Effects of Chlorpyrifos and Chlorpyrifos-methyl on the Outgrowth of Axon-like Processes, Tubulin and GAP-43 in N2a Cells

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ABSTRACT

The aim of this work was to study the neurodegenerative effects of the organophosphate (OP) pesticides chlorpyrifos (CPF) and chlorpyrifos-methyl (CHM) on cultured mouse N2a neuroblastoma cells. CPF or CHM, at a subcytotoxic concentration of 3μM, were added to the cells either at the time of the induction of cell differentiation (co-differentiation) or 16 h after the induction of differentiation (post-differentiation). CPF and CHM were similar in inhibiting significantly the outgrowth of axon-like processes from N2a cells after only 4 h exposure under both co- and post-differentiation exposure conditions. Densitometric scanning of Western blots of extracts of cells treated with CPF or CHM for 4 h revealed significantly decreased cross-reactivity with a monoclonal antibody recognizing the protein GAP-43 under post- but not under co-differentiation exposure conditions. Exposure to CPF or CHM for 4 h under post-differentiation conditions also resulted in reduced fluorescence of N2a cell body staining with anti-GAP-43. Cross-reactivity of Western blots with a monoclonal antibody recognizing α-tubulin was not significantly affected by OP treatment. These data indicate that a disturbance in GAP-43 may be important in the retraction of axons in pre-differentiated N2a cells and support the notion that the mechanisms involved in CPF- and CHM-induced inhibition of axonal outgrowth may be different under co- and post-differentiation exposure conditions.

INTRODUCTION

Chlorpyrifos (CPF) and chlorpyrifos-methyl (CHM) are broad-spectrum organophosphate (OP) pesticides. CPF is widely used in agriculture, veterinary medicine and in the household. This compound has been shown to have developmental neurotoxicity (Campbell et al. 1997; Crumpton et al. 2000). In addition, under certain conditions it is capable of inducing a characteristic delayed-onset neuropathy known as organophosphate-induced delayed neuropathy (OPIDN) (Richardson 1995).

In order to study the molecular basis of CPF-induced neurotoxicity in more detail, our own work has focussed on the effects of subcytotoxic concentrations of CPF on differentiating mouse N2a neuroblastoma cells(Sachana et al. 2001). In this cellular system, it is possible to determine the effects of CPF on the outgrowth and retraction of axons by adding the OP at the point of induction of cell differentiation (co-differentiation) or following 16 h of cell differentiation (post-differentiation), respectively. Axon numbers were significantly lower than controls following 4h exposure to 3 μM CPF under both co-differentiation and post-differentiation conditions, indicating that this compound not only inhibited axon outgrowth but also caused retraction of pre-formed axons in this cellular system (Sachana et al. 2001).

Impairment of axonal outgrowth in cell culture models by OPs has been associated with a number of mechanisms including the inhibition of the enzyme neuropathy target esterase (NTE) (Li and Casida 1998; Sachana et al. 2001) as well as the disruption of cytoskeletal proteins (Flaskos et al. 1998; Schmuck and Ahr 1997). In our work, CPF was found to inhibit neuropathy target esterase (NTE) activity in N2a cells after 4 h under co-differentiation conditions (Sachana et al. 2001), in agreement with the developmental neurotoxicity reported for this compound (Campbell et al.

1997; Crumpton et al. 2000). In contrast, 4 h exposure of pre-differentiated N2a cells to CPF had no effect on NTE activity (Sachana et al. 2001). Similarly, exposure of N2a cells to CPF for 4 h caused an increase in the levels of the heat-shock protein HSP-70 under co- but not under post-differentiation conditions (Sachana et al. 2001). These data indicate that the mechanism(s) associated with the CPF-induced retraction of axons in N2a cells are different from those involved in the inhibition of axon outgrowth by the same compound.

Of further interest to the current work is the toxicity of CHM, the dimethyl ester of CPF, which has been mainly used in the control of insect pests on certain stored grains. There is a paucity of available data concerning the neurotoxic properties of this OP, as indicated in a recent review published by the U.S. Environmental Protection Agency (EPA 2000).

The aim of this study is to study the ability of CPF and CHM to inhibit axon outgrowth and to cause axon retraction in differentiating N2a cells. At a molecular level, special emphasis has been placed on the effects of these OPs on the levels and the intracellular distribution of the proteins α-tubulin and GAP-43, both of which are important in axonal outgrowth and/or stabilization (Meiri et al. 1986, 1998; Keith 1990; Knoops and Octave 1997). This would help to define further the different mechanisms underlying the ability of certain OPs to cause axon retraction and to inhibit axon outgrowth.

MATERIALS AND METHODS

Materials

The mouse N2a neuroblastoma cell line was purchased from ICN (Thane, UK). All sterile plasticware was obtained from Scientific Laboratory Supplies (Nottingham, UK). All cell culture media components, mouse anti-α-tubulin (B512) and mouse anti-GAP-43 (GAP-7B10) monoclonal antibodies were acquired from Sigma Aldrich Chemical Co. (Poole, UK). Rabbit anti-mouse horseradish peroxidase (HRP) and fluorescein isothiocyanate (FITC) conjugates were bought from Dako Ltd. (Cambridge, U.K.). The OP pesticides, chlorpyrifos and chlorpyrifos-methyl (purity 99%) were obtained from Riedel de Haen (Seelze, Germany). All other chemicals were purchased from Sigma Aldrich Chemical Co (Poole, UK).

Cell Maintenance and Differentiation

N2a cells were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with serum, glutamine and antibiotics, as described previously (Flaskos et al. 1998; Fowler et al. 2001). Cells were plated out in growth medium at a density of 50,000 cells/ml and allowed to grow for 24 h. In co-differentiation (CD) experiments, cell differentiation was induced by the addition of 0.3 mM dibutyryl-cAMP (dbcAMP) in serum-free medium with or without 3 μM CPF or CHM, previously dissolved in ethanol. This was followed by incubation for 4 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The final ethanol concentration in the medium of OP-treated and untreated control cells was 0.5% v/v. In the case of post-differentiation (PD) experiments, cells were allowed to differentiate under the influence of dbcAMP in serum-free medium for 16 h prior to exposure to CPF or CHM for 4 h.

Determination of Cell Viability

The effects of 3 μM CPF and CHM on cell viability were determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazan crystals (Mosmann 1983). This spectrophotometric assay was conducted on N2a cells cultured in 24-well plates and treated with the OPs for 4 h under both differentiation conditions described above.

Outgrowth of Axon-like Processes

N2a cells seeded in 24-well plates and treated with CPF, CHM or ethanol were fixed with 90% (v/v) methanol in phosphate buffered saline (PBS) at –20^{o}C for 20 min. They were subsequently stained with Coomassie brilliant blue R-250 (1.25% w/v in 40% v/v methanol and 20% v/v glacial acetic acid in distilled water) for 1 min at room temperature. Stained cells were washed with distilled water and left to air dry for 24 h. From each well, 5 randomly chosen fields were viewed with the aid of an inverted light microscope at 200× magnification. The total number of cells and axon-like processes in each field were counted and the number of axons per 100 cells was determined. An axon-like process was considered to be an extension with a length greater than two cell body diameters (Keilbaugh et al. 1991; Flaskos et al. 1998; Fowler et al. 2001).

Gel Electrophoresis and Western Blotting

For immunoblotting analysis the N2a cells were seeded in a volume of 10 ml of growth medium in culture flasks at a density of 50,000 cells/ml and induced to differentiate, as described above. Cultures differentiated in the presence or absence of CPF or CHM were solubilised in 1ml of electrophoresis sample buffer and separated by SDS-PAGE (Laemmli 1970). Equal amounts (approximately 20 μg) of cell protein were loaded and the protein was estimated using the method of Lowry et al (1951),

with minor modifications. Following electrophoresis, the separated proteins were blotted onto nitrocellulose membrane filters (Towbin et al. 1979). The resultant Western blots were checked for efficient transfer of proteins and the correct sample loading, by staining with 0.05% copper pthalocyanine in 12mM HCl. They were then destained with 12mM NaOH, prior to blocking with 3% BSA in Tris buffered saline (TBS: 10 mM Tris, 140 mM NaCl), pH 7.4. The blots were then probed with monoclonal antibodies that recognize α-tubulin (clone B512, diluted 1:2000 in 3% BSA/TBS) and GAP-43 (clone GAP-7B10, diluted 1:1000 in 3% BSA/TBS) followed by incubation with HRP-conjugated rabbit anti-mouse IgG. Antibody reactivity was revealed by enhanced chemiluminescence (ECL, Amersham, UK).

Quantification of Antibody Reactivity on Western Blots

Densitometric scanning of Western blots was performed by using the Quantiscan image analysis system (Version 1.5; Biosoft) interfaced to a high-resolution CCD video camera. Blots were digitized and the protein levels were quantified by directly relating antibody reactivity to specific protein level. Using the Quantiscan software program, individual lanes were superimposed over the region of interest, from which a peak profile could be constructed. All peak areas were expressed as a percentage of the corresponding control value, using the same threshold for background subtraction.

Indirect immunofluorescence

The cells were seeded on to glass coverslips and induced to differentiate for 16h, prior to treatment for 4 h with 3 μM CPF or CHM. They were then fixed at -20 °C for 10 min in 90% methanol, blocked by incubation in 3 % BSA in TBS followed by incubation overnight at 4 °C with the primary antibody B512 (diluted 1:200) or GAP-7B10 (diluted 1:50 in 3% BSA/TBS). After washing in TBS to remove unbound primary antibody, cells were incubated with the secondary antibody, FITC-conjugated

rabbit anti-mouse IgG (diluted 1:50 in 3% BSA/TBS). Cells were then visualized with a Leica confocal laser scanning microscope in order to determine whether exposure to OPs induced changes in the intracellular distribution of tubulin or GAP-43.

Statistical Analysis

Statistical analyses of the data were performed using Mann-Whitney *U*-test for non-parametric distribution or using the Student's *t*-test, as appropriate, using 95% confidence limits. Data were expressed as mean ± S.E.M. values.

RESULTS

In order to compare the ability of CHM to cause neurodegenerative effects to that of CPF, the effects of these compounds on the outgrowth of axon-like processes in differentiating N2a cells were studied in parallel. Both CHM and CPF were used at a concentration of 3 μM, which was shown to have no effect on the viability of N2a cells, under both differentiation conditions (Table 1). At this concentration, both OPs were found to cause a significant reduction in the outgrowth of axon-like processes by N2a cells compared to the controls within 4 h. These effects were noted either when the OPs were added at the time of induction (CD) or 16 h after the induction of cell differentiation (PD) (Table 2).

Western blotting analysis was carried out to investigate the molecular mechanism(s) involved in the retraction of axon-like processes by the two OPs. For this purpose, blots were probed with antibodies recognizing α-tubulin and the axon growth-associated protein GAP-43, both of which are proteins known to be associated with axon outgrowth and/or stabilization (Meiri et al. 1986, 1998; Keith 1990; Knoops and Octave 1997). Immunoblot analysis revealed a decrease in reactivity of cell extracts with GAP-7B10 antibody, following 4-h post-differentiation exposure to CHM or CPF (Figure 1d, left panel). Indeed, densitometric analysis of cells exposed to 3 μM CHM or CPF, under the above experimental conditions, showed that GAP-7B10 reactivity was significantly reduced ($p < 0.05$) by 53.9% ± 4.8 and 34.3% ± 5.4, respectively, compared with controls (Figure 1d, right panel). However, a slight but not significant increase was observed in reactivity with GAP-7B10 under co-differentiation conditions (Figure 1c). Exposure to both compounds had no effect on reactivity with B512 anti-tubulin antibody under co-differentiation conditions (Figure

1a) and caused slight but not significant reductions under post-differentiation conditions (Figure 1b).

Exposure to 3 μM CHM or CPF for 4 h post-differentiation resulted in weaker fluorescence of GAP-43 staining over the nucleus of N2a cells compared to the controls (Figure 2d, e, f). In control N2a cells, the protein GAP-43 recognized by GAP-7B10 staining was evident in the cell body and partially obscured the nucleus (Figure 2d). No major changes in the level or distribution of B512 staining in OP-treated cells compared to controls were detected under the same experimental conditions (Figure 2a, b, c).

DISCUSSION

The data presented in this work demonstrate that both CPF and CHM are capable of inhibiting the formation and maintenance of axons in cultured mouse N2a neuroblastoma cells after only 4 h exposure.

Effects of CPF and CHM on axon outgrowth

The inhibitory effects of CHM on axon outgrowth, studied for the first time in the present work, are similar to those of CPF as well as to those of other neuropathic OPs tested previously on the same cell line (Fowler et al. 2001, Sachana et al. 2003). Altered expression and distribution of GAP-43 and α-tubulin have been previously related to the neurodegenerative properties of various chemical compounds (Di Luca et al. 1994; Hargreaves 1997). However, the data obtained in the present work indicate that, although the test OPs both inhibited the formation of axons in N2a cells, α-tubulin and GAP-43 levels were not significantly affected. In previous work, it was also found that CPF had no effect on neurofilament heavy chain levels under the same exposure conditions but caused a significant increase in the levels of HSP-70 (Sachana et al. 2001).

Effects of CPF and CHM on axon maintenance

Morphological assessment of pre-differentiated cells indicated that exposure to both compounds caused retraction of pre-formed axons. As in the case of axon outgrowth experiments, analysis of Western blots probed with monoclonal antibody B512

indicated that there was no significant change in the levels of total α-tubulin compared to untreated controls. By contrast, analysis of blots probed with monoclonal antibody GAP-7B10 suggested that exposure to both OPs caused a significant reduction in the levels of GAP-43 compared to controls.

This may reflect an OP-induced reduction in synthesis or increased turnover of GAP-43 under post differentiation exposure conditions. The observed retraction of axons after exposure of the pre-differentiated N2a cells to CPF and CHM is consistent with the fact that GAP-43 levels increase during axon outgrowth and are important in the maintenance of axons (Skene 1989). The reduced staining intensity noted in the cell bodies of the OP-treated pre-differentiated N2a cells probed with GAP-7B10 is consistent with the decrease in the levels of GAP-43 detected on Western blots of extracts from cells exposed to CPF and CHM under the same conditions. This further suggests that exposure to these OP compounds could lead to a disturbance in GAP-43 synthesis or turnover and, possibly, to reduced transport of this protein from the cell body to the axon and dendrites.

Proposed mechanism of toxicity

The present study provides the first evidence for an early biochemical change that is linked to the CPF- and CHM-induced retraction of axons in pre-differentiated N2a cells. These findings add further support to our previous data indicating that the mechanisms involved in the CPF-induced retraction of axons in N2a cells may be different under co- and post-differentiation conditions (Sachana et al. 2001). For example, in previous studies showing the induction of axon retraction by CPF in pre-differentiated cells, NTE activity and levels of HSP-70 and the cytoskeletal protein

neurofilament heavy chain (NF-H) were unaffected, whereas they were reduced or increased respectively under co-differentiation exposure conditions (Sachana et al. 2001).

The observed retraction of axons after exposure of the pre-differentiated N2a cells to CPF and CHM is consistent with the fact that GAP-43 levels increase during axon outgrowth and are important in the maintenance of axons (Skene 1989). The reduced levels of GAP-43 following post-differentiation exposure to the two OPs could be due to decreased synthesis or increased turnover of the protein. This perturbation in GAP-43 levels may be mechanistically important as it could play a role in the morphological effects of CPF and CHM on the maintenance of axon-like processes. For example, consistent with the proposed role of GAP-43 as a cytoskeleton-membrane linker that plays an important role in axon development and stability (Skene 1989; Meiri et al. 1986, 1998), reduced levels of GAP-43 could destabilise the axon by reducing cytoskeleton-membrane interactions.

By contrast, the blot and staining pattern of α-tubulin in N2a cells were not found to be significantly affected under any conditions by CPF and CHM, suggesting that microtubule disruption was not responsible for the observed inhibition of axon outgrowth and the induction of axon retraction. This is consistent with the finding that tubulin synthesis shows small or no specific increase during axon outgrowth (Joshi and Baas 1993). Altered α-tubulin mRNA expression has been reported in the central nervous system of hens and rats following exposure to the OP compounds sarin and diisopropyl phosphorofluoridate (DFP) (Damodaran et al. 2001; 2002). The latter OP has very potent OPIDN-inducing properties and it has been suggested that the observed disturbance in α-tubulin may be associated with the development of OPIDN

or could, alternatively, represent the consequence of important changes occurring during the progression of OPIDN (Damodaran et al. 2001).

The investigation of the neurotoxic properties of CHM undertaken in this study demonstrates that this OP not only exerts effects comparable to those of CPF on axon outgrowth but also that this OP is similar to CPF in terms of its effects on the levels and distribution of GAP-43 and α-tubulin under all conditions studied. CHM and CPF have been recently shown to have similar relative enzyme inhibitory potencies for acetylcholinesterase versus NTE and similar abilities to cause OPIDN (Kropp and Richardson 2003). Our present data suggest that, in serum withdrawal-mediated axonal outgrowth in N2a cells *in vitro*, there exists a similar pattern of effects for the two OPs at a molecular level.

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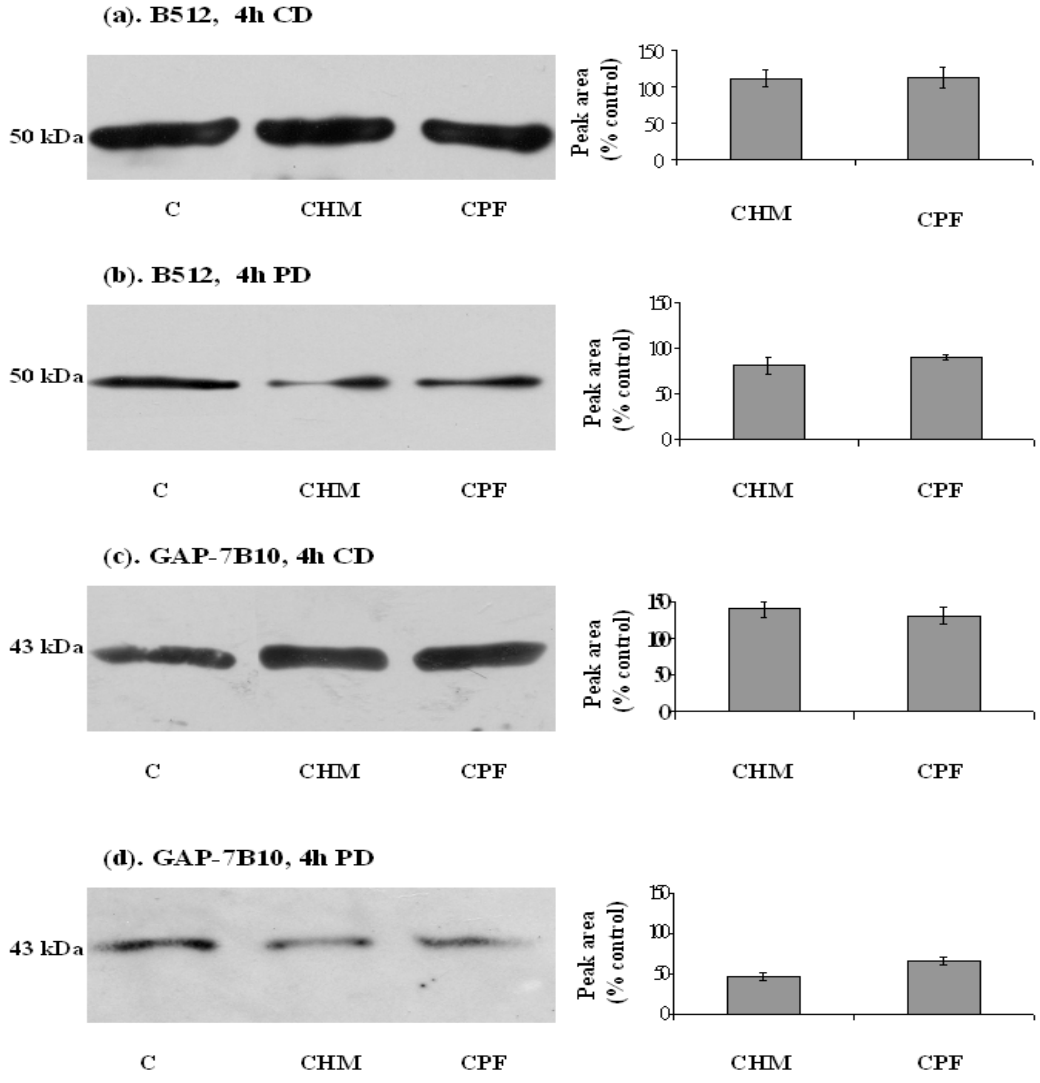

Fig. 1: Western blotting analysis of extracts from N2a cells exposed to subcytotoxic levels of CHM and CPF.

Left panel: N2a cells were incubated in presence or absence of CHM or CPF under co- or post-differentiation conditions, before solubilisation of the cell monolayer in electrophoresis sample buffer. Equal cell protein amounts were separated by SDS-PAGE and then transferred onto nitrocellulose membrane filters. Blots were then probed with antibodies that recognize α-tubulin (clone B512) (a, b) and GAP-43 (clone GAP-7B10) (c, d). **Right panel**: Digital images of probed blots were analysed densitometrically using Quantiscan image analysis software. Data are mean ± S.E.M. of three separate experiments. The results are expressed as a percentage of the corresponding control (defined as the 100% value).

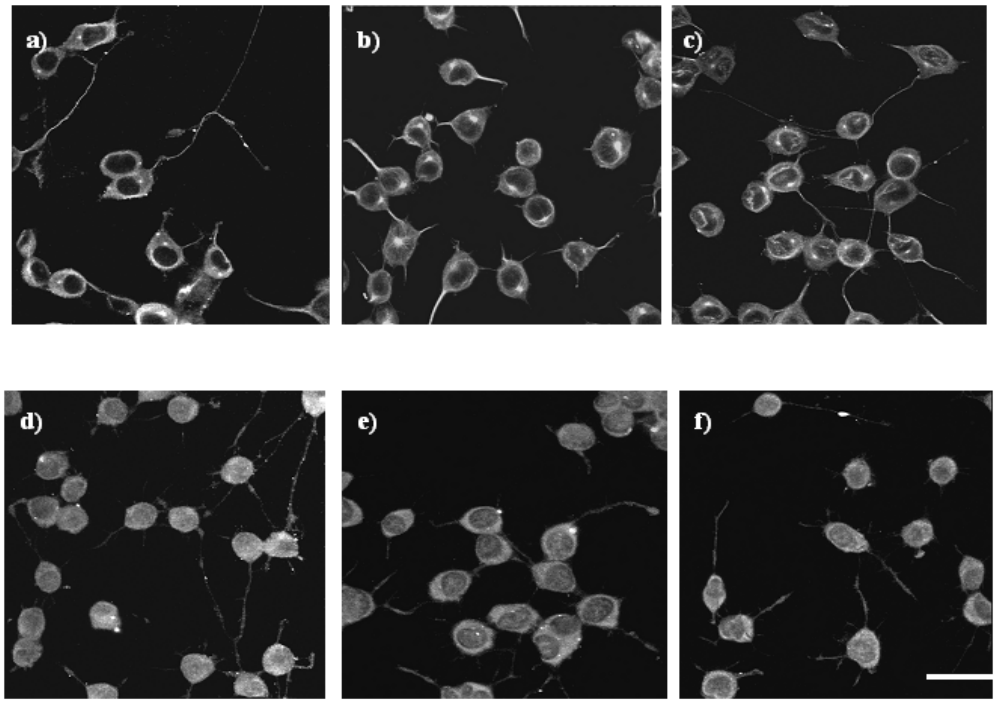

Fig. 2: Immunostaining of N2a cells exposed to subcytotoxic levels of CHM and CPF. N2a cells were incubated in the presence or absence of CHM or CPF under post-differentiation conditions, before they were fixed and processed for immunofluorescence using an antibody that recognizes α-tubulin (clone B512) and GAP-43 (clone GAP-7B10). N2a cells were viewed with a Leica confocal laser scanning microscope. Shown are control cells (a, d), CHM-treated cells (b, e) and CPF-treated cells (c, f) stained with B512 (a, b, c) and GAP-7B10 (d, e, f). White scale bar = 30 μm.

Differentiation conditions and exposure time (h)	MTT reduction (A_{570})		
	Control	Chlorpyrifos - methyl	Chlorpyrifos
4 h CD	0.41 ± 0.02	0.43 ± 0.01	0.47 ± 0.03
4 h PD	0.56 ± 0.05	0.70 ± 0.01	0.74 ± 0.04

TABLE 1: Effects of CHM and CPF on MTT reduction in N2a cells.

N2a cells were induced to differentiate in the presence or absence of 3 μM CHM or CPF (co-differentiation, CD) or they were treated with CHM or CPF after 16 h of differentiation (post-differentiation, PD), and the level of MTT reduction was determined. Results are expressed as mean absorbance ± S.E.M. (n = 8 culture wells, from cells grown on at least two separate occasions).