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Organophosphates modulate tissue transglutaminase activity in differentiated C6 neural cells

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Abstract. – OBJECTIVE: The organophosphate compounds chlorpyrifos (*O, O*-diethyl O-[3,5,6-trichloro-2-pyridinyl] phosphorothioate, CPF) and phenyl saligenin phosphate (PSP) have been widely implicated in developmental neurotoxicity and neurodegeneration. However, the underlying mechanism remains unclear. Transglutaminase (TG)2 is a calcium ion (Ca^{2+})-dependent enzyme with an important role in neuronal cell outgrowth and differentiation and in neurotoxin activity and is modulated by organophosphates.

MATERIALS AND METHODS: We studied TG2 activity modulation by CPO and PSP during differentiation in C6 glioma cells. We studied the effects of CPO or PSP treatment with or without the TG2 inhibitor Z-DON and identified potential TG2 protein substrates via mass spectrometry.

RESULTS: PSP and CPO did not affect cell viability but affected TG2 activity in differentiating cells. Our results indicate that the organophosphate-induced amine incorporation activity of TG2 may have a direct effect on neuronal outgrowth, differentiation, and cell survival by modifying several essential microtubule proteins, including tubulin. Inhibiting TG2 reduced neurite length but not cell survival.

CONCLUSIONS: TG2 inhibitors can protect against organophosphate-induced neuropathy and could be used for developing novel therapeutic strategies for treating brain cancer and neurodegenerative disorders.

Key Words:
Organophosphate toxicity, Neurite outgrowth, Covalent adduct, Tissue transglutaminase.

Introduction

Chlorpyrifos (*O*, *O*-diethyl O-[3,5,6-trichloro-2-pyridinyl] phosphorothioate, CPF), also known as Dursban®, is an organophosphate compound that has been extensively studied in developmental neurotoxicity. *In vitro* studies[1-6] have shown that CPF inhibits neurite outgrowth, neuronal cell replication, and DNA synthesis in neural cell lines.

CPF mainly targets the enzyme acetylcholine esterase (AChE) in the peripheral and central nervous systems (CNS and PNS, respectively), but is not a very powerful inhibitor *in vivo*. However, it can undergo oxidative desulfuration, catalyzed mainly by cytochrome P450 oxidase, to become chlorpyrifos oxon (CPO), a more powerful AChE inhibitor[7,8]. CPF interferes with the AChE transduction cascade[3] and transcriptional events, influencing the nuclear transcription factors AP-1 and SP1 in the differentiation of neuronal cells[4,9] and neurotransmission[10]. However, CPF has neurotoxic effects on the developing rodent brain at concentrations that do not inhibit AChE, and inhibits protein and DNA synthesis, thereby affecting brain development[11].

In vitro studies on glial cells suggest that CPF affects cell replication and differentiation in such brain cells[6]. Further, CPF inhibits DNA synthesis and affects the acetylcholine signaling pathway[4], which controls the construction of cyclic AMP (cAMP), a vital switch signal that modulates cell differentiation[12]. Our group showed that CPF and its metabolite CPO inhibit glial cell differentiation in sodium butyrate (NaB)-treated C6 glioma cells[13]. Glial cells exposed to CPF show increased reactive oxygen species (ROS) formation[14]. ROS production is not related with direct chemical activities; instead, it relates to the influence of

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CPF on cell metabolism[9]. Cell culture studies[15,16] suggest that ROS disrupts calcium ion (Ca^{2+}) homeostasis.

Transglutaminases (TG) constitute a Ca^{2+}-dependent enzyme family, catalyzing protein post-translational modification[17,18,19]. TG are related with the maintenance of tissue and with preventing and protecting against physical damage; nevertheless, they are incorrectly activated in some pathologies[20,21]. TG2 dysregulation is associated with various human diseases; increased TG2 activity is associated with renal fibrosis, liver cirrhosis and fibrosis, diabetes, celiac disease, some cancers, and neurodegenerative diseases. TG activity is markedly increased in a variety of neurodegenerative diseases, rendering it a possible treatment target.

TG2 has a vital function in numerous processes in normal neural tissues. It is involved in CNS stabilization and synaptic plasticity via the construction of crosslinks resistant to protease[22]. TG also has a role in suppressing neuronal release of catecholamine, producing negative feedback that inhibits unnecessary transmitter release. TG2 is also important in axonal regeneration, where its activity is significantly upregulated[23]. A potential role of TG2 in neuronal cell differentiation is modulating their ability to activate AChE directly or indirectly, resulting in cAMP production and subsequent activation of cAMP response element-binding protein (CREB)[24,25]. cAMP/CREB acts as a transcription factor that modulates synaptic plasticity, cell survival, and cell differentiation[26].

Further, TG2's presence and its crosslinking action have been described in neurite adhesion sites or growth cones from cerebellar granule neurons, in which there are several TG2 substrates[27]. Some have proposed that TG2 could also be involved in cytoskeletal stabilization by covalently crosslinking microtubules with other cellular components. The formation of neurite adhesion sites and cytoskeletal stabilization are critical processes in neuronal development. TG2 is also present in rat neurons and astrocytes[28].

TG2 is Ca^{2+}-dependent; therefore, its activity can be disturbed by modified Ca^{2+} homeostasis and by the presence of ROS, which can be induced by organophosphates[29,30]. Phenyl saligenin phosphate (PSP), an OP that can inhibit neuropathy target esterase (NTE) activity and neurite outgrowth in differentiating mouse N2a neuroblastoma cells[31], decreases TG2 activity *in vitro* in N2a cells and increases TG2 activity in human HepG2 liver carcinoma cells after 24-h exposure. Further, N2a cells that had been treated with PSP had decreased TG2 protein levels, but that in PSP-treated HepG2 cells was increased, indicating that the protein level alterations might be related to changes in activity[32]. TG2 also acts in the outgrowth of neurites and the differentiation of nerves either through its catalytic activity or expression. Almami et al[33] studied this role along with its potential involvement in neurotoxicity by examining TG2 activity variation by exposing differentiating mouse N2a cells to the organophosphates PSP and CPO.

Neurotoxins affect TG activity in neuronal and hepatic cells, making TG2 a possible target for organophosphate toxicity. Glial cells play an important role in neurological disorders, and the effects of CPF and CPO on them have been studied. Both toxins inhibit C6 cell differentiation, used as a model of glial cell differentiation, although the glial cell type into which C6 cells differentiate in the presence of NaB is unclear[34]. The role of TG2 in C6 cell differentiation is also unclear, although it is essential for neuronal differentiation[23,24,35]. Therefore, the phenotype of the glial model used, the role played by TG2 in its differentiation, and how CPF and CPO affect TG2 in glial cells are of great interest.

Considering the increasing evidence of TG2 function in cell differentiation and outgrowth via either catalytic activities or expression, and its role in the neurotoxin mechanism of action, the present study was aimed at elucidating TG2 action regulation by organophosphates (CPO and PSP) during C6 cell differentiation. We also aimed to identify the TG2 protein substrates in the cells.

Materials and Methods

Dulbecco's Modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich (Nottingham, UK); DMEM containing 4.5 g/L glucose without L-glutamine was purchased from Lonza (Vivier, Belgium). Plastic cell culture products were purchased from Scientific Laboratory Supplies (Nottingham, UK)[36]. The rat C6 cells were purchased from ECACC (Sigma-Aldrich, Poole, UK).

C6 Cell Maintenance and Differentiation

C6 cell monolayers were cultured in T75 flasks in growth medium [20 mL DMEM supplemented

with 10% w/v fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 units/mL), and streptomycin (100 µg/mL)] at 37°C and 5% CO_2/95% air in a humidified incubator. The cells were subcultured when they were 60-80% confluent[37]. An appropriate volume of diluted cell suspension was seeded in the appropriate tissue culture vessels for the experiments. For the cell viability studies, 24-well culture dishes were seeded with 0.5 mL cell suspension per well (25,000 cells/well). For the sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and AChE activity assays, T25 or T75 flasks were seeded with 10 mL (500,000 cells) or 40 mL (2 million cells) cell suspension, respectively, and allowed 24-h recovery before cell differentiation was induced[5]. After recovery, the growth medium was replaced with filter-sterilized serum-free medium[21] containing 2 mM NaB with or without organophosphates. The cells were then incubated for 24 h.

Organophosphate Treatment of C6 Cells

Stock concentrations of PSP or CPO were dissolved in dimethyl sulfoxide (DMSO) and diluted to a final concentration of 10 µM in serum-free medium that had been warmed prior and that contained dibutyryl cAMP (0.3 mM). The C6 cells were plated in T75 flasks at a density of 50,000 cells/mL. Control cells were treated with a corresponding volume of dimethyl sulfoxide (DMSO) (0.05% [v/v]). The flasks were then incubated for 24 h in a humidified atmosphere of 95% air/5% CO_2 at 37°C. In some experiments, and to verify TG2 involvement, the cells were incubated with 100 µM Z-DON[33,38] (Zedira GmbH, Darmstadt, Germany) for 1 h before differentiation was induced.

Cell Extraction

For measuring TG2 activity, treated C6 cells were rinsed two times with chilled phosphate-buffered saline (PBS, 2 mL), and lysed with ice-cold lysis buffer (500 µL; 50 mM Tris-HCl [pH 8.0], 0.1% [v/v] protease inhibitor cocktail, 0.5% [w/v] sodium deoxycholate[34]). We clarified cell lysates by 20-min centrifugation at 4°C at 14 000 ×*g*. We collected the supernatants and stored them at −20°C. We measured the protein concentration using the bicinchoninic acid (BCA) protein assay (Sigma-Aldrich, Poole, UK) as described previously[39] before the TG2 activity assay. For Western blotting, the cells were rinsed two times with chilled PBS (2 mL), and lysed boiling PBS (500 µL) containing 0.1% (w/v) SDS. We boiled the suspension at 100°C for 5 min and centrifuged it for 30 min at 100,000 ×g, and collected and stored the supernatants at -20°C.

In vitro TG2 Biotin-X-Cadaverine Incorporation Assay

We performed the TG2 biotin-X-cadaverine incorporation assay as described by Lilley et al[40] Briefly, 96-well microtiter plates (Maxisorp Nunc, Saint Neots, UK) underwent overnight coating at 4°C with 250 µL N,N′-dimethyl casein (10 mg/mL in 100 mM Tris-HCl, pH 8.0). We washed the plate two times with distilled water and blocked with bovine serum albumin (BSA, 250 µL, 3% [w/v]) in 0.1 M Tris-HCl at pH 8.0, and then incubated at 37°C for 30 min. We washed the plate two times before the application of 150 µL Tris-HCl (100 mM, pH 8.0) that contained 225 µM biotin-X-cadaverine (a TG substrate commonly used in amine incorporating activity; (Invitrogen, Inchinnan, Renfrew, UK), 2 mM 2-mercaptoethanol, and either calcium chloride (6.67 mM) or EDTA (13.3 mM) for TG2 inactivation. We initiated the reaction by adding 50 µL sample, positive control (guinea pig liver TG2), or negative control (Tris buffer). Following 1-h incubation at 37°C, we washed the plates as described earlier. We added Tris-HCl (200 µL, 100 mM, pH 8.0) that contained ExtrAvidin HRP (1:500) to each well and incubated the plates for 45 min at 37°C, then washed them as before. We developed the plates with fresh developing buffer (200 µL, 7.5 µg/mL 3, 3′, 5, 5′-tetramethylbenzidine and 0.0005% [v/v] H_2O_2 in 100 mM sodium acetate, pH 6.0) and incubated them at room temperature for 15 min. We terminated the reaction via the addition of 50 µL sulfuric acid (5.0 M) and read the absorbance at 450 nm. One TG2 unit was described as an absorbance change at 450 nm per 1 h. We performed each experiment in triplicate.

In-Situ TG2 Biotin-X-Cadaverine Incorporation Assay

C6 cells (50,000 cells/mL; 300 µL medium/well) were seeded in LabTek 8-well chamber slides (Nalgene Nunc International, New York, NY, USA) and cultured for 24 h in fully supplemented DMEM. Then, we treated the cells for 1 h with Z-DON, and induced differentiation by adding serum-free medium that contained 10 µM PSP or CPO for 24 h. After 20-h differentiation, biotin-X-cadaverine [1 mM, 5-(((N-(biotinoyl)

amino)hexanoyl)amino) pentylamine, trifluoroacetic acid [TFA] salt, 1:100 [v/v] in DMEM; (Invitrogen, Inchinnan, Renfrew, UK) was added to the slides[33,38]. Following stimulation, we fixed the cells for 15 min with 3.7% (w/v) paraformaldehyde in PBS for 15 min at room temperature and permeabilized them for 15 min with 0.1% (v/v) Triton X-100 in PBS at room temperature. Every step was followed by washing with PBS for 3 × 5 min. Finally, we blocked the cells at room temperature with 3% (w/v) BSA for 1 h. Transamidated and crosslinked cadaverine substrates detected by staining with 1:200 (v/v) ExtrAvidin-FITC (green fluorescence). Nuclei were stained blue with DAPI (Invitrogen, Inchinnan, Renfrew, UK) and viewed at 400× magnification under a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

TG2 Protein Substrates

We measured biotin-X-cadaverine incorporation into proteins serving as TG2 substrates and in cellular proteins acting as substrates for endogenous TG2-catalyzed polyamine incorporation reactions. After the C6 cells had been stimulated, 1 mM biotin-X-cadaverine (1:100 (v/v) in DMEM) was added to the flask in the last 4 h of differentiation. The cell extracts (40 µg per lane) underwent SDS-PAGE and were transferred to nitrocellulose membranes. We probed the biotinylated proteins with ExtrAvidin HRP and visualized them with enhanced chemiluminescence (ECL). In some experiments, we enriched the biotinylated proteins with CaptAvidin agarose (Life Technologies Ltd., Paisley, UK) before the SDS-PAGE[33,38].

Measurement of Cell Viability

The C6 cells were plated in 24-well flat-bottom plates (15,000 cells/well) and cultured for 24 h in fully supplemented DMEM. After treatment, cell viability was determined by measuring the metabolic reduction of MTT to a colored formazan product. The cells were incubated for 1 h in 0.5 mg/mL MTT, after which the medium was removed and replaced with 200 µL DMSO. Absorbance was measured at 570 nm.

Proteomic Analysis of TG2 Biotinylated Substrate Proteins

C6 cell proteins were extracted after pretreatment with 1 mM biotin-X-cadaverine. We purified biotin-X-cadaverine-labeled proteins using CaptAvidin agarose (Life Technologies Ltd., Paisley, UK) before SDS-PAGE[33,38]. Protein bands were visualized using InstantBlue protein staining (Slough, UK). Differentially expressed TG2 biotinylated substrate proteins were removed and underwent in-gel digestion according to the method by Aldubyan et al[41]. In brief, the bands were removed from the gel and minced with a sterile scalpel. Then, the pieces underwent destaining with fresh 100% methanol and $(NH_4)HCO_3$ (50 mM, 1:1 [v/v]). For high-sequence coverage, the gel pieces underwent reduction and alkylation using freshly prepared 25 mM dithiothreitol (DTT) in 50 mM $(NH_4)HCO_3$ and were incubated with freshly prepared 55 mM iodoacetamide in 50 mM $(NH_4)HCO_3$ solution. The pieces were digested with trypsin (Trypsin Gold; Promega, Southampton, UK) overnight at 37°C. We terminated the reaction by adding 0.5% (v/v) TFA to a final concentration of 0.5% (v/v) and recovered the digested proteins by centrifugation. The supernatant containing the digestion solution was transferred to a new tube.

Mass Spectrometry and Protein Identification

The enriched biotin-X-cadaverine proteins were profiled using LC–MS/MS. In brief, samples were injected by trap-elute (4 µL) using an Eksigent 425 LC system (Shimadzu, Manchester, UK) (trap: YMC-Triart C18 0.3 × 5 mm, 300 µm ID; analytical column: YMC-Triart C18 150 × 0.3 mm, 3 µm, 5 µL/min) into a Sciex TripleTOF 6600 mass spectrometer in information-dependent acquisition (IDA, top 30) mode via gradient elution (mobile phase A: 0.1% formic acid; B: acetonitrile in 0.1% formic acid) over 87 min (2% to 30% B over 68 min; 40% B at 72 min, and then column wash and re-equilibration[33,38]). We processed the MS raw data using ProteinPilot 5.02 (Sciex, Warrington, UK) against the SwissProt mouse database (January 2019) and applied a 1% false discovery rate (FDR) protein cutoff. Statistical analysis (one-way analysis of variance [ANOVA] followed by Tukey's multiple comparison test and two-way ANOVA for group comparison) was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

Statistical Analysis

We report quantitative data as the mean ± SEM of a minimum of three independent experiments. We determined the statistical significance of differences with ANOVA (one- or

two-way), and then performed Tukey's post hoc test and/or Bonferroni's multiple comparison test, using (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered a significant difference.

Results

Effect of TG2 Inhibitor on C6 Cell Viability

To determine the toxicity of Z-DON, an irreversible TG2 inhibitor, in C6 cells, the cells were pre-treated with 50–150 μM Z-DON for 1 h and subjected to the 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. All concentrations of Z-DON alone had no significant effect on C6 cell viability (Figure 1).

Effect of Organophosphates on TG2 Activity in Differentiated C6 Cells

The C6 cells were treated with 2 mM NaB with or without 10 μM CPO or PSP[33] (Figure 2), and biotin-X-cadaverine incorporation, representing the acyl acceptor probe of TG2, was assayed. The data suggested a significant change in C6 cell TG2 activity in the presence of 10 μM PSP (n = 3, $p < 0.01$) or CPO (n = 3, $p < 0.05$) compared to that in undifferentiated control cells but not in untreated differentiated cells. Z-DON-treated cells in the presence of PSP (n = 3, $p < 0.001$) or CPO (n = 3, $p < 0.01$) showed significantly decreased TG2 activity compared to Z-DON-untreated cells.

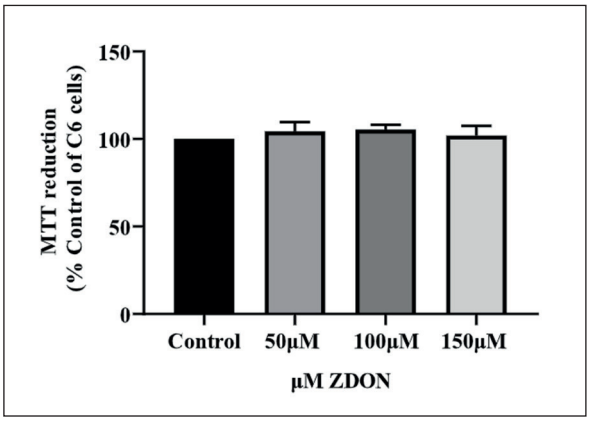

Figure 1. MTT determination of C6 cell viability. C6 cells were preincubated with 50–150 μM Z-DON for 1 h. Unstimulated cells were used as the control. Data points represent the mean ± SEM of three independent experiments. Data were analyzed using Bonferroni's multiple comparison test.

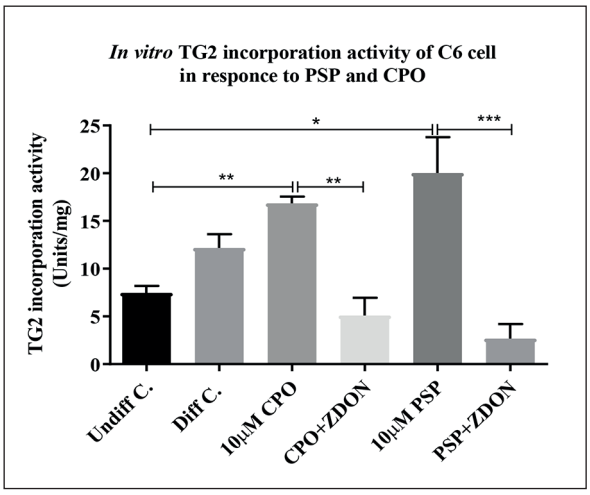

Figure 2. Biotin-X-cadaverine incorporation assay of C6 cell lysates. C6 cells were pretreated with 100 μM Z-DON for 1 h, and differentiation was induced using 2 mM NaB in the presence of 10 μM CPO or PSP for 24 h. Data are the mean ± SEM for activity specific for TG2 from three independent experiments. Data were analyzed using Tukey's multiple comparison test. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Undiff C, Undifferentiated cells.

Detection of TG2 Activity and Protein Substrates after CPO and PSP Exposure in the Presence or Absence of Z-DON

To identify TG2 biotinylated protein substrates in CPO- or PSP-treated cells, whole cell extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and visualized by ExtrAvidin horseradish peroxidase (HRP) probing. CPO and PSP treatment increased the incorporation of biotin-X-cadaverine into several proteins of different molecular masses in the cells. As expected, Z-DON pretreatment inhibited biotin-X-cadaverine incorporation into the TG2 protein substrates.

Effect of PSP and CPO on TG2 Activity in Differentiating C6 Cells

There was elevated biotin-X-cadaverine incorporation in numerous proteins in cells that had been treated with CPO (n = 3, $p < 0.05$) or PSP (n = 3, $p < 0.001$) compared to the undifferentiated control cells (Figure 3). These proteins had molecular weights (MW) of 25–150 kDa. The biotin-X-cadaverine-labeled proteins also showed reduced biotinylation in the presence of Z-DON, and the activity returned to that at baseline as in the control samples.

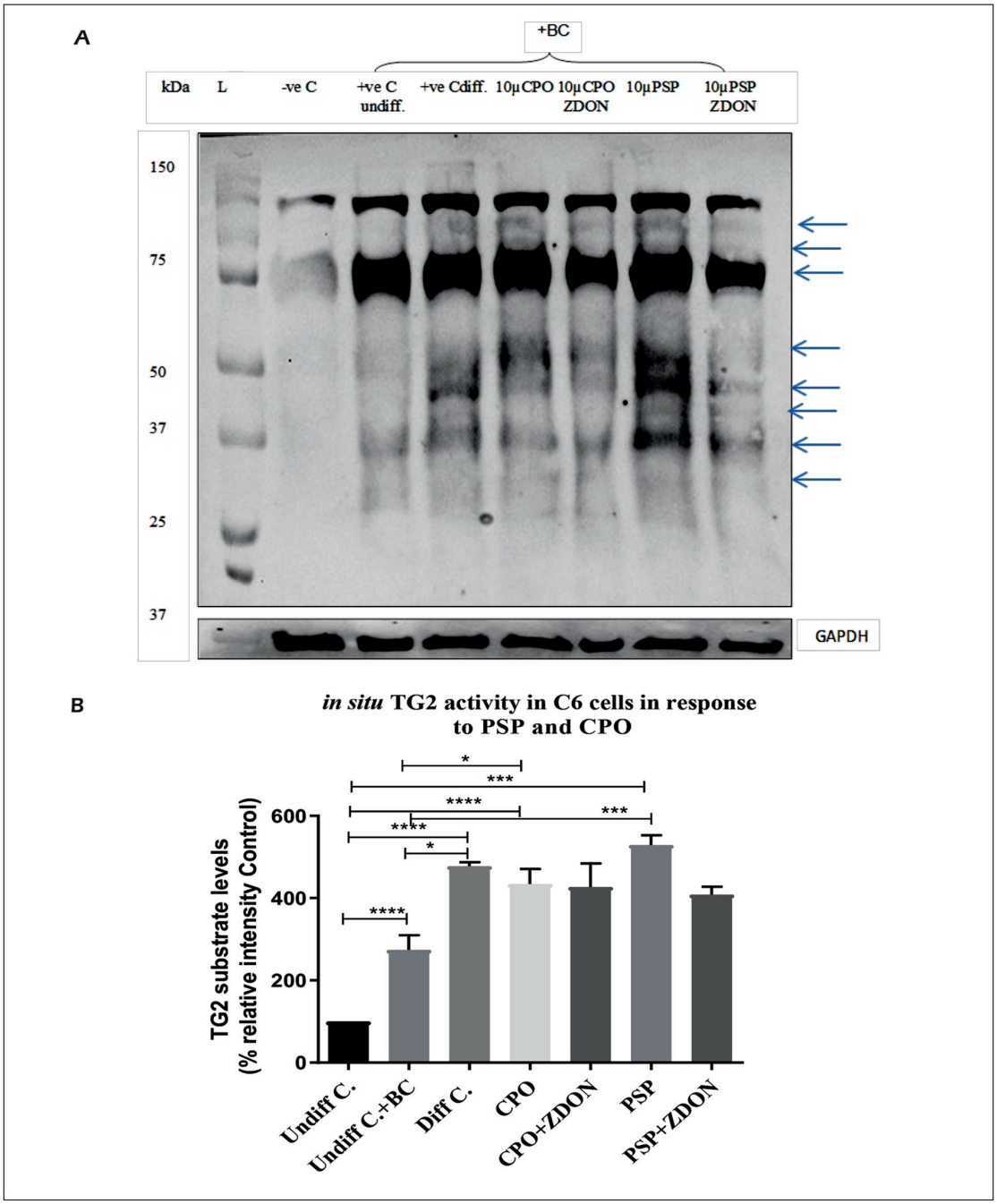

Figure 3. Detection of *in situ* TG activity and protein substrates in PSP- and CPO-treated C6 cells. **A,** Detection of TG2 transamidating activity and protein substrates. Samples were next analyzed on a separate blot. Anti-GAPDH antibody was the protein loading control. The arrows indicate prominent proteins labeled with biotin-ExtrAvidin. **B,** Densitometry of every lane (total labeled protein) was performed with GelQuant software (Life Technologies Ltd., Paisley, UK). The data are the percentage of basal TG2 substrate proteins after normalization to GAPDH. *$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Visualization of In Situ TG2 Activity after CPO and PSP Exposure in Differentiated C6 Cells

We investigated TG2 activity in intact cells. We developed an assay using a biotinylated probe (biotin-X-cadaverine) to assess *in situ* TG2 activity in differentiated C6 cells in response to CPO and PSP exposure[33].

We tested specific TG2 inhibitors to confirm that TG2 is responsible for CPO- and PSP-stim-

ulated TG activity in differentiated C6 cells. The cells underwent 1-h incubation with 100 μM Z-DON prior to 24-h stimulation with 10 μM CPO or PSP. Z-DON completely blocked CPO- and PSP-induced TG amine incorporation activity (Figure 4), confirming the involvement of TG2.

These results show that both CPO and PSP induced increases in biotin-X-cadaverine incorporation into the TG2 endogenous protein substrates. This biotinylation in living cells was reduced in the presence of Z-DON. These data are in agreement with the TG2 transamidation activity observed in the *in vitro* assay (Figure 2).

In situ TG2 activity was confirmed by fluorescein cell-penetrating staining of substrates of TG2. Z-DON significantly inhibited the increased TG activity in differentiated cells induced by CPO and PSP.

Effect of Z-DON on Neuroprotection in PSP- and CPO-Treated Differentiated C6 Cells

To determine the possible role of TG2 in C6 cell neuroprotection, we tested the effect of Z-DON in PSP- and CPO-induced differentiated C6 cell death. Cellular viability was determined by MTT reduction assay; C6 cells were preincubated with or without 100 μM Z-DON for 1 h prior to 10 μM PSP or CPO treatment and induction of differentiation.

PSP and CPO induced significantly reduced cell viability ($p < 0.0001$ *vs.* untreated differentiated C6 cells [control]) (Figure 5). Pretreating the cells with Z-DON significantly reversed the PSP- and CPO-induced cell death ($p < 0.0001$ *vs.* PSP- and CPO-treated cells).

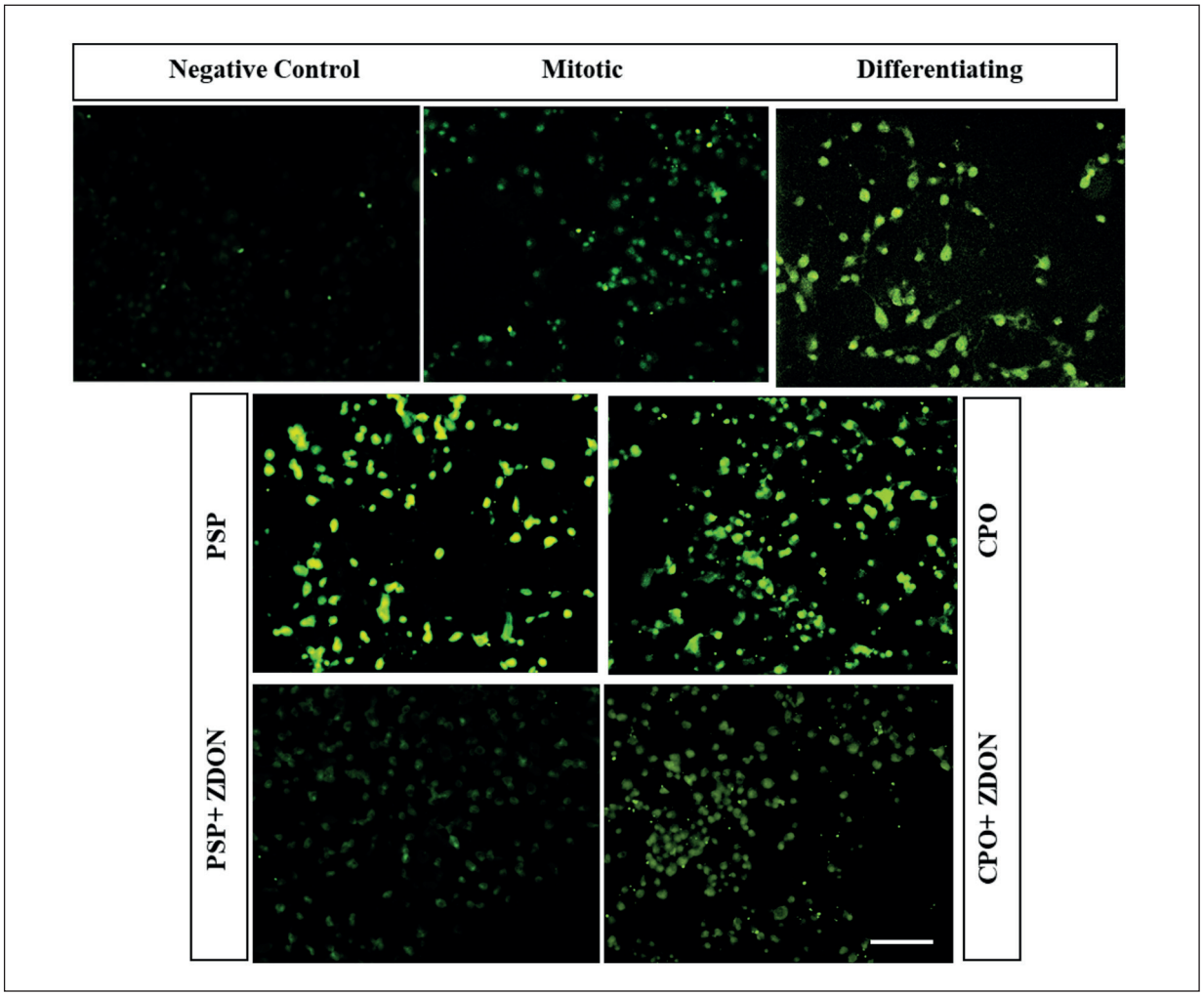

Figure 4. Visualization of TG2-mediated biotin-X-cadaverine incorporation into intracellular proteins with ExtrAvidin-FITC (*green*). Untreated cells were used as the negative control in the absence or presence of BTC (*mitotic*). Nuclei were stained with DAPI (*blue*). Magnification, 20×. The images shown are representative of three independent experiments.

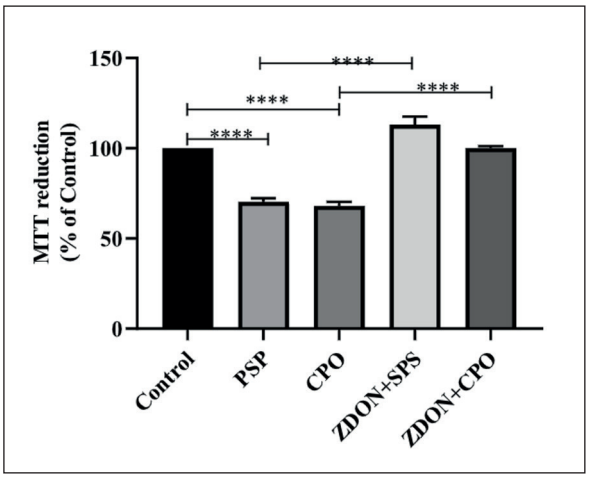

Figure 5. MTT assay showing the reduction in differentiated C6 cell viability induced by PSP and CPO. Z-DON pretreatment significantly reversed the organophosphate-induced cell death. Unstimulated cells were used as the control. The MTT assays were performed in duplicate. Data points represent the mean ± SEM of three independent experiments. Data were analyzed using Bonferroni's multiple comparison test. ****$p < 0.0001$ *vs.* untreated cells and *vs.* PSP- and CPO-treated cells.

Identification of TG2 Substrates in Differentiated C6 Cells

Based on a MW ladder, the protein bands showing TG2 biotin-X-cadaverine incorporation activation or inhibition were removed from the gels according to their MW (Figures 3 and 6). The proteins were removed from the gel and digested with trypsin after reduction and alkylating. The samples were directly analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), in which multiple proteins were identified within a single protein band. Table I lists the proteins with a high score and sequence coverage (SC) following peptide mapping.

The biotin-X-cadaverine-labeled TG2 substrate proteins were analyzed by MS. The data of the identified proteins are reported according to the high score and SC percentage (SC%). The MW are indicated in kDa.

Nearly 26 proteins in the seven excised bands were identified as TG2 substrates and had MW of 30–150 kDa. These targets include cytoskeletal organizing proteins, chaperone and folding proteins, Ca^{2+}- and phospholipid-binding proteins, and proteins involved in vesicle transport processes.

Based on the TRANSDAB database (http://genomics.dote.hu/wiki/index.php/), some of the proteins identified have been described as substrates of TG2[42]. However, they have not been identified in differentiated C6 cells, which renders them novel TG2 substrates; these proteins include heat shock protein (HSP)90 β; stress-70 protein, mitochondrial; actin; tubulin; elongation factor 2; kinesin-1 heavy chain; vimentin; and heterogeneous nuclear ribonucleoprotein. Some of these TG2 substrates have been identified in cardiomyocytes[38,43].

Tubulin as a Target for TG2 Activity

TG2 could be involved in neuroprotection, and this role seems to be modulated by its amine-incorporation activity on several substrate proteins. It is therefore important to confirm whether selected target proteins are colocalized with TG2 activity in intact cells. Here, tubulin α/β, the main building blocks of microtubule filaments and therefore cytoskeleton organizing proteins,

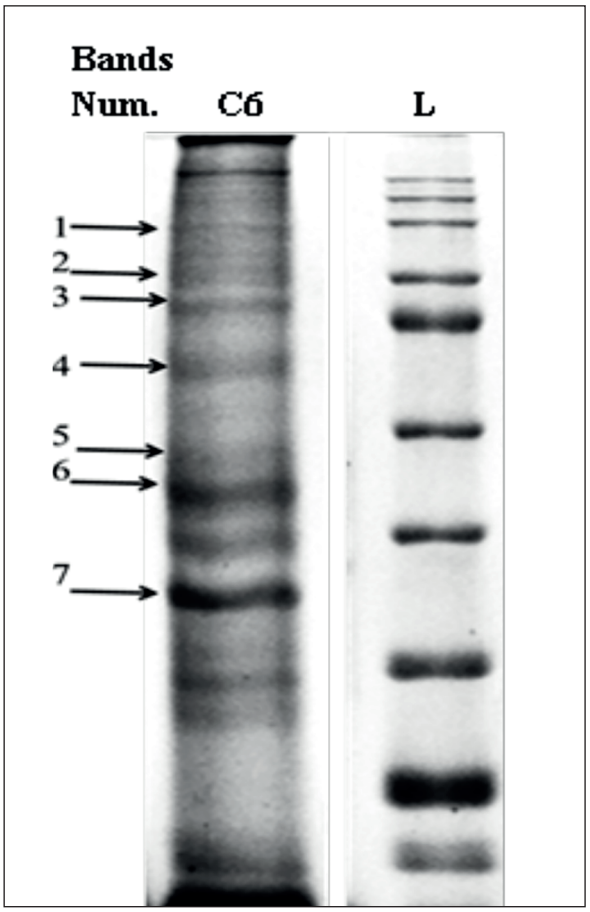

Figure 6. Blot showing treated C6 cell proteins fractionated using CaptAvidin beads. Aliquots of bound proteins (40 µg) were loaded onto 12% gels stained with InstantBlue protein stain. The arrows indicate the 7 excised bands selected for protein identification that range from 100-40kDa. L, Ladder.

Table I. TG2 substrate proteins identified in differentiated C6 cells by MS.

Band	Accession number(s) substrates	% Coverage (95)	Proteins identified as potential TG2	Peptide matches	Approx. mass (kDa)
1	Q61768	6.54	Kinesin-1 heavy chain*	7	109
2	P58252	12.12	Elongation factor 2*	7	95
	P11499	9.81	HSP90 β	7	83.281
3	P48678	41.05	Prelamin A/C*	28	74
	P20029	32.21	78-kDa glucose-regulated protein*	18	72.422
	Q9D0E1	22.09	Heterogeneous nuclear ribonucleoprotein*	17	34.196
	P26041	24.09	Moesin*	16	67.767
	P38647	21.65	Stress-70 protein, mitochondrial*	7	73.461
4	P48678	41.05	Prelamin A/C	28	74.238
	P20029	32.21	78-kDa glucose-regulated protein	18	72.422
	P26041	24.09	Moesin	16	67.767
5	P63260	18.67	Actin, cytoplasmic 2*	11	41.793
	P60710	18.67	Actin, cytoplasmic 1	11	41.737
	P27773	13.86	Protein disulfide isomerase A3*	6	56.678
	P52480	14.50	Pyruvate kinase	6	57.845
6	P20152	42.06	Vimentin	25	54
	P99024	25.23	Tubulin β-5 chain	17	50
	P68372	25.17	Tubulin β-4B chain*	16	49.831
	Q9D6F9	19.82	Tubulin β-4A chain*	13	49.586
	Q9CWF2	22.47	Tubulin β-2B chain*	17	49.953
	Q7TMM9	22.47	Tubulin β-2A chain*	17	49.907
	P68373	19.82	Tubulin α-1C chain*	9	49.909
	P05213	19.73	Tubulin α-1B chain*	9	50.152
	P68369	19.73	Tubulin α-1A chain*	9	50.136
7	P63260	41.87	Actin, cytoplasmic 2*	30	41.793
	P60710	41.87	Actin, cytoplasmic 1	30	41.737

*Indicates unknown TG2 substrates according to the TRANSDAB database of Csősz et al[42].

were among the TG2 substrates identified (Table I), suggesting TG2's role in the dynamic regulation of microtubule assembly in differentiated C6 cells and the associated cytoskeleton proteins.

Immunohistochemistry using anti-tubulin antibody yielded evidence of direct tubulin biotin-X-cadaverine labelling in PSP- and CPO-treated differentiated C6 cells.

C6 cells were cultured in chamber slides and underwent 1-h treatment with 100 μM Z-DON before 24-h incubation with 10 μM PSP or CPO and 2 mM NaB. After 20 h, we added biotin-X-cadaverine (1 mM), and untreated cells in the absence (undifferentiating) or presence (differentiating) of NaB were used as the controls. The TG2-mediated biotin-X-cadaverine incorporation into intracellular proteins was visualized using ExtrAvidin-FITC staining; nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Figure 4). Microtubules were detected using mouse anti-tubulin monoclonal antibody and visualized with anti-mouse Alexa 568 secondary antibody (Figure 7).

To confirm that tubulin is a TG2 substrate, we performed immunofluorescence staining to confirm the colocalization of tubulin and TG2 activity in the presence or absence of PSP and CPO (Figure 7). We observed colocalization of tubulin and TG2 incorporation activity in the PSP- and CPO-treated cells (red + green = yellow/orange), which was more concentrated around the nuclei. However, too many cells were detached, likely owing to cell death induced by the organophosphate treatment.

Discussion

Our results reveal a clear role for TG2 activity in organophosphate-mediated neurotoxicity and therefore its potential use in neuroprotection. In addition to its amine incorporation activity, TG2 activates the crosslinking, deamidation, and activation of protein disulphide isomerase, protein kinase, and esterase[38-43]. However, the amine incorporation activity plays a critical role in cell physiological functions and disease progression[44,45]. For example, TG2 shows an optimum amount of activity under normal conditions, regulating cell growth, proliferation, differentiation

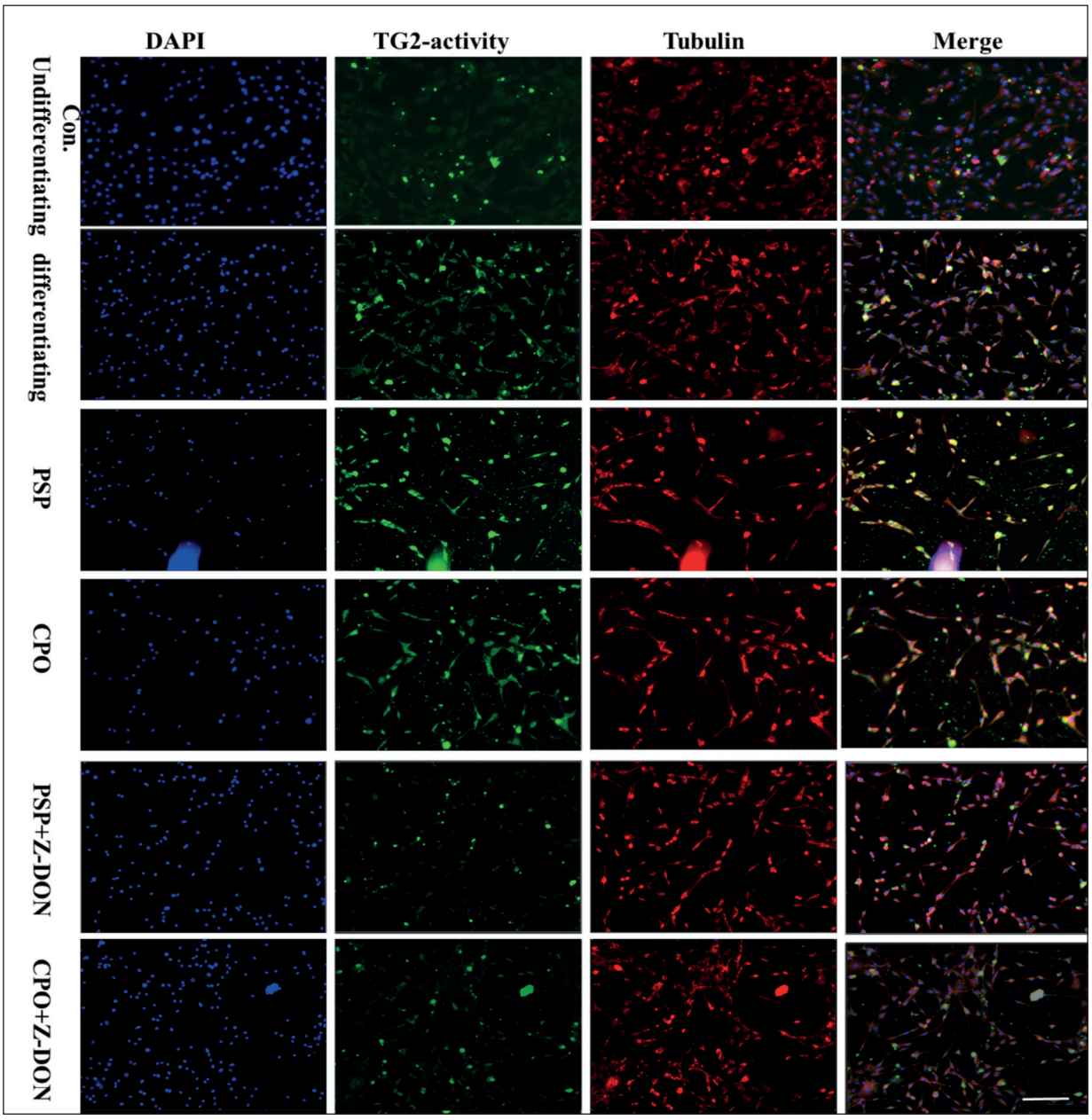

Figure 7. Colocalization of tubulin with TG2 activity, indicating its function as a TG2 cytoskeleton substrate. Microtubules were detected using mouse anti-tubulin monoclonal antibody and visualized with anti-mouse Alexa 568 secondary antibody (*red*). The merge photograph shows the colocalization of tubulin (*red*) and TG2 activity (green), appearing as yellow. The magnification was 20x.

signaling, and homeostasis, and therefore promoting cell survival[46,47]. However, in extremely stressful environments, upregulated TG2 activity can induce cell death[48,49]. In the present study, organophosphate-treated C6 cells showed significantly increased TG2 amine incorporation activity (Figures 2 and 3), and this activation was correlated with decreased cell viability and toxicity (Figure 5).

Our group has reported TG2 activation by organophosphates in N2a cells, revealing a novel covalent adduct between TG2 and organophosphates[33]. Further, CPO can exert neurotoxicity by directly inhibiting extension outgrowth in differentiated C6 cells and therefore potential alteration in the related microtubule proteins[13]. Different types of neuronal-like cells show phenotypic alteration after organophosphate exposure[50,51].

Some organophosphates can reduce neurite-like process length in N18 cells and promote their formation in C6 cells, while others cannot[52]. These conflicting observations could be due to differences in experimental parameters such as organophosphate concentration, exposure time, and cell type. Here, the organophosphate-treated cells showed reduced cell viability in comparison to the controls (differentiated cells) and cells treated with organophosphates plus Z-DON. Moreover, double-staining (Figure 7) revealed increased neurite length in organophosphate-treated cells compared to the other treated cells, and some reduction in attached cells. Z-DON reversed this effect, suggesting that TG2 may have a role in neuroprotection.

TG2 plays a role in N2a cell survival, and neurite outgrowth occurs *via* TG2 activation by nerve growth factor[33]. It would be of interest to confirm this protection by using other end points such as extracellular signal-regulated kinases, ROS, or by inhibiting NTE. Further investigation is needed to confirm neurite length reduction by TG2 inhibitors *via* specific markers such as MAP2 dendritic marker, βIII-tubulin, and nestin[53,54], and applying specific assays for measurement.

Many intracellular and extracellular proteins can serve as TG2 substrates for the amine incorporation activity essential to cellular biological functions[38,43]. Moreover, TG2 has pathological and protective roles in different diseases[55-57]. Therefore, the recognition of proteins that act as TG2 substrates is essential to biomedical research for developing novel drug targets or diagnostic markers.

In the present study, 26 proteins were identified as TG2 substrates in C6 cells. Based on the TRANSDAB database, some of these proteins have been described as substrates of TG2, but not in differentiated C6 cells[42]. This includes 78-kDa glucose-regulated protein Hspa5 chaperone, a quality control protein in the lumen of the endoplasmic reticulum involved in misfolded protein degradation and correct protein folding[58]. It also has an important function in neurological disorders and acts as a sensor of stress and in embryonic development of the CNS[59]. It also has an important role in protection against neuronal apoptosis[60]. In an earlier study of N2a cells, our group demonstrated an increased regulation of a 78-kDa glucose-regulated protein in response to diazinon[61]. This protein was identified as a TG2 substrate in H9c2 cardiomyocytes[33,38].

HSP90 β and stress-70 cognate are chaperone proteins involved in cell protection, protein folding, and transporting, and are upregulated in mouse brain after sarin insult[62]. Actin and tubulin (microtubule proteins) are the major protein components of the cytoskeleton and are significantly expressed in differentiated neuroblastoma cell lines[63] and differentiated human mesenchymal stem cells[64]. The effect of CPO on organophosphorylated tubulin β-5 and its role in neurotoxicity has also been studied in mouse brain[65]. Moreover, tubulin polyamination by TG2 is essential for stabilizing microtubules and therefore maintaining neuronal structure and functions[66,67]. Tubulin β-5 and the other α/β tubulin chains appear to be novel TG2 substrates. Moesin plays an important role in actin rearrangement in the cytoskeleton and modulates neuronal morphogenesis and long-term memory formation[68].

Kinesin-1 is a microtubule molecular motor and is involved in transporting cellular organelles and proteins. The most prevalent post-translational interaction of tubulin is that of tyrosinated α-tubulin with kinesin-1 in glioblastoma cells, indicating potential application as a novel therapeutic target[69]. Elongation factor 2 is a protein involved in regulating protein synthesis during the elongation stage in different cell lines[70]. Its overexpression has been linked with cardiovascular and neurodegenerative diseases and cancer, which makes it a promising therapeutic target[71,72].

Prelamin is a precursor to mature lamin A, a nuclear cytoskeleton protein of the intermediate filament family of proteins. Its overexpression is involved in cell survival, stiffness, and migration[70]. Vimentin is another intermediate filament protein and is an astrocyte-specific marker in healthy human brain[73]; it is upregulated in intracerebral hemorrhage-induced brain injury[74]. Vimentin downregulation decelerated glial scar formation and depressed immune response, indicating its role in inflammatory responses[75]. Moreover, it appears to play many other roles in regulating astrocyte structure through its involvement in nestin and synemin translation[76].

Conclusions

Our results indicate that the organophosphate-induced amine incorporation activity of TG2 may have a direct effect on neuronal outgrowth, differentiation, and cell survival. This enzymatic activity affects several essential microtubule proteins, and its inhibition reduced neurite length but not cell survival. These results

are of interest because they indicate that TG2 inhibitors protect against the neuropathic effects of organophosphates and facilitate the development of novel therapeutic strategies for treating brain cancer and neurodegenerative disorders.

Conflict of Interest
The Authors declare that they have no conflict of interests.

Ethical Approval
The work does not require ethical approval.

Data Availability
All data are available upon reasonable request.

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