
# Testing of developmental neurotoxicity by *in vitro* Methods

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# 1. INTRODUCTION

Microtubules (MTs) are cytoskeletal polymers made of α- and β-tubulins and are the largest filamentous structures of the eukaryotic cytoskeleton. Although their structure is evolutionary conserved, MTs have a roles in various cellular processes like cell mitosis, motility, transport, secretion, shape, differentiation etc. (Song et al., 2015). Their functional diversity might be regulated by post-translational modification of the tubulin subunits like acetylation, phosphorylation, polyglutamylation etc. This is important because different types of MTs have specific roles in cells (Song et al., 2014). In neurons, MTs play a role in neuronal polarization, remodeling of both dendritic and axonal spine, the trafficking of cargo molecules etc. Such mechanism can be achieved by spatial and temporal generation of PTMs, commonly known as "tubulin code". Microtubule polyglutamylation plays an important role because it changes the charges of the tubulins in order to regulate electrostatic interactions between MTs and microtubule associated proteins (MAPs). Hence, polyglutamylated chains on MTs are hotspots for MAPs like MAP2 or Tau protein. Tubulin tyrosine-like ligases (TTLL) are modifying enzymes that are involved in addition of glutamate side chains of different lengths on several sites within the carboxy-terminal tails of tubulins (Janke et al., 2008). Polyglutamylation occurs in two steps, initiation and elongation. First step is addition of glutamayl unit by the formation of covalent bond between its amino group and δ-carboxyl group of glutamate residue that is incorporated inside the carboxy-terminal tail of tubulin unit. Elongation is a series of reactions in which new glutamyl units are incorporated inside the glutamate side chain (Janke, 2005).

Glufosinate ammonium (PPT), is a broad-spectrum herbicide originally found in AgrEvo's Basta® formulation and used as crop desiccant due to its phytotoxic effect in plants by acting as a competitive and irreversible inhibitor of glutamine synthetase (GS) in plants (Nunes et al., 2010.). Since PPT is structural analogue of neurotransmitter glutamate (**Fig.1**), intraperitoneal administration of PPT and its first metabolite 4-methyl-phosphinico-2-oxo-butanoic acid (PPTO) cause serious neurotoxic consequences in mammals (convulsions, memory loss, seizures, circulatory failure etc.) (Song et al., 2015). Furthermore, it has been reported that low dose exposure of PPT in mice pups induce alterations in neuroblasts migration to olfactory bulb (consequence is ectopic migration or accumulation of neuroblasts in the subventricular zone) and its disabilities to perform chain-like migration (A. Herzine, unpublished article). Therefore, this study suggested that PPT and PPTO inhibit TTLL, as well as their functional antagonists cytosolic carboxypeptidases (CCPP), leading to MT stabilization and violation of several cellular functions.

The specific aims of this research after pesticide treatment were as follows:

1. detection of MAPs involved in cytoskeleton network and by Western blot (WB);
2. comparison of morphological differences between control samples and pesticide-treated samples;
3. determine MAPII/polyglutamylated MT co-localization by immunocytochemistry method (ICC);

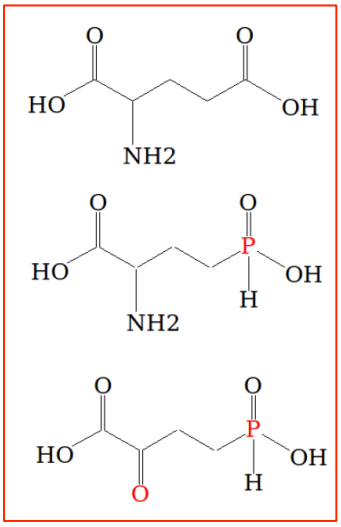


**Figure 1. Chemical structure of glutamate, PPT and PPTO** (taken from www.fao.org/fileadmin/templates/agphome/.../Pests.../Glufosinate.pdf **).**

## 2. MATERIALS AND METHODS

### 2.1 Protein isolation and determination of protein concentration

To determine protein concentration, firstly the cells were washed two times with ice-cold PBS. Then approximately 200 µL ice-cold RIPA lysis buffer was mixed with 5 µL of protein inhibitor and 5 µL of phenylmethanesulfonylfluoride (PMSF). The cells were scraped off the dish, collected, resuspended and then placed in 1.5 mL microfuge tube. After centrifugation at constant agitation for 30 minutes was finished, the supernatant was removed from the pellet and placed in a fresh tube. 4 L of 2 mg/mL of BSA solution was prepared in order to make a bovine serum albumin (BSA) calibration curve and were diluted in a range from 2 mg/mL to 0.125 mg/mL. 20 µL of each concentration of BSA solution was mixed with 1 mL of Bradford assay. After that, the same procedure was performed for protein samples. The colour was left to develop for 5 minutes and then the solutions were put into a UV spectrophotometer at 595 nm. The absorbance was measured for every sample in order to make the BSA standard curve which was used for protein concentration determination.

### 2.2 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Firstly, 12% acrylamide running gel (distillated water; 0.8% BisAcrilamide; 1.5 M Tris-HCl, pH 8.8; 10% SDS; TEMED(Sigma); 10% APS) and 4% acrylamide stacking gel (distillated water; 0.8% BisAcrilamide; 0.5 M Tris-HCl, pH 6.8; 10% SDS; TEMED; 10% APS) were prepared. The protein samples were prepared by mixing Laemmli (0.5M Tris-HCl, pH 6.8; 10% SDS, 100% glycerol; bromphenol blue; 14.5M β-mercaptoethanol), 30 μg of protein samples and RIPA buffer. The protein extracts were denatured at 95°C for 10 minutes before being loaded on the polyacrylamide gel for separation that was conducted at 130V for approximately 2 hours in running buffer 1x (Tris-HCl; glycine; 10% SDS, distillated water).

### 2.3 Western blot

Transfer cassette, fiber pads, filter papers and nitrocellulose membrane were placed in transfer buffer in order to make a WB sandwich. The transparent part of the case has been placed down and then fiber pad and filter paper were added on it. Membrane was placed on the filter paper and gel was separated from the glass plates and placed on the membrane. Another filter paper and fiber pad were added onto the gel and then cassette was closed. The cassette was placed into the transfer tank and wet transfer was run for 1 hour at 100 V constant voltage. After the transfer has finished, the membranes were washed with Tris buffered saline supplemented with 0.1% Tween® 20 (TBST buffer 1x) 3 times for 5 minutes, were put into the blocking solution (5% milk mixed with TTBS buffer 1x) and left for 2 hours at slight agitation. Next step was incubation of the membranes overnight at 4 °C with gentle rocking and with primary antibodies diluted in the blocking solution (anti-α/β-TubulinIII, 1/ 10 000; anti-α-MAPII, 1/ 5000; anti-α-Tau, 1/ 5000). The next day washing steps were repeated and membranes were incubated in secondary antibody for two hours (anti-mouse IgG- HRP, 1/10 000). Three washes for 5 min were performed in TBST buffer 1x after incubation with the secondary antibodies. The proteins of interest were revealed by chemiluminescence following addition of substrate to the membranes (reagent kit ECL ™ Western Blotting Detection Reagent (AmershamTM, GE Healthcare)) and the immunopositive signals were membranes were developed in the dark room on X-ray films.

### 2.4 Immunocytochemistry

Images provided by project leader were analysed in Image J program. After the results have been measured, the mean and standard error of the mean (SEM) have been calculated. Data analysis was done by using GraphPad Prism 5.03 and the difference in samples data were analysed by one-way (ANOVA). When ANOVA showed significant difference between overall samples, this difference between sample groups were assessed by using the *post hoc* test (Bonferroni test).

## 3. RESULTS

### 3.1 Changes in protein localization and cell shape in pesticide-treated differentiated C6 cells

MAPII and Tau are proteins that can bind to polyglutamylated sites in order to induce stabilization of MTs. MAPII is more prevalent in dendritical, while Tau is more abundant in axonal parts of the neuron-like cells. The fluorescence of MAPII and polyglutamylated α-tubuline were analyzed in the ICC images in order to obtain information about their co-localization, fluorescence intensity, morphological changes (**Fig. 2**).

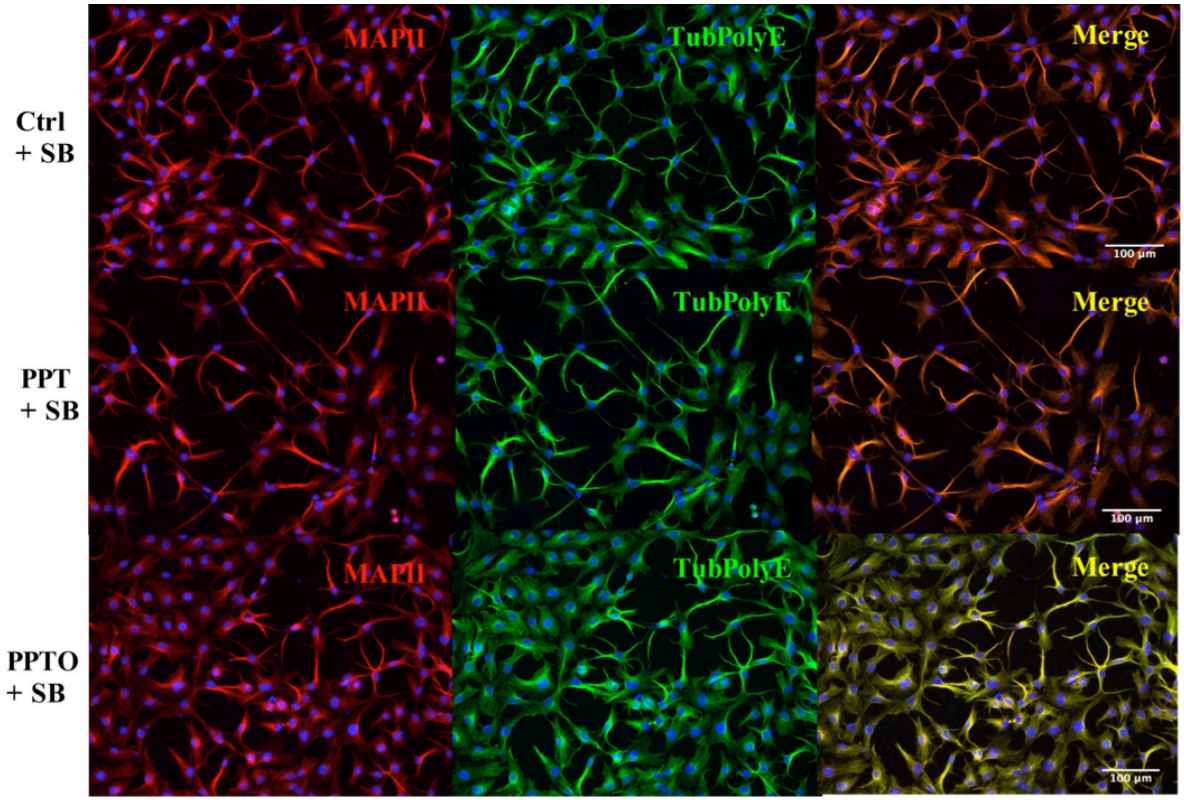


**Figure 2. MAPII co-localization with polyglutamylated tubulins and pesticides-induced morphological changes in C6 cells.** C6 cells were cultured in 24-well plates and allowed to grow until reaching 60% of confluence. In the same time, cells were exposed to SB (2mM) and pesticides (10µM) for 48 hours and co-localization pattern and morphological changes were analyzed using upright fluorescence microscope. Pictures were taken with a magnification of ×20 for MAPII detection. Nucleus was counterstained with DAPI (blue).

MAPII had 0.71 and 0.95-fold decrease in fluorescence in PPT and PPTO samples respectively compared to the control samples. The 0.44-fold reduction of polyglutamylation signal in PPT-treated samples and 3.05-fold increase of polyglutamylation in PPTO-treated samples compared to differentiated control was measured (**Fig. 3**).

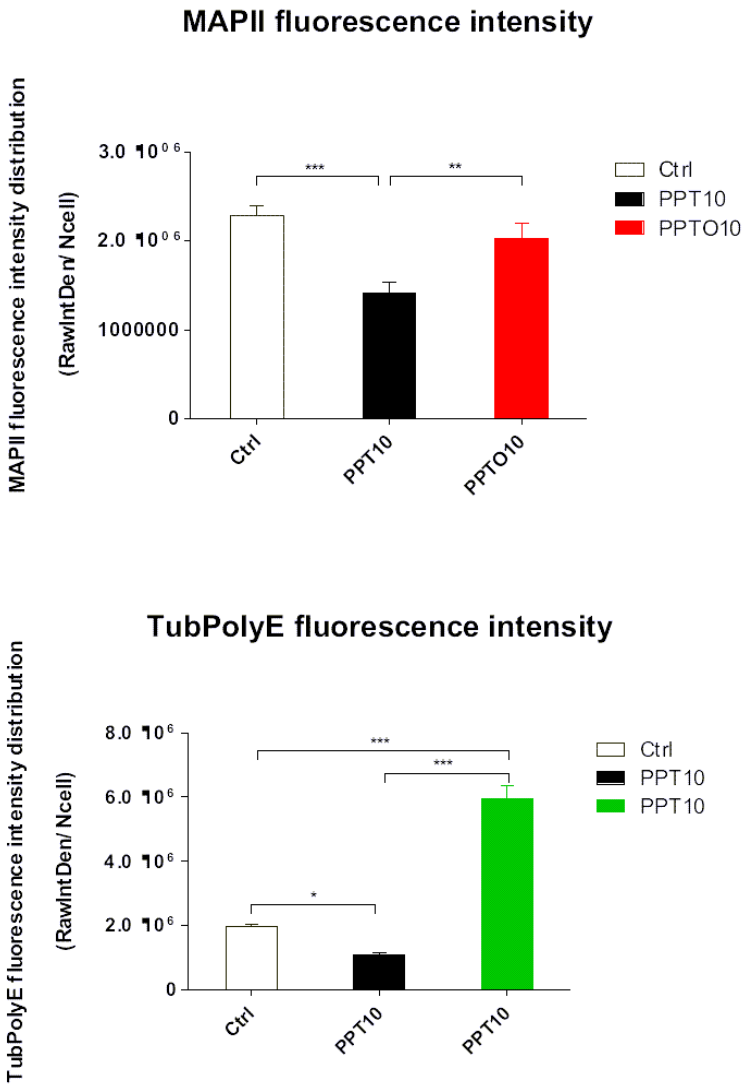


**Figure 3. These graphs show the fluorescence intensity of the MAPII and α-tubulin proteins in control samples, 10 µM PPT and 10µM PPTO samples.** Statistical analysis: ANOVA with Bonferroni *post hoc* test. Significance difference: $P < 0.05$*, $P < 0.01$**, $P < 0.001$***.

Afterwards the percentage of dividing cells among the total amount of glioblastoma cells was calculated for control, PPT and PPTO samples. More cells were captured in division in PPT (1.75%) and PPTO (2.39%) treated samples compared to control samples (0.23%) (**Fig. 4**).

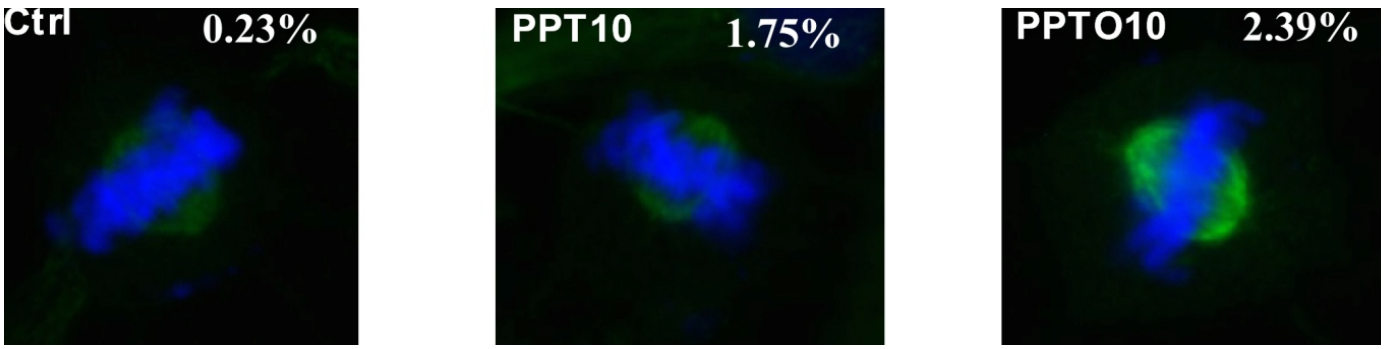

**Figure 4. Detection of polyglutamylated mitotic spindle (green) after 48 hours of PPT- and PPTO treatment in C6 cells.** The green colour (FITC) stands for polyglutamylation, the blue (DAPI) indicates the cell nucleus.

### 3.2 Analyzis of protein quality by WB

After protein isolation, WB was performed with the aim of measuring the relative abundance of α-tubuline, MAPII and Tau proteins. The amount of α-tubuline and MAPII was detected to be roughly the same as in control samples, 10 μM PPT and 100 μM PPT (**Fig. 5 and 6**). Tau protein was found to be more abundant in both PPT samples (**Fig. 7**). Semi-quantitative analysis of proteins of interest with housekeeping protein could not be conducted due to insufficient lab conditions.


**Figure 5. Western Blot results showed stabile expression of α,β-Tubulin III in C6 cells.**


**Figure 6. WB α-Map II protein levels in C6 neuron-like cells.**


**Figure 7. Western Blot results for α-Tau protein in C6 cell line.**

# 4. DISCUSSION AND PERSPECTIVES

Our data analysis of ICC indicates that PPT and PPTO treatments have a significant effect on polyglutamylation levels. The measured polyglutamylation level in PPT treated cells is slightly lower than in control samples, but interestingly in PPTO treated cells the polyglutamylation level is significantly higher than in the other samples. This results could be due to the fact, that PPT can bind to TTLLs and after PPT-polyglutamylase complex binds to the glutamate chain, the enzyme is irreversibly blocked and hinders any further modification of the chain. The cells were treated with sodium-butyrate (SB), which is a substance that promotes cell differentiation. Initially all cells had a low amount of polyglutamylation, but after SB treatment in the control and PPTO samples the level of polyglutamylation arose. However due to the blocked chains the polyglutamylation level stayed the same in the PPT treated samples. We used B3 antibodies which could only bind to glutamate chains comprised of more than 3 units, therefore we could only detect a small amount of fluorescence in PPT samples. PPTO is a structural analogue of PPT, it only differs by one atom and it has an extra negative charge. According to our hypothesis PPTO can bind more strongly to polyglutamylases (and decarboxylases) and therefore can fully inhibit the binding of polyglutamylases to MTs. In this way the glutamate chains remain free for uninhibited enzyme binding. Due to the presumable overexpression of polyglutamylases caused by SB not all polyglutamylases are inhibited and the glutamate chains are elongated faster than broken down by decarboxylases.

The fluorescence of MAPII had a major decrease in the PPT samples. This is probably due to the fact that only a small amount of MAPII could bind to polyglutamate chains, because of the inhibitory effect of PPT. The analysis of the colocalization of MAPII and tubulin subunits in the ICC confirmed this theory. However the fluorescence intensity cannot give us precise information of the real abundance of proteins.

During the analysis of the ICC pictures the number of cells in division were counted. The results show that more cells were in division in PPT and PPTO samples, than in the control. Dividing cells form mitotic spindles which are made of MTs and this can partly explain the increase of fluorescence in PPT and PPTO samples. These findings support our hypothesis, because the difference in mitotic activity can be explained by the higher polyglutamylation levels of PPT and PPTO treated samples. The polyglutamylate chains bind proteins which can then induce stabilization of MTs. The mitotic spindle cannot be disassembled when it is hyperglutamylated, hence the dividing cells get blocked in the metaphase of mitosis.

Our WB measurements were not precise because we had an insufficient amount of primary and secondary antibodies. Furthermore they were carried out in poor lab conditions, so we cannot

draw solid conclusions from the results. MAPII, α-tubulin and Tau proteins were isolated from glioblastoma cells and their relative abundance was seen by the intensity of the bands.

The amount of MAPII was found to be approximately the same in control and PPT samples. This is in contradiction with the ICC measurement of MAP fluorescence, but as mentioned before the fluorescence intensity cannot provide precise information of the amount of proteins. Tau proteins were found to be more abundant in PPT samples, than in the control. The explanation could be that SB treated cells need to stabilize their MTs in order to complete their differentiation. PPT samples have a constant low level of polyglutamylation, therefore the MTs are not sufficiently stabile and the cells produce more Tau proteins to stabilize the MTs.

The α-tubulin abundance was equal in all samples as expected. This indicates that polyglutamylation is responsible for the difference of the measured amount of Tau in PPT and control samples.

## 5. REFERENCES


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