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Diazoxon disrupts the expression and distribution of βIII-tubulin and MAP 1B in differentiating N2a cells

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Abstract: This study aimed at assessing the effects of diazoxon (DZO), a major metabolite of the insecticide diazinon (DZ), on key cytoskeletal proteins in differentiating N2a neuroblastoma cells. Initial experiments established that sub-lethal concentrations of 1, 5 and 10 μM DZO produced profound inhibition of neurite outgrowth. Densitometric scanning of probed immunoblots of N2a cell lysates demonstrated that DZO had no effect on total β-tubulin levels. However, probing with a monoclonal antibody that recognised specifically the βIII-tubulin isotype revealed that 10 μM DZO induced a significant reduction in the levels of this particular form. Levels of polyglutamylated tubulin were not altered. Exposure to 10 μM DZO also decreased the expression of microtubule associated protein 1B (MAP 1B). However, DZO had no effect on the expression of MAP tau. DZO also failed to affect the levels neurofilament light (NFL) and neurofilament medium (NFM) chain levels. Indirect immunofluorescence demonstrated that the staining of neurites in treated cells was weaker than in the controls for βIII-tubulin. In conclusion, DZO disrupts the microtubule (MT) network affecting the expression and distribution of two specific MT proteins known to be important in neuritogenesis. DZO may contribute to the developmental neurotoxicity seen following exposure to DZ.

Keywords: Diazoxon; N2a neuroblastoma; Neurite outgrowth inhibition; βIII-tubulin; MAP 1B; Organophosphorothionate insecticide

Abbreviations: AChE, acetylcholinesterase; CP, chlorpyrifos; DMEM, Dulbecco's modified Eagle’s medium; DMSO, dimethyl sulphoxide; DZ, diazinon; DZO, diazoxon; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; MAP 1B, microtubule associated protein 1B; MT, microtubule; NF, neurofilament; NFL, neurofilament light chain; NFM, neurofilament medium chain; OP, organophosphorus ester; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; TRITC, tetramethyl rhodamine isothiocyanate.

Introduction

Organophosphorus esters (OPs) comprise the largest group of insecticides globally employed; however, due to their toxicity, they can cause damaging effects on mammals, birds and the environment in general. OPs can induce in adults a number of distinct neurotoxic effects [1], among which the most frequently encountered is acute neurotoxicity due to the inhibition of acetylcholinesterase (AChE). In recent years, however, an increasing number of in vivo studies indicate that OP insecticides, and particularly the extensively used organophosphorothionates chlorpyrifos (CP) and diazinon (DZ), can also cause developmental neurotoxicity [2, 3]. This toxicity is related to the ability of these OPs to interfere with a number of neurodevelopmental events including the process of neuronal differentiation and axon formation [4, 5].

Phosphorothionates are known to be metabolically converted in vivo to their desulphurated, oxon analogues by specific mixed function oxidases (cytochrome p450s), mainly in the liver [6]. In vitro studies using a variety of cell culture systems demonstrate that the oxon metabolites of both CP and DZ have the capacity to directly disrupt the process of neurite outgrowth, a widely employed marker of neuronal differentiation in vitro. Thus, the oxon metabolite of CP, chlorpyrifos oxon (CPO) inhibits the outgrowth of axonal processes in primary cultures of embryonic rat superior cervical [7] and dorsal root [8] ganglia neurons. CPO can also impair the extension of neurites in the rat PC12 pheochromocytoma [9] and mouse N2a neuroblastoma [10] cell lines. The ability to disrupt the process of neurite development in the N2a cell line is also exhibited by the oxon metabolite of DZ, diazoxon (diazoxon, DZO).

The neuronal cytoskeleton, and in particular microtubules (MTs) and neurofilaments (NFs), is one of the most important endogenous factors that control normal neurodevelopment including the process of axonal/neurite outgrowth [11]. This study sought to assess in differentiating mouse N2a neuroblastoma cells the effects of DZO on the expression and distribution of a number of MT and NF proteins which are neurodevelopmentally important and may be involved in neurite outgrowth and stability.

DZO was employed at three different concentrations, 1, 5 and 10 μM, which were found to cause extensive impairment of neurite outgrowth. These DZO concentrations were previously shown to be non-cytotoxic in our laboratory using the same differentiating cell line [12].

Materials and methods

Materials. The mouse neuroblastoma cell line was obtained from ICN (Thane, UK). Cell culture plastic materials were supplied by Scientific Laboratory Supplies (Nottingham, UK). Cell culture reagents, as well as mouse monoclonal antibodies to total α-tubulin (B512), total β-tubulin (TUB 2.1), β III-tubulin (SDL.3D10) and polyglutamylated tubulin (clone B3) were purchased from Sigma-Aldrich Co. Ltd (Poole, UK). Mouse monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-32233), rabbit polyclonal antibody against MAP 1B (H-130; sc-25729) and goat polyclonal antibodies against tau (C17; sc-1995), NFL (N-14; sc-12966) and NFM (G-20; sc-16143) were obtained from Santa Cruz Biotechnology (CA, USA). Anti-phosphorylated NFH mouse monoclonal antibody SMI34 was supplied by Sternberger Monoclonals Inc. (Berkeley, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit and anti-goat secondary antibodies were bought from DakoCytomation (Ely, UK). Fluorescein isothiocyanate (FITC)-conjugated goat anti rabbit immunoglobulin and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse immunoglobulin were also purchased from DakoCytomation. Diazoxon (purity 97.6 %) was supplied by Chem Service Inc. (West Chester, PA, USA). All other chemicals and reagents were the best grade available from Sigma-Aldrich Co. Ltd (Poole, UK).

Growth and maintenance of N2a cells. N2a cells were maintained as monolayers in the logarithmic phase of growth at 37°C in a humidified atmosphere of 5% CO_2 and 95% air employing growth medium comprising Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v foetal calf serum, 1 mM glutamine, penicillin G (100 U/ml) and streptomycin (100 μg/ml), as described previously [14, 15] and sub-cultured at 60-80% confluence.

Induction of N2a cell differentiation and DZO treatment. N2a cells, between passage 10 and 20, were detached by pipette and harvested by centrifugation. Cell pellets were resuspended in 1 ml growth medium and cell number determined in a haemocytometer chamber. For determination of neurite outgrowth, N2a cells, detached at 60-80% confluence, were plated out in 24-well culture dishes at a density of $5x10^4$ cells/ml, with each well receiving 0.5 ml of cell suspension. The cells were allowed to recover for 24 h in a CO_2 incubator and then, to induce differentiation, the growth medium was carefully withdrawn and replaced with serum-free medium containing 0.3 mM dibutyryl cAMP and with or without DZO. Cells were returned to the incubator and allowed to differentiate for 24 h. DZO was freshly prepared as 200-fold concentrated stock solutions in dimethyl sulphoxide (DMSO) and added to the serum-free medium. The final DMSO concentration in the medium of DZO-treated and untreated control cells was 0.5% v/v. In the present study, DZO was employed at final concentrations of 1, 5 and 10 μM. These concentrations have been previously shown to non-cytotoxic towards the differentiating N2a cells, as determined by two different cell viability assays [12].

Assessment of neurite outgrowth. Following differentiation for 24 h, N2a cell monolayers were fixed with 90% methanol in Tris-buffered saline (TBS; 10 mM Tris, 140 mM NaCl, pH 7.4) and stained with Coomassie Brilliant Blue R-250. They were then washed with TBS and viewed with the aid of an inverted light microscope [14]. Alternatively, they were stained with anti-tubulin or anti-NFH antibodies as described below. From each well, five randomly selected fields giving a total cell count of 200-300 cells/well were examined. In each well the total number of cells and the total number of axon-like processes were counted and the mean number of axon-like processes per 100 cells was calculated. An axon-like process was defined as an extension with a length greater than two cell body diameters [16].

Gel electrophoresis and Western blotting. For Western blotting analysis, N2a cells were seeded in T25 culture flasks in a volume of 10 ml growth medium at a density of $5x10^4$/ml. They were then induced to differentiate in the absence or presence of 1, 5 or 10

μM DZO, as described above. At the end of the differentiation period and prior to cell lysis samples were checked microscopically for proper neurite development (untreated control cells) and neurite outgrowth inhibition (DZO-treated cells). Intact cell monolayers were then solubilised by boiling in 1 ml electrophoresis sample buffer containing 62.5 mM Tris, 2% w/v sodium dodecyl sulphate (SDS), 10% w/v β-mercaptoethanol and 0.002% w/v bromophenol blue. The resultant cell lysates were subsequently subjected to gel electrophoresis in the presence of sodium dodecyl sulphate (SDS/PAGE) employing either a 7.5% w/v or a 10% w/v polyacrylamide resolving gel, as appropriate, overlaid with a 4% w/v stacking gel [17]. Separated proteins were then transferred electrophoretically onto nitrocellulose membrane filters [18]. The resultant Western blots were checked for equal protein loading and efficient transfer by staining with 0.05% w/v copper pthalocyanine [19] and were then blocked with 3% w/v non-fat milk powder (Marvel) in phosphate-buffered saline (Marvel/PBS) containing 0.05% w/v Tween-20 for at least 1 h at room temperature. The blots were then probed overnight at $4^{o}C$ with appropriate dilutions of primary antibodies in Marvel/PBS. The antibodies included the mouse anti-total β-tubulin (dilution 1:1000), mouse anti-β-tubulin III (1:1000), anti-polyglutamylated tubulin, rabbit anti-MAP1B (1:200), goat polyclonal anti-tau (dilution 1:500), goat anti-NFL (dilution 1:200), goat anti-NFM (1:100) and anti-GAPDH (1:1000). Following six 10-min washes with PBS containing 0.05% w/v Tween 20 (PBS/Tween), the blots were probed with appropriate dilutions of HRP-conjugated anti-mouse, anti-rabbit or anti-goat secondary antibodies for 3 h at room temperature. After six further washes with PBS/Tween, antibody reactivity was visualised with enhanced chemiluminescence reagent (ECL). For quantification of antibody reactivity, densitometric scanning of images from probed Western blots was performed by using the Quantiscan image analysis system (Version 3; Biosoft), as described previously [19]. The band densities for total β-tubulin, βIII-tubulin, polyglutamylated tubulin, MAP 1B, tau, NFL and NFM were normalised to band densities for GAPDH.

Indirect immunofluorescence staining of N2a cell monolayers. For this, 200 μl of cell suspension at 10^{4} cells/ml were seeded into cell culture chamber slides. Following recovery, cells were induced to differentiate for 24 h in the absence and presence of 10

μM DZO, after which cell monolayers were fixed for 10 min at -20ºC in 90% methanol in PBS, blocked and incubated with either anti-α-tubulin, anti-βIII-tubulin, anti-MAP 1B or anti-phosphorylated NFH (all diluted at 1:200 in 3 % w/v BSA in PBS) and either FITC- or TRITC-conjugated secondary antibodies (diluted 1:100 in 3% w/v BSA in PBS) as described previously [20], except that the anti-fade mountant contained the nuclear staining agent DAPI (Vector Laboratories Inc.)

Statistical analysis. Quantitative data were expressed as mean ± SEM for at least 3 independent experiments. Statistical analysis for multiple comparisons was performed using one way ANOVA with a post-hoc Tukey test, using 95 % confidence limits. Otherwise the Mann Whitney U-test was employed.

3. Results

Preliminary experiments showed that exposure for 24 h to DZO concentrations of 1, 5 and 10 μM used in this study produced approximately 50 – 75 % inhibition of neurite outgrowth (data not shown), which was similar to earlier data [12]. This was further confirmed by measurement of axon-like processes in cells stained with antibodies to total α-tubulin and phosphorylated NFH (Fig 1).

In order to reveal the nature of the biochemical phenomena that underlie these potent effects of DZO on cell morphology, Western blots of extracts of N2a cells exposed to the above oxon concentrations for 24 h were probed with a number of antibodies that are known to recognise key proteins of the neuronal cytoskeleton, since the latter is important in cell morphology and the process of neurite outgrowth. As can be seen in the probed blots in fig. 2, the cross-reactivity of N2a cell lysates with a monoclonal antibody that recognises total β-tubulin was apparently unaffected by treatment with DZO, at all concentrations employed. In contrast, probing of these blots with a monoclonal antibody that recognises specifically isotype III of β-tubulin revealed that extracts of N2a cells that had been exposed to higher concentrations of DZO exhibited decreased cross-reactivity compared to untreated controls. On the other hand, cross-reactivity with an antibody

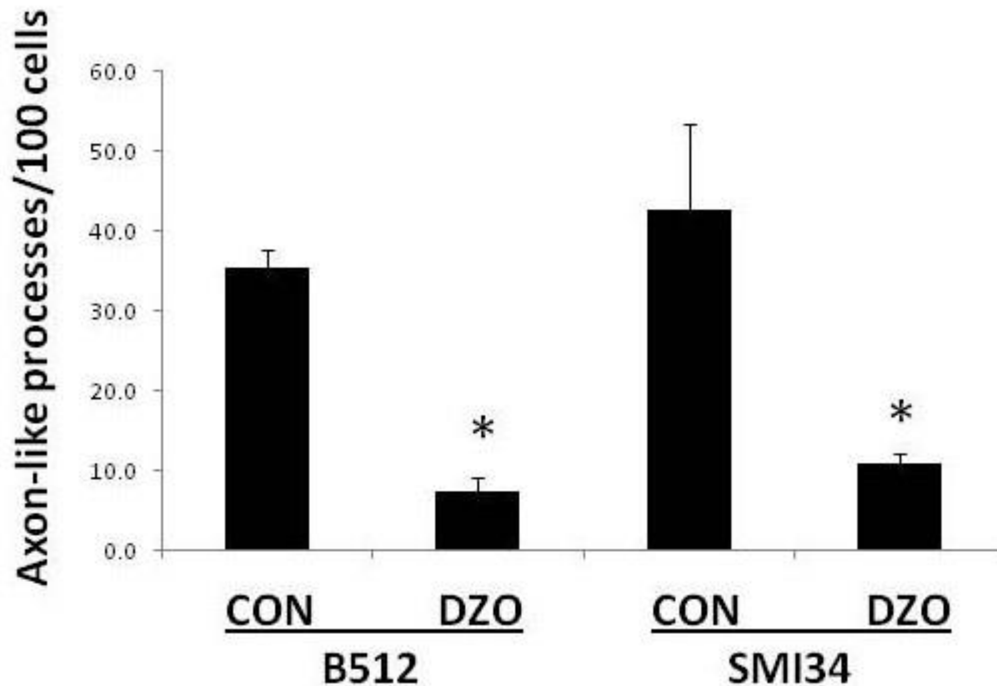

Fig. 1. Effects of diazoxon on neurite outgrowth. N2a cells were induced to differentiate in the absence (control) and presence of 10 μM DZO , stained with antibodies to total α-tubulin (B512) and phosphorylated NFH (SMI34) and assessed for neurite outgrowth as described in Materials and methods. Shown are mean numbers of axon like neurites per 100 cells ± S.E.M. Asterisks indicate significant differences compared to the corresponding control ($p<0.05$).

recognising polyglutamylated tubulin seemed to be unchanged following DZO treatment. By contrast, cross-reactivity of blots with an antibody recognising MAP 1B appeared to be reduced in DZO-treated cells compared to controls. On the other hand, exposure of N2a cells to all DZO concentrations for 24 h had no apparent effect on the levels of cross-reactivity with an antibody recognising tau protein. Finally, probing of blots with antibodies recognising the NF proteins NFL and NFM demonstrated no apparent differences in cross-reactivity between DZO treated samples and controls (data not shown).

Quantification of the observed alterations in antibody reactivity was subsequently carried out employing Quantiscan image analysis software. Densitometric analysis confirmed the

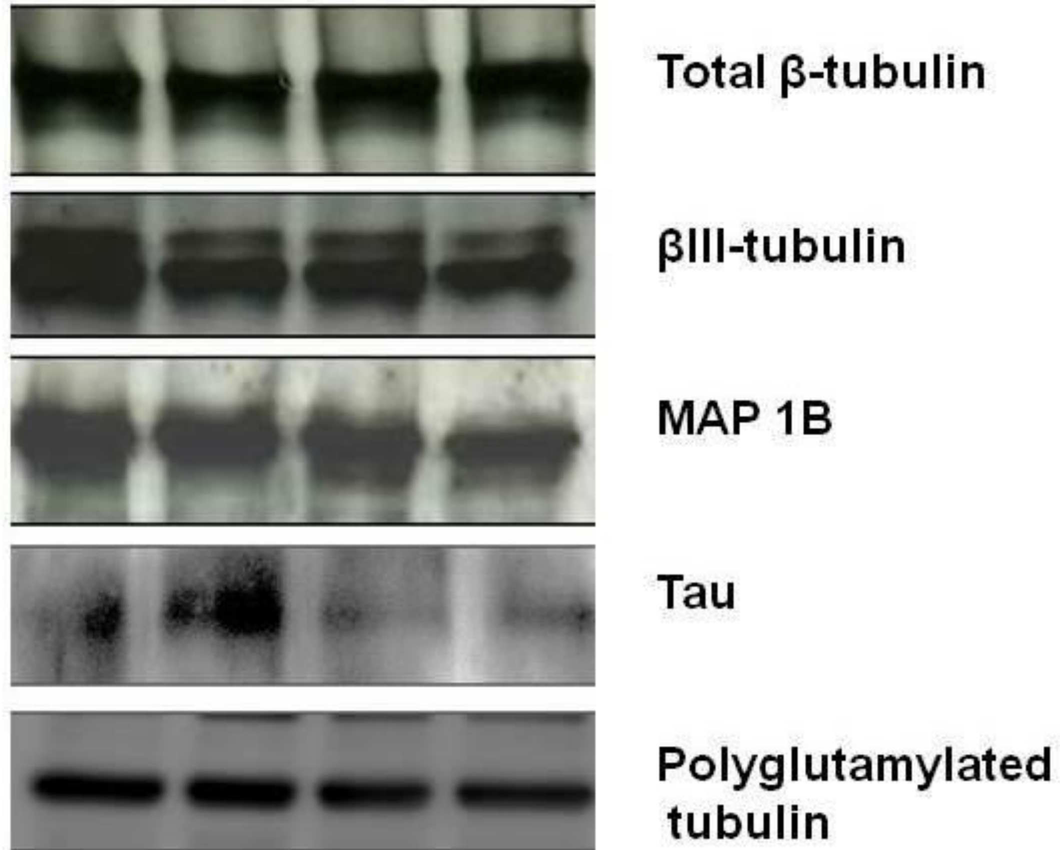

Fig. 2. Western blotting analysis of proteins in control and DZO-treated cell extracts. N2a cells were induced to differentiate for 24 h in the absence or presence of 1, 5 and 10 μM DZO prior to extraction of the cell monolayers. Proteins were then separated by SDS-PAGE, subjected to Western blotting and the resultant blots probed with monoclonal antibodies against total β-tubulin, βIII-tubulin and MAP1B, as described in Materials and methods. Shown are representative digital images taken using the FUJIFILM LAS-3000 ECL camera. Blots probed with antibodies against the two neurofilament proteins for which the densitometric analysis demonstrated no changes, are not shown.

lack of statistically significant changes in the levels of total β-tubulin, polyglutamylated tubulin and tau (table 1) as well as in those of NFL and NFM (not shown) following treatment of N2a cells with 1-10 μM DZO. In contrast, the quantitative analysis revealed that exposure of differentiating N2a cells to 10 μM DZO for 24 h caused a statistically significant 38.6 percent reduction in the levels of the βIII-tubulin isotype, whereas a reduction of 23.6 percent induced by 5 μM DZO was not statistically significant. Densitometric analysis also demonstrated that treatment of N2a cells for 24 h with 10 μM

Antigen	Densitometric peak area (% Control ± S.E.M.)		
	1 μM DZO	5 μM DZO	10 μM DZO
Total β-tubulin	98.4±10.9	114.8±4.7	99.0±5.2
βIII-tubulin	114.9±9.1	76.4±9.7	61.4±13.1*
Polyglutamylated tubulin	107.0±22.3	90.2±23.1	90.6±24.7
MAP 1B	76.0±12.6	75.0±13.4	51.3±12.5*
Tau	149.2±56.9	129.7±61.5	133.3±14.1

Table 1. Densitometric analysis of antibody reactivity on Western blots.
Immunoblots probed with antibodies against total β-tubulin, β-III tubulin, polyglutamylated tubulin, MAP1B and tau were incubated with Luminol reagent and the resultant enhanced chemiluminescence was detected as described in Materials and methods. Band densities for all proteins above were normalised to band densities for glyceraldehyde-3-phosphate dehydrogenase. Values are expressed as a percentage of their corresponding control ± S.E.M. for 3 or 4 independent experiments, each experiment involving a different initial cell population. Asterisks indicate values that were significantly different from their corresponding controls (*$p<0.05$).

DZO resulted in a statistically significant 48.7 percent decrease in the levels of MAP 1B, whereas 1 and 5 μM DZO produced slight but not statistically significant decreases of 24 and 25 percent, respectively.

As shown in fig. 3, indirect immunofluorescence staining with monoclonal antibodies B512 and SMI34 showed that there were less axon-like outgrowths in DZO treated cells than in control cells (see also fig. 1). Furthermore, the intensity of antibody staining in neurites and cell bodies was relatively weak in DZO treated cells compared to the control for anti-βIII-tubulin but not for anti-α-tubulin, with evidence of aggregation in the former. Fewer neurites were also detectable in DZO treated cells stained with anti-MAP1B,

although the staining intensity was very weak even in control cells (not shown). Staining with the anti-phosphorylated NFH antibody SMI34 also confirmed the appearance of cell body aggregate staining in DZO treated cells, indicative of neurofilament disruption.

Discussion

The densitometric data obtained in this study indicate that concentrations of DZO which severely impair neurite outgrowth in N2a cells have no effect on the levels of total β-tubulin. The fact that total α-tubulin levels are also unaffected under identical conditions [12] suggests that DZO exposure has no effect on the overall levels of α and β subunits. A similar lack of effect was observed on total α-tubulin following exposure of differentiating N2a cells to the parent compound DZ [20].

However, β-tubulin exists as a number of distinct isotypes [21], of which class III β-tubulin (βIII-tubulin) is the only one that is present almost exclusively in neuronal cells [22] and is one of the earliest neuronal cytoskeletal proteins to be expressed during development [23,24]. Western blotting analysis of this β-tubulin isotype in particular revealed that, in contrast to the lack of its effect on total β-tubulin, DZO, at a neurite-inhibitory dose of 10 μM, induced a significant reduction in the levels of βIII-tubulin.

These data suggest that this effect is specific for βIII-tubulin since the monoclonal antibody employed for the analysis is known to recognise specifically an epitope located on this isoform and not to cross-react with other tubulin isotypes [25]. The observation of decreased βIII-tubulin levels at a DZO concentration that has been shown in this study to severely impair neurite outgrowth in N2a cells is consistent with the findings of studies in other laboratories showing that synthesis of the βIII-tubulin isotype is crucially involved in microtubule formation during neuritogenesis in vivo and in differentiating mouse neuroblastoma cells [22, 26].

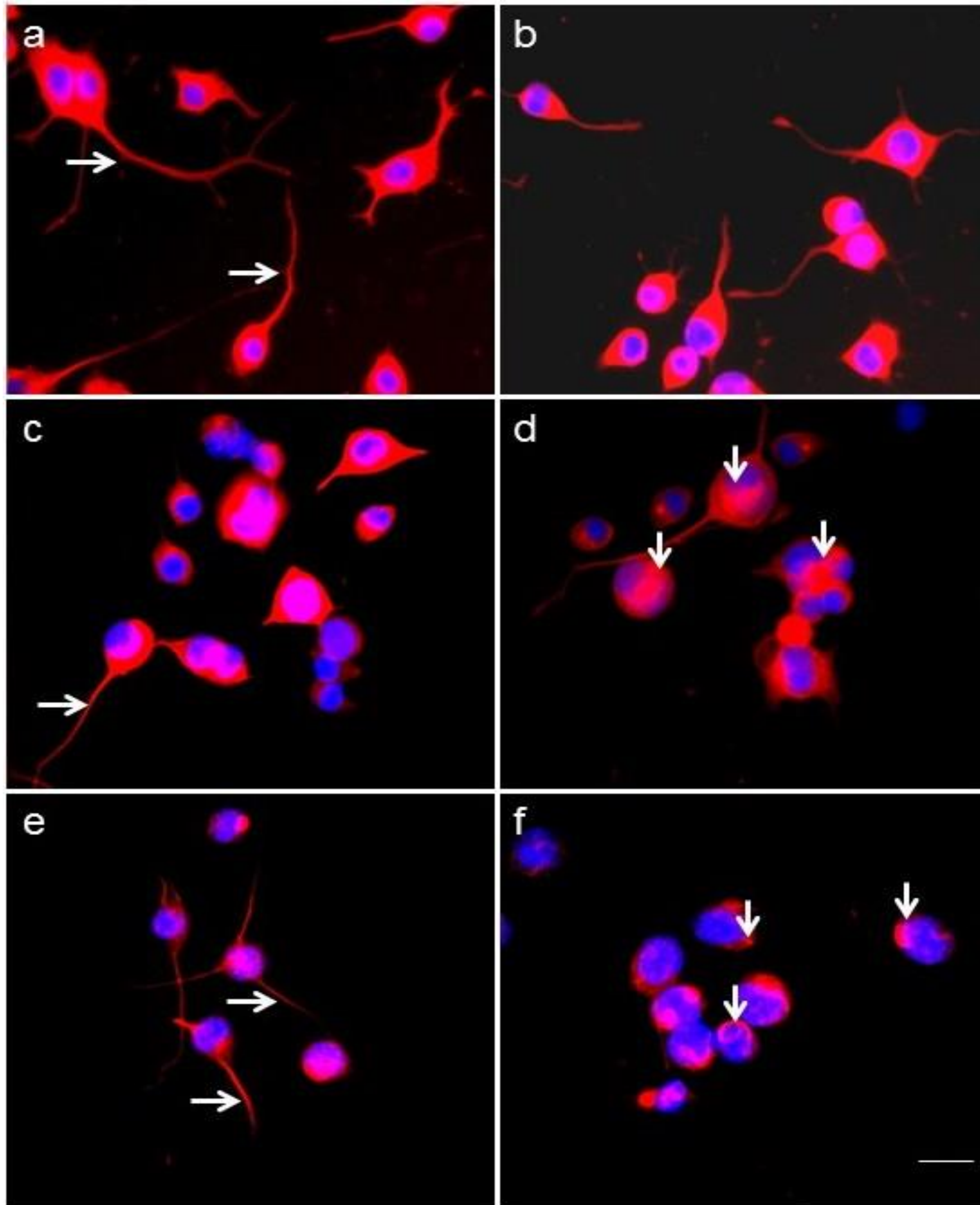

Fig. 3. Indirect immunofluorescence staining of N2a cells induced to differentiate in the absence and presence of DZO. N2a cells were induced to differentiate for 24 h in the absence (a, c, e) and presence (b, d, f) of 10 µM DZO prior to indirect immunofluorescence staining of the cell monolayers as described in Materials and methods. Shown are typical images of staining with anti-α-tubulin (a, b), anti-βIII-tubulin (c, d) and anti-phosphorylated NFH (e, f). Horizontal arrows indicate typical neurites. Downward vertical arrows indicate aggregated staining in cell bodies. Bar represents 20 µm.

Reduced levels of βIII-tubulin could reflect increased degradation by proteases such as calpain, which has been shown to be activated in response to OP treatment in vivo [27]. Alternatively, the reduction in βIII-tubulin levels could be due to decreased synthesis, as phosphorothionates are known to inhibit the synthesis of both DNA and proteins in vivo [28]. The lack of a significant change in the total levels of β-tubulin in N2a cells treated with 10 μM DZO despite a significant reduction in the levels of the βIII-tubulin isotype can be attributed to the fact that there is a total of five different β-tubulin isotypes in differentiating mouse neuroblastoma cells [26] and that in these cells the βIII-tubulin is not the main isotype present [29].

Immunoblot analysis also indicated that DZO exposure reduces the levels of the MT stabilising protein MAP 1B. Similarly to βIII-tubulin, MAP 1B is not uniquely present in the nervous system but is more abundantly expressed in neurons, especially during development [30]. It is the first MAP to be expressed in neurons [31] and plays a key role in neuronal differentiation and the early stages of neuritogenesis [32] when it can act concomitantly or in coordination with βIII-tubulin [22]. Our present findings showing that doses of DZO which cause neurite inhibition, apart from decreasing the levels of βIII-tubulin, also reduce at the same time the levels of MAP 1B are consistent with the above data. On the other hand, the fact that the levels of another MAP, tau, were not affected by neurite inhibitory concentrations of DZO, is in line with previous data showing that the retraction of neurites in neuroblastoma cells is not necessarily linked to changes in the amount of tau [33].

Tubulin (poly)glutamylation is a posttranslational modification which is important for binding of tau and other MAPs to tubulin [34]. Glutamylated tubulin accumulates in neuronal cultures and brain during development and may have a role in neurite outgrowth. However, the results suggest that the neurite inhibitory doses of DZO used in this study had no significant effect on the glutamylation state of tubulin. It is worth noting that the level of α-tubulin tyrosination, another posttranslational modification of tubulin linked to microtubule stability, was also found to be unaffected in N2a cells by DZO treatment [12].

The reduced numbers of long neurites in immunofluorescently stained DZO treated cells confirms our finding that 10 μM DZO causes profound inhibition of neurite development, as shown in the morphological analysis presented in fig. 1. However, the reduced intensity of staining of those neurites present with antibodies to βIII-tubulin but not total α-tubulin, suggested a redistribution of βIII-tubulin. Reduced levels of these proteins in developing neurites may be a key factor in the inhibition of neurite outgrowth by DZO.

In contrast to the considerable changes that DZO induces in the levels of two neurodevelopmentally relevant proteins of the MT system, the effects of this compound on the levels of NFL and NFM, two proteins of the NF network, were not significant. The protein levels of NFH, whose expression in growing neurites occurs later than that of NFL and NFM [11] has also been found to be unaffected by DZO treatment under identical conditions [12]. On the other hand, since the phosphorylation of NFs is an important factor in the regulation of their known ability to modulate axon girth and axon/neurite stabilisation [35], the possibility of neurite-inhibitory doses of DZO exerting a significant effect on NFL and NFM phosphorylation cannot be precluded, particularly in view of our previous data showing that DZO under identical conditions affects the phosphorylation of NFH [12]. Indeed, in the current study, immunofluorescence staining of cells with monoclonal antibody SMI34 (which recognises phosphorylated NFH) confirmed that there was a major redistribution of this network in N2a cells treated with 10 μM DZO. This finding is in good agreement with previous studies using the parent compound DZ [20] and suggests that either reduced levels of NFH or its altered phosphorylation status may be associated with pesticide induced disruption of the NF network.

The significant effects of DZO on the levels of βIII-tubulin and MAP 1B are caused by a sub-cytotoxic concentration of 10 μM and such changes in two neurodevelopmentally important cytoskeletal proteins may be a primary cause of the inhibition of neurite outgrowth induced in N2a cells. On the other hand, DZO concentrations of 1 and 5 μM have no significant effect on the above two proteins, although they produce considerable

neurite outgrowth impairment. Neurite inhibition by these concentrations of DZO in N2a cells has been previously shown to be associated with significant alterations in the levels of the axon growth associated protein GAP-43 and the extent of NFH phosphorylation [12].

Comparison of the effects of DZO with those of its parent phosphorothionate DZ shows that the pattern of cytoskeletal changes related to neurite inhibition is not identical for the two compounds. For example, exposure to both DZ [20] and DZO decreases the levels of MAP 1B in N2a cells. Furthermore, both compounds impair neurite outgrowth without inducing any change in the levels of total α-tubulin [12, 20]. On the other hand, in contrast to DZ, which has been shown to have no effect on the developmentally relevant βIII-tubulin isotype in N2a cells [36], DZO induces a significant reduction in the levels of this protein, in line with a number of studies indicating a greater range and/or potency of neurodevelopmental effects of the oxon metabolites compared to their parent phosphorothionates [13]. The difference between DZ and DZO is also extended to their effects on proteins of the NF network, as our previous N2a data indicate distinct actions of neurite-inhibitory doses of the two compounds on the levels and phosphorylation of NFH [12, 20]. Moreover, the differential effects of DZ and DZO on cytoskeletal proteins are not confined to differentiating N2a cells, but also occur in differentiating cultured cells of glial origin [20, 37]. Thus the greater potency of DZO to inhibit neurite outgrowth in differentiating neuronal cells [12,20] may be related to its ability to disrupt additional cytoskeletal protein targets to those affected by DZ.

The above morphological and biochemical effects of DZO noted in vitro are induced by concentrations similar to those likely to occur in vivo in the developing organism. For example, in a prospective cohort study with randomised sampling, one third of foetal meconium samples contained detectable amounts of diazinon with a median value of 12.96 μg/ml, which is the equivalent of approximately 40 μM [45]. Although studies have not been conducted specifically with DZO, toxicokinetic data obtained for CPO indicate that oxon metabolite levels in the low micromolar range are attainable in the

developing human [13]. Therefore the above mentioned DZO-specific cytoskeletal changes are relevant to developmental toxicity in humans.

DZO and oxon metabolites in general have a considerably higher aqueous solubility compared to their parent phosphorothionates [38] which could potentially affect their penetration into the foetus. However, data showing significant inhibition of cholinesterase in foetal tissues after in vivo administration of phosphorothionate insecticides [39] indicate that exposure of the foetus to the oxon occurs. In humans, it is thought that the foetus is mainly exposed to oxons formed in maternal tissues. On the other hand, placental metabolism is unlikely to contribute significantly to oxon formation, since CYP2B6, the main CYP responsible for oxon generation in humans [40, 41], is expressed to a limited extent in human placenta [42]. However, although relatively low levels of CYP2B6 are found in the human foetus compared to later developmental stages [43], the levels of the OP degrading enzyme paraoxonase 1 (PON1) are also very low [44] during early development, which could allow some conversion of CPF to CPO within the foetus and/or for the oxon metabolite to accumulate. Additionally, polymorphisms in specific coding and regulatory sequences of the PON1 gene are a major cause of reduced serum PON1 activity and would result in increased risk of susceptibility to the toxicity of the oxon metabolite [44].

In summary, the results of this study indicate that exposure of differentiating cultured cells of neuronal origin to the oxon metabolite of the insecticide DZ causes significant reductions in the levels and distribution specifically of βIII-tubulin and MAP 1B, two proteins of the MT network that are known to be important for axonogenesis. These effects are induced by concentrations of DZO which are sub-cytotoxic and toxicologically relevant and they are not related to acetylcholinesterase inhibition [12]. Certain effects produced by DZO are not seen with its parent phosphorothionate DZ. Taken together with our other findings in differentiating C6 cells [37], the present data imply differential targeting by DZO and DZ of key cytoskeletal proteins during neuronal and glial cell differentiation and indicate that the oxon metabolite may contribute

significantly to the developmental neurotoxicity produced following exposure of the developing organism to the insecticide.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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