co-precipitate in untreated PC12 cells, after geldanamycin treatment their interaction was no longer observable and it remained disrupted even if the proteasome inhibitor was added together with geldanamycin. The marked reduction in the amount of the associated chaperone was not due to its reduced overall levels as shown by Western blot of the lysates.

5. Inhibition of the proteasome function can restore NGF-induced TrkA phosphorylation in geldanamycin-treated PC12 cells.

In order to evaluate if the TrkA protein saved by MG-132 from degradation was still responsive to NGF we performed Western blotting with an antibody specific to the phosphorylated form of TrkA following geldanamycin and MG-132 treatments. The NGF-inducibility of TrkA phosphorylation effectively blocked by geldanamycin could successfully be restored by the simultaneous inhibition of the proteasome. In fact, NGF stimulation of TrkA phosphorylation appeared to be somewhat stronger in lysates derived from cells treated with both geldanamycin and MG132. This observation is in accordance with data reported by Song et al. (2009, 2011) about the effect of MG-132 on TrkA.

6. Reduced ERK 1 and 2 phosphorylation following geldanamycin treatment.

Next by further Western blotting we examined if NGF activation of TrkA could successfully be transmitted onto downstream members of protein kinase cascades in cells treated with both geldanamycin and MG-132. The phosphorylation of ERK 1 and 2 as an easy measure of detecting NGF's downstream effect revealed that the lower dose and shorter duration of geldanamycin treatment not able to initiate apoptosis, could already weaken the level of ERK 1 and 2 phosphorylation induced by NGF.

7. Inhibition of the proteasome function restores the NGF-inducible ERK1/2 phosphorylation in geldanamycin-treated PC12 cells.

Western blots also shown that simultaneous addition of the proteasome inhibitor MG-132 could restore the NGF-inducibility of ERK1/2 in geldanamycin-treated cultures when compared to treatments with geldanamycin and NGF only. The somewhat elevated baseline phosphorylation of ERK upon inhibition of the proteasome can be attributed to the ERK phosphorylation inducing ability of MG-132 itself. Reprobing the membrane for total ERK1/2 excluded the possibility of the observed changes in ERK1/2 phosphorylation being due to loading differences of the lanes.

8. Phosphorylation of Akt is inhibited following geldanamycin treatment.

Not only the NGF activation of ERK1/2 but that of protein kinase B/Akt was also compromised in geldanamycin-treated PC12 cultures. The baseline phosphorylation of Akt was also reduced after geldanamycin treatment to barely detectable. (Western blot)

9. Inhibition of the proteasome function does not restore NGF-inducible protein kinase B/Akt phosphorylation in geldanamycin-treated PC12 cells.

Our Western blots have shown that that he proteasome inhibitor MG-132, when applied together with geldanamycin under the same conditions that could salvage the NGF activation of ERK1 and 2, could not restore activation of protein kinase B/Akt upon NGF treatment. Baseline Akt phosphorylation, however, was elevated after pretreatment with the proteasome inhibitor as also reported earlier by Moises et al. (2009).

10. The intracellular distribution of TrkA is altered in geldanamycin- and MG-132-treated PC12 cells (immunocytochemistry).

The proteasome inhibitor MG-132, when applied together with geldanamycin, could efficiently save a substantial portion of TrkA protein as seen on Western blots. The intracellular localization of salvaged TrkA, nevertheless, was dramatically different when compared to the TrkA immunoreactivity pattern of untreated PC12 cells. In untreated cells a substantial portion of TrkA was in the immediate vicinity of the plasma. The TrkA signal of cells treated with the Hsp90 inhibitor geldanamycin was barely detectable at the level of individual cells. In cultures treated with both geldanamycin and the proteasome inhibitor MG-132 the TrkA signal regained in strength but was no longer shifted towards the plasma membrane, instead it was evenly distributed throughout the entire cytoplasm. Proteasome inhibition alone had no effect on the intracellular pattern of TrkA immunoreactivity when compared to untreated controls. (The intracellular localization of p-TrkA, unfortunately, could not be determined, because the antibody used for Western blot was not suitable for immunocytochemical detection of the target protein as stated by the manufacturer and also supported by our repeatedly failed trials.)

11. The intracellular distribution of Hsp90 is altered in geldanamycin- and MG-132-treated PC12 cells (immunocytochemistry).

Having seen the changes in the distribution of TrkA we decided to examine the intracellular localization of Hsp90, too. In untreated cells and upon geldanamycin treatment the Hsp90 immune signal had an even distribution predominantly over the cytoplasm. After combined treatment with geldanamycin and MG-132, however, Hsp90 was markedly shifted towards the periphery of the cytoplasm. Proteasome inhibition alone had no effect on the intracellular pattern of Hsp90 immunoreactivity when compared to untreated controls. The overall level of Hsp90 was not affected by MG-132 and/or geldanamycin treatments as shown by Western blot.

12. The kinetics of ERK1/2 phosphorylation induced by the MG-132 proteasome inhibitor in PC12 cells.

The phosphorylation of ERK1/2 upon MG-132 treatment alone was further studied by our workgroup also inspired by Hashimoto et al. We first applied MG-132 for increasing periods of time and then analyzed ERK phosphorylation by Western-blot using an anti-phospho-ERK1/2 antibody The increased phosphorylation of ERK1/2 compared to the

untreated control was already detectable after 15 minutes and it was found to be prolonged rising ot a peak at 6 hours of pretreatment. After 24 hours the level of activation was still higher than in the untreated cultures. The total ERK1/2 content of the different samples was detected on the same membranes and used for normalization during the densitometric analysis.

13. The phosphorylated ERK1/2 is translocated into the nuclei of PC12 cells upon MG-132 proteasome inhibitor treatment.

We studied the alteration of the intrcellular distribution of phospho-ERK1/2 after MG-132 pretreatment by fluorescent immunocytochemistry. The weak immune signal was mostly cytoplasmic in untreated cultures. The 6 hours long treatment with the MG-132 proteasome inhibitor increased the amount of activated ERK1/2 in the nucleus and in the cytoplasm, similarly to the 3 hours long NGF treatment. This nuclear translocation and sustained activation of phospho-ERK1/2 confirms the observations of MG-132 inducing neurite growth in PC12 cells similarly to NGF.

Discussion

During our research we found that the treatment of wild type PC12 rat pheochromocytoma cells with geldanamycin leads to reduced levels of TrkA protein well before the onset of programmed cell death. Drug sensitivity of cell lines including PC12 cells may vary due to clonal differences, passage number and culturing conditions. The dose of geldanamycin used in our experiments was just a tenth of that inducing apoptosis in the experiments of others. Furthermore, our treatment time was also reduced by about one third. Despite of the milder treatment, the NGF stimulation of ERK1/2 and protein kinase B/Akt were both severely compromised under such conditions.

The simultaneous treatment with the proteasome inhibitor MG-132 helped to keep the level of TrkA higher than that seen in the cultures treated with geldanamycin alone and it also rendered the phosphorylation of the receptor upon NGF stimulation again possible. NGF activation of TrkA in cultures treated with both geldanamycin and the proteasome inhibitor was in fact somewhat stronger than that seen in PC12 cells without pretreatment. The ability of the proteasome inhibitor MG-132 to potentiate the effect of NGF has been reported by Song et al. (2009, 2011). Nevertheless, the activating effect of NGF on TrkA could only be

further transmitted onto ERK1/2 but not towards protein kinase B/Akt in cultures pretreated with both geldanamycin and MG-132.

The intracellular distribution of the TrkA immune signal in untreated PC12 cells was cytoplasmic, as expected, with a substantial portion of the immunoreactivity being strongly shifted towards the periphery of the cytoplasm into the immediate vicinity of the plasma membrane. According to the Western blot results the TrkA immune signal has become much weaker in the presence of geldanamycin but has regained strength if the proteasome inhibitor MG-132 was also present during treatment with the Hsp90 antagonist. Despite its regained intensity the original, membrane-associated fraction of the TrkA signal was not restored by MG-132 in geldanamycin-treated cells. This newly acquired intracellular position of TrkA raises the possibility that it might also have altered downstream signaling connections. Interestingly, the Hsp90 immunoreactivity showed a shift into the opposite direction, towards the membrane in cells treated with both geldanamycin and MG-132. Currently we can only speculate about the significance of this latter observation. One possibility could be that MG-132, being a rather hydrophobic compound reaches preferentially the peripheral regions of the cytoplasm by diffusion. Here, it could lead to the accumulation of Hsp90's protein partners compromised by geldanamycin but saved by MG-132 from the proteasome. The accumulation of these proteins might result in a compensatory shift of Hsp90 capacity in the same direction. The phenomenon will be worth further investigating.

Pre-organized molecular complexes are known to increase the efficiency of intracellular signal transduction markedly. The altered intracellular localization of TrkA in cells treated with both geldanamycin and MG-132 raises the possibility that a substantial portion of TrkA may no longer be close enough to the plasma membrane to effectively stimulate all of its downstream cascades. Jullien et al. (2003) reported that gp140 TrkA is the only form of TrkA readily detectable at the PC12 cell surface. In their studies treatment by NGF produced a clearing of more than 70% of the receptor from the cell surface within 15 minutes of treatment. With similar results Grimes et al. (1996) have found that 66% of the receptor initially labeled at the PC12 cell surface was found inside the cells after 20 minutes of NGF treatment. Currently we can't tell exactly why the TrkA receptors saved by proteasome inhibition in geldanamycin-treated PC12 cells did not return to their membrane-associated original location. There are several points at which inhibition of the proteasome could interfere with the regular and quite dynamic trafficking of TrkA. In experiments of Sommerfeld et al. (2000) the brain-derived neurotrophic factor (BDNF) receptor TrkB was downregulated following the binding of BDNF. This could be prevented by the inhibition of

the proteasome. TrkA is rapidly and transiently ubiquitinated upon addition of NGF and it has been shown that TrkA is deubiquitinated by proteasomes prior to degradation by lysosomes whereby the scaffold protein p62 acts as a shuttling protein in the internalization and transport of TrkA. The kinetics of receptor trafficking post-internalization occurs as a sequel from early into late endosome, then into multivesicular bodies and proteasomes, finally degradation in the lysosomes or, alternatively, TrkA can also return to the membrane from early endosomes through recycling endosomes.

According to a study by Zhang et al. (2000) neuronal differentiation of PC12 cells is promoted by catalytically active Trk-s within endosomes in the cell interior. In contrast, survival responses are initiated by activated receptors at the cell surface where they orchestrate prolonged activation of protein kinase B/Akt. Thus, interaction between TrkA and intracellular signaling molecules are dictated by both phosphotyrosine residues of the activated receptors and by the intracellular location of the latter. The activated receptor subunits with their phosphorylated tyrosines build scaffolding for the assembly of a multi-enzyme signaling complex. The altered intracellular distribution of TrkA could explain, at least from the viewpoint of protein localization, why its activation by NGF could not lead to subsequent phosphorylation of protein kinase B/Akt under such conditions.

Our research group further analyzed the effects of MG-132 as we confirmed that the proteasome inhibitor, similarly to NGF, induces a prolonged ERK1/2 phosphorylation parallelly causing a nuclear translocation as well. This behavior of ERK leads to neurite growth and neural differentiation, it was therefore not surprising when MG-132 treatment also led to the appearance of cellular processes in these cell cultures. We studied further which elements of the NGF receptor-ERK cascade way are necessary for the described activation by paying special attention to the non-receptor tyrosine-protein kinase Src.

Our experiments shed light on the fact that protein-protein interactions, the creation of multiprotein complexes, especially the formation of heteromultimers containing the Hsp90 is essential for signaling processes. We believe that our observations may help to better understand the mechanisms of action of geldanamycin and MG-132, two compounds with derivatives possessing strong human therapeutic potential.

Acknowledgements

I thank my supervisor, György Sétáló Jr., without whom this thesis could not have been written. He was already my supervisor in the Student Research Program, and so allowed me to start my work in this field, and since then helped me with his counsel during our close cooperation in our workgroup.

I am very grateful to the head of the department and the program, József Szeberényi, who made my work in the Department of Medical Biology possible. I received a lot of help from him during the writing of the thesis as well when he applied his usual thoroughness pinpointing my mistakes.

I thank Oktávia Tarjányi very much for allowing me to complete my thesis to a whole complete achievement during our efficient cooperation.

I am grateful to Hajnalka Ábrahám, Éva Mikó, and Erika Pétervári for their attentive work during the pre-evaluation of my thesis.

I thank for the great amount of excellent laboratory work to the assistant of our research group, Mónika Vecsernyés. Her tireless technical support made it possible to balance my time between my different research and educational obligations.

I am grateful to Judit Bátor for sharing her experience about the PhD procedures and for helping with the elimination of errors.

I thank for all the help, advices and support I received from the rest of the colleagues in the Department of Medical Biology.

I am really grateful to my many research cooperation partners and also happy for being able to participate in so many different interesting projects revealing numerous biological problems which never cease to impress me.

And at the last but not the least I owe deep gratitude to my family, foremost my wife Zita Farkas who establishes the safety of home for us and for our two children providing a solid background allowing me to pursue my research in proper times undisturbed.

Publications

Peer-reviewed articles in the topic of the thesis (IF: 5,538)

Berta Gergely, Harci Alexandra, Tarjányi Oktávia, Vecsernyés Mónika, Balogh András, Pap Marianna, Szeberényi József, Sétáló György Jr: Partial rescue of geldanamycin-induced TrkA depletion by a proteasome inhibitor in PC12 cells. Brain Research 1520: pp. 70-79. (2013) **IF: 2.879**

Tarjányi Oktávia, Berta Gergely, Harci Alexandra, Bacsa Eszter, Stark Borbála, Pap Marianna, Szeberényi József, Sétáló György Jr: The role of Src protein in the process formation of PC12 cells induced by the proteasome inhibitor MG-132. Neurochemistry International 63:(5) pp. 413-422. (2013) **IF: 2.659**

Additional peer-reviewed articles (IF: 23,893)

Szeberényi József, Bátor Judit, Berta Gergely, Fábián Zsolt, Kiss Katalin, Komáromy László, Pap Marianna, Sétáló György Jr: Experiments in Molecular Cell Biology: A Problem-oriented Basic Science Course in a Medical Curriculum. In: Crabtree H, Darvill A, Holland K, MacKay S, McLoughlin M, Oakley D, Supyk J (edit): Problem-based Learning 2004: A Quality Experience. Salford: Univ. of Salford, 2006. pp. 80-89.

Talabér Gergely, Boldizsár Ferenc, Bartis Domokos, Pálinkás László, Szabó Mariann, Berta Gergely, Sétáló György, Németh Péter, Berki Tímea: Mitochondrial translocation of the glucocorticoid receptor in double-positive thymocytes correlates with their sensitivity to glucocorticoid-induced apoptosis. International Immunology 21:(11) pp. 1269-1276. (2009) **IF: 3.403**

Bátor Judit, Varga Judit, Berta Gergely, Barbakadze Tamar, Mikeladze David, Ramsden Jeremy, Szeberényi József: Sodium nitroprusside, a nitric oxide donor, fails to bypass the block of neuronal differentiation in PC12 cells imposed by a dominant negative Ras protein. Cellular & Molecular Biology Letters 17:(3) pp. 323-332. (2012) **IF: 1.953**

Hafner Dóra, Bodnár Zsófia, Horvatovich Katalin, Berta Gergely, Kovács Melinda: Preliminary investigations into the effect of feeding mannan oligosaccharide (MOS) on the genotoxic effect of T-2 toxin in rabbits measured by comet assay. Acta Agriculturae Slovenica 100:(Suppl. 3) pp. 351-355. (2012)

Hamar János, Sólymár Margit, Tanai Edit, Cséplő Péter, Springó Zsolt, Berta Gergely, Debreceni Béla, Koller Ákos: Bioassay-comparison of the antioxidant efficacy of hydrogen sulfide and superoxide dismutase in isolated arteries and veins. Acta Physiologica Hungarica 99:(4) pp. 411-419. (2012) **IF: 0.882**

Kiszler Gábor, Várhalmi Eszter, Berta Gergely, Molnár László: Organization of the sensory system of the earthworm Lumbricus terrestris (Annelida, Clitellata) visualized by DiI. Journal Of Morphology 273:(7) pp. 737-745. (2012) **IF: 1.602**

Halász Melinda, Polgar Beáta, Berta Gergely, Czimbalek Lívia, Szekeres-Barthó Júlia: Progesterone-induced blocking factor differentially regulates trophoblast and tumor invasion by altering matrix metalloproteinase activity. Cellular And Molecular Life Sciences 70:(23) pp. 4617-4630. (2013) **IF: 5.615**

Horvatovich Katalin, Hafner Dóra, Bodnár Zsófia, Berta Gergely, Hancz Csaba, Dutton Mike, Kovács Melinda: Dose-related genotoxic effect of T-2 toxin measured by comet assay using peripherial blood mononuclear cells of healthy pigs. Acta Veterinaria Hungarica 61:(2) pp. 175-186. (2013) **IF: 1.173**

Karsai Gergely, Pollák Edit, Wacker Matthias, Vömel Matthias, Selcho Mareike, Berta Gergely, Nachman Ronald J., Isaac R. Elwyn, László Molnár, and Wegener Christian: Diverse in- and output polarities and high complexity of local synaptic and non-synaptic signaling within a chemically defined class of peptidergic Drosophila neurons. Frontiers In Neural Circuits 7: Paper 127. 22 p. (2013) **IF: 3.333**

Dénes Viktória, Czotter Nikoletta, Lakk Mónika, Berta Gergely, Gábriel Róbert: PAC1-expressing structures of neural retina alter their PAC1 isoform splicing during postnatal development. Cell And Tissue Research 355:(2) pp. 279-288. (2014) **IF: 3.677**

Kellermayer Zoltán, Fisi Viktória, Mihalj Martina, Berta Gergely, Kóbor József, Balogh Péter: Structural characteristics and cellular constituents of the follicular depositionof marginal zone macrophage receptor MARCO in the mouse spleen. Journal of Histochemistry and Cytochemistry 62(6):436-449 (2014) **IF: 2,255**

Posters, oral presentations, abstracts in the topic of the thesis

György Sétáló ifj., Gergely Berta, József Szeberényi: The role of 90 kDa chaperones in PC12 cells. FEBS Special Meeting 2003 on Signal Transduction, Bruxelles, 3-8 July 2003.

György Sétáló Jr., Gergely Berta, Borbála Stark, Oktávia Tarjányi and József Szeberényi: The effect of proteasome inhibition on PC12 rat phaeochromocytome cells. 30th

FEBS Congress 9th IUBMB Conference, Budapest, 2-7 July 2005. Published abstract: FEBS Journal Volume 272 Supplement 1 July 2005 pp315

Ifj. Sétáló György, Berta Gergely és Szeberényi József: Geldanamycin hatása PC12 sejtekben. XI. Sejt- és Fejlődésbiológiai Napok, Siófok, 15-17 April 2003.

Berta Gergely, Ifj. Sétáló György, Stark Borbála és Szeberényi József: Geldanamycin és MG-132 hatásai patkány feokromocitóma (PC12) sejtekben. XII Sejt- és Fejlődésbiológiai Napok, Pécs, 16-18 April 2004.

Berta Gergely, Stark Borbála, Ifj. Sétáló György és Szeberényi József: Proteaszómagátlás hatásának vizsgálata PC12 patkány feokromocitóma sejtekben. XIII. Sejt- és Fejlődésbiológiai Napok, Eger, 10-12 April 2005.

Stark Borbála, Berta Gergely, Tarjányi Oktávia, Harci Alexandra, Ifj. Sétáló György és Szeberényi József: Proteaszóma gátlás hatása PC12 patkány feokromocitóma sejtek differenciációjára és túlélésére. 36. Membrán-Transzport konferencia, Sümeg, 23-26 May 2006.

Stark Borbála, Berta Gergely, Tarjányi Oktávia, Harci Alexandra, Varga Judit, Ifj. Sétáló György és Szeberényi József: MG-132 jelátviteli és morfológiai hatásai patkány feokromocitóma tenyészetekben. A Magyar Biokémiai Egyesület 2006. évi Vándorgyűlése, Pécs, 30 August- 2 September 2006.

Stark Borbála, Harci Alexandra, Balogh András, Berta Gergely, Ifj. Sétáló György, Szeberényi József: Egy peptidil-aldehid proteaszóma gátló (MG-132) jelátviteli hatásainak vizsgálata patkány feokromocitóma (PC12) sejtekben. Semmelweis Egyetem PhD Tudományos Napok, Budapest, 12-13 April 2007.

Harci Alexandra, Berta Gergely, Stark Borbála, Ifj. Sétáló György, Szeberényi József: Patkány feokromocitóma (PC12) sejtek differenciációjának vizsgálata proteaszóma gátló (MG-132) és Src-inhibitorok (PP1, PP2) jelenlétében. Semmelweis Egyetem PhD Tudományos Napok, Budapest, 12-13 April 2007.

Harci Alexandra, Berta Gergely, Stark Borbála, Ifj. Sétáló György és Szeberényi József: Proteaszóma gátló (MG-132) és Src-inhibitorok (PP1, PP2) differenciációs hatásainak vizsgálata patkány feokromocitóma (PC12) sejtekben. XIV. Sejt- és Fejlődésbiológiai Napok, Balatonfüred, 15-17 April 2007.

Stark Borbála, Harci Alexandra, Berta Gergely, Ifj. Sétáló György és Szeberényi József: Egy peptidil-aldehid Proteaszóma gátló (MG-132) jelátviteli hatásainak vizsgálata patkány feokromocitóma (PC12) sejtekben. XIV. Sejt- és Fejlődésbiológiai Napok, Balatonfüred, 15-17 April 2007.

Berta Gergely, Stark Borbála, Tarjányi Oktávia, Harci Alexandra, Ifj. Sétáló György és Szeberényi József: MG-132 proteaszóma gátló differenciációs és apoptotikus hatása PC12 sejtekben. A Magyar Humángenetikai társaság VII. Kongresszusa, Pécs, 11-13 July 2008.

Harci Alexandra, Berta Gergely, Stark Borbála, Ifj. Sétáló György, Szeberényi József: Patkány feokromocitóma (PC12) sejtek differenciációjának vizsgálata proteaszóma gátló (MG-132) és Src-inhibitorok (PP1, PP2) jelenlétében. PhD Tudományos Napok, Budapest, 30-31 March 2009.

Gergely Berta, Alexandra Harci, Oktávia Tarjányi, Mónika Vecsernyés, András Balogh, Marianna Pap, József Szeberényi and György Sétáló, Jr.: Partial rescue of geldanamycin-induced TrkA depletion by a proteasome inhibitor in PC12 cells. 2nd International Doctoral Workshop on Natural Sciences, Pécs, 11-12 September 2013.