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Chlorpyrifos and Cypermethrin Induce Apoptosis in Human Neuroblastoma Cell Line SH-SY5Y

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Abstract: Our previous *in vivo* studies showed that chlorpyrifos (CPF) and cypermethrin (CM) in a mixture dermally administered, strongly inhibited cholinesterase activity in plasma and the brain and were very toxic to the rat central nervous system. In this work, the mechanisms of neurotoxicity have not been elucidated. We used human undifferentiated SH-SY5Y cells to study mechanisms of pesticide-induced neuronal cell death. It was found that chlorpyrifos (CPF) and its mixture with cypermethrin (CPF+CM) induced cell death of SH-SY5Y cells in a dose- and time-dependent manner, as shown by MTT assays. Pesticide-induced SH-SY5Y cell death was characterized by concentration-dependent down-regulation of Bcl-2 and Bcl-xL as well as an increase in the caspase 3 activation. Pan-caspase inhibitor Q-VD-OPh produced a slight but significant reversal effect of pesticide-induced toxicity indicating that the major caspase pathways are not integral to CPF- and CPF+CM-induced cell death. Furthermore, signal transduction inhibitors PD98059, SL-327, SB202190, SP600125 and mecamylamine failed to attenuate pesticides effect. Atropine exhibited minimal ability to reverse toxicity. Finally, it was shown that inhibition of TNF-α by pomalidomide attenuated CPF-/CPF+CM-induced apoptosis. Overall, our data suggest that FAS/TNF signalling pathways may participate in CPF and CPF+CM toxicity.

The toxic responses of pesticides on cellular and molecular level can be studied in cultured cells using standard methods. Most of the pesticides do not produce the same pattern of toxic responses because of the involvement of different toxicological mechanisms and different lines of cultured cells used in studies.

The SH-SY5Y cell line has been widely used in experimental neurological studies, including analysis of neuronal differentiation, metabolism and function related to neurodegenerative and neuroadaptive processes, neurotoxicity and neuroprotection [1]. Consequently, this cell line is a reliable model for studying the neurotoxic effect of pesticides and for elucidating the mechanisms of induced neurotoxicity from the aspect of apoptosis [1].

Insecticides that are currently available on the market show very good efficiency in pest fighting, a low environmental cumulation and quite low toxicity to human beings. Actually, the most popular classes of insecticides are synthetic pyrethroids and organophosphates. In this study, we focused on two insecticides representing these groups, used as single compounds or as a mixture of both chlorpyrifos and cypermethrin. The application of pesticides in mixture may result in decreased toxicity of mixture components, additive or synergistic toxicity. Although the effects of individual pesticides on health have been studied for decades, the neurotoxicity of mixtures is still poorly understood.

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Chlorpyrifos (CPF), *O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate, an organophosphorus insecticide, is one of the most extensively used insecticides [2].

Chlorpyrifos is known to induce acute and chronic neurotoxicity in people and animals primarily by inhibiting cholinesterase activity [3]. Besides causing *in vivo* toxicity by AChE inhibition, CPF may also induce other activities within exposed cells. Consequently, recent research has focused on identifying chlorpyrifos toxicity independent of its effect on AChE inhibition. The *in vitro* and *in vivo* studies showed that CPF at low concentrations induced oxidative stress [4], disturbed neurotransmission [5], inhibited replication in cells of the nervous system [6], disrupted neuronal differentiation [7] and induced neurobehavioural changes (e.g. weakness of psychomotor function, reduction of Mental Development Index (MDI) scores in children) [2]. Moreover, CPF and its metabolite chlorpyrifos oxon (CPO) were able to induce apoptosis in several types of cells [8,9] including neuronal cells [10,11].

Cypermethrin (CM) [(RS)-α-cyano-3-phenoxybenzyl(1RS)-*cis-trans*-3-(2,2-dichloro-vinyl)-2,2-dimethylcyclopropanecarboxylate] is a synthetic pyrethroid used as an insecticide in large-scale commercial agricultural applications as well as in consumer products for domestic purposes. CM is considered as the most safest pesticide used worldwide [12], but evidence has shown that CM could induce various toxic effects including developmental neurotoxicity [12,13], oxidative stress [14] and apoptosis [13]. The potential developmental neurotoxicity of CM has also been investigated *in vitro* using neuronal cell lines, including human neuronal cell SH-SY5Y [15]. The neurotoxic responses of cypermethrin are mainly mediated by the

modulation of ion channels, including sodium and chloride channels. As one of the primary targets for cypermethrin is insect voltage-gated sodium channel (VGCC), it is expected that mammalian sodium channels and receptors regulated by it can also act as primary targets for toxicity in human beings. Other main channels and receptors, which are influenced by cypermethrin, include voltage-gated calcium channels (VGCC), potassium channels, GABA receptors, glutamate receptors, acetylcholine receptors and ATPases [16].

It has been demonstrated that many of the toxic effects elicited by exposure to pesticides are mediated by the regulation of apoptosis and redox (reduction/oxidation) signalling [10]. Pesticides have been shown to induce apoptosis by activating a wide variety of signalling pathways mediated by mitochondria, DNA damage (intrinsic pathways) as well as by activation/modulation of death receptors (extrinsic pathways) [17]. The extrinsic pathway is activated by the ligation of death receptors. Death receptor ligands characteristically initiate signalling via receptor oligomerization, which in turn results in the recruitment of specialized adaptor proteins and activation of caspase cascade causing the apoptotic cells death [18].

The mammalian family of mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signalling associated with a variety of cellular activities including cell proliferation, differentiation, survival and death. These signalling pathways have been implicated also in the regulation of neuronal apoptosis [19]. The mammalian MAPK family consists of extracellular signal-regulated kinases (ERK) 1/2, p38 MAPK, mitogen-activated protein kinase kinases (MEK1,2) and the c-Jun NH2-terminal kinase (JNK). JNK and p38 are preferentially activated by cell stress-inducing signals, such as oxidative stress, environmental stress and toxic chemical insults [19].

The current studies were designed to assess the cytotoxicity of chlorpyrifos and cypermethrin, alone and in combination, as well as to investigate the molecular mechanism of pesticide-induced cell death in the SH-SY5Y cell line.

Materials and Methods

Reagents. Chlorpyrifos and cypermethrin were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Mecamylamine hydrochloride, atropine, pomalidomide (3-amino-thalidomide), and MAPK inhibitors, PD98059, SL 327, SB202190, SP600125 and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), were obtained from Sigma.

Q-VD-OPh, (N-(2-quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methylketone), was purchased from BioVision (Mountain View, CA, USA). Monoclonal anti-Fas IgG (CD95/Apo-1) clone Dx2 antibody, monoclonal anti-CD95 antibody (SAB4700005) and Anti-TNF Receptor 2 antibody (SAB4502989) were obtained from Sigma. Cell Death Detection ELISA kit, version 11, was purchased from Roche (Roche Diagnostics, Mannheim, Germany). Unless otherwise stated, all other reagents were purchased from the Sigma-Aldrich Chemical Company.

Reagents working solutions. Chlorpyrifos and cypermethrin stock solutions (100 mM) were prepared in ethanol and stored at 4°C. Working solutions were prepared by dissolving an appropriate stock solution in culture medium. In each case, CM in mixture with CPF was in concentration 1 out of 10 of concentration of CPF, as in the commercial preparations used for plant protection.

Atropine and Mecamylamine were dissolved in ethanol to concentration 100 mM and stored at 4°C. Working solutions were prepared by dissolving appropriate stock solutions in culture medium.

Pan-caspase inhibitor, Q-VD-OPh (1 mM) and all MAPK inhibitors (10 mM) and pomalidomide (10 mM) stock solutions were prepared in DMSO and stored at 4°C. Before the experiments, its working solutions were prepared by dissolving a stock solution in culture medium. Both anti-CD95 and anti-TNF antibodies were dissolved directly into media. All solutions used in the experiments were prepared in medium supplemented with 2% FBS. Final ethanol and DMSO concentrations in medium did not exceed 0.05%.

Cell culture. Human undifferentiated neuroblastoma cell line SH-SY5Y was purchased from ECACC (European Collection of Cell Cultures), Salisbury, UK. Cells were grown in 1:1 mixture of Ham's F12 nutrient and Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% foetal bovine serum (FBS), 1% non-essential amino acid solution, penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Quantification of cell death and apoptosis. The effect of pesticides on SH-SY5Y cells viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolism assay [20].

SH-SY5Y cells at 2×10^5 cells/mL were exposed to serial dilutions of tested compounds for 24, 48 and 72 hr at 37°C in 5% CO_2. Then, 10 μL of MTT dye was added, and the cells were incubated for a further 4 hr at 37°C. Then, 100 μL of SDS buffer, pH 7.4 (10% SDS in 0.01 N HCl), was added and mixed thoroughly with the cells. The absorbance was measured at 570/620 nm using microplate reader (BioTek ELx800, Highland Park, Winooski, VT, USA).

Apoptosis of cells was measured using the Cell Death Detection ELISA^{plus} kit (Roche Diagnostics GmbH) that quantifies histone-associated DNA fragments (mono and oligonucleosomes).

SH-SY5Y cells (2×10^5 cells/mL) were exposed to various concentrations of the investigated compounds: CPF (17.5, 25, 30 μM), CM (1.75, 2.5, 3 μM) or CPF+CM (17.5+1.75, 25+2.5, 30+3.0 μM) at 37°C for 24 hr. The procedure was performed according to the manufacturer's protocol. Briefly, the culture supernatants and lysate of cells were prepared and incubated in the microtitre plate coated with antihistone antibody. After colour development, the results were analysed spectrophotometrically using a microplate reader (ELx800) at 450 nm wavelength.

Inhibitory studies were conducted by incubating SH-SY5Y cells (2×10^5 cells/mL) with 25 μM CPF or with CPF+CM (25+2.5 μM) in the presence and absence of 5 μM Q-VD-OPh (pan-caspase inhibitor), 20 μM PD98059 (ERK inhibitor), SL-327 (MEK inhibitor), SB202190 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), 25 μM atropine (muscarinic cholinergic receptor antagonist), 25 μM mecamylamine (nicotinic cholinergic receptor antagonist), 1 μM pomalidomide (TNF-α inhibitor) and 1 μg/mL SAB4700005 (anti-CD95, clone Dx-2 antibody), 2 μg/mL anti-CD95 (CD95/Apo-1 clone Dx2) antibody and 1 μg/mL SAB4502989 (anti-TNF-R2 antibody).

After 24 hr of incubation in standard conditions, apoptosis was determined with the Cell Death Detection ELISA^{plus} kit, as described above.

Western blot analysis. SH-SY5Y cells were seeded on 6-well microplates at 2.5×10^5 cells/mL density. On the following day, the culture medium was removed and cells were exposed to 25 μM CPF alone or to a mixture of CPF and CM in a proportion of 25:2.5 μM

(v/v), in the fresh medium supplemented with 15% FBS. SH-SY5Y cells were exposed to tested compounds for the time intervals of 1–24 hr (1, 3, 6, 24 hr).

After treatment, cells were washed with ice-cold PBS, harvested and lysed in RIPA buffer 1% NP40 (Tergitol), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 1 mM Na_3VO_4, 20 mM NaF, 0.5 mM DTT, 1 mM PMSF, protease inhibitors mixture in PBS, pH 7.4 and centrifuged at 14 000 × *g* for 10 min. Protein content in supernatants was determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). RIPA cells lysates were solubilized in sample buffer (30% glycerol, 10% SDS, 0.5 M Tris-HCl, pH 6.8, 0.012% bromophenol blue, 5% β-mercaptoethanol) and boiled for 5 min. Equal amounts of proteins were electrophoresed on 12% SDS-PAGE and transferred to PVDF membrane. After blocking for 1 hr at room temperature with 5% non-fat dry milk in TBS-0.1% Tween-20 (TBS-T), membranes were probed at 4°C overnight with primary antibodies directed against β-actin, caspase-3, cleaved caspase-3, Bcl-2 and Bcl-xL (Cell Signalling Technology, Beverly, MA, USA), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signalling Technology) for 1 hr at room temperature and visualized using enhanced chemiluminescence (Pierce Biotechnology). Serial exposures were made on Kodak BioMax Light film (Eastman Kodak Company, Rochester, NY, USA). For stripping, membranes were incubated with stripping buffer, ReBlot Plus Strong Antibody Stripping Solution (Millipore, Billerica, MA, USA), then washed, blocked and probed with the relevant antibody, as described above. The amount of protein was densitometrically determined using Image J software (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis. The data were presented as the mean value and standard error of the mean (S.E.M.). Statistical analysis was performed with the one-way ANOVA with Tukey's post hoc test (GraphPad Prism 5, GraphPad Software, Inc, San Diego, California, USA). Significance was accepted at $p < 0.05$.

The IC_{50} value was calculated using computerized linear regression analysis of quantal log dose-probit functions, according to the method of Litchfield and Wilcoxon [21].

Results

Pesticide-induced cell death in neuroblastoma SH-SY5Y cells. To examine the toxic effects of CPF and CM, SH-SY5Y cells were treated with varying concentrations of CPF (5–500 μM) or CM (0.5–50 μM) and assayed for cell viability at various times after treatment using the MTT metabolism assay. CPF reduced MTT metabolism in a concentration- and time-dependent manner (fig. 1), suggesting that CPF decreases neuroblastoma SH-SY5Y cells viability. In contrast, CM at concentrations from 0.5 to 25 μM used in these conditions had no effect on MTT metabolism whereas at the concentration of 50 μM reduced it ($p < 0.05$) (results not shown).

To determine the effect of CM on cytotoxicity of CPF in SH-SY5Y cells, we compared the effects of CPF in a mixture with CM (at a concentration of 1/10 CPF) with the effects of CPF alone. As shown in fig. 1, the MTT metabolism after 24, 48 and 72 hr exposure with the mixture of CPF+CM was significantly reduced compared with that observed in the presence of CPF alone, indicating that CM had an additional effect on the cytotoxic effect of CPF on SH-SY5Y cells. The IC50 values at 24, 48 and 72 hr for CPF and CPF+CM were 313, 182, 51 μM (for CPF) and 103, 66, 44 μM (for CPF+CM), respectively.

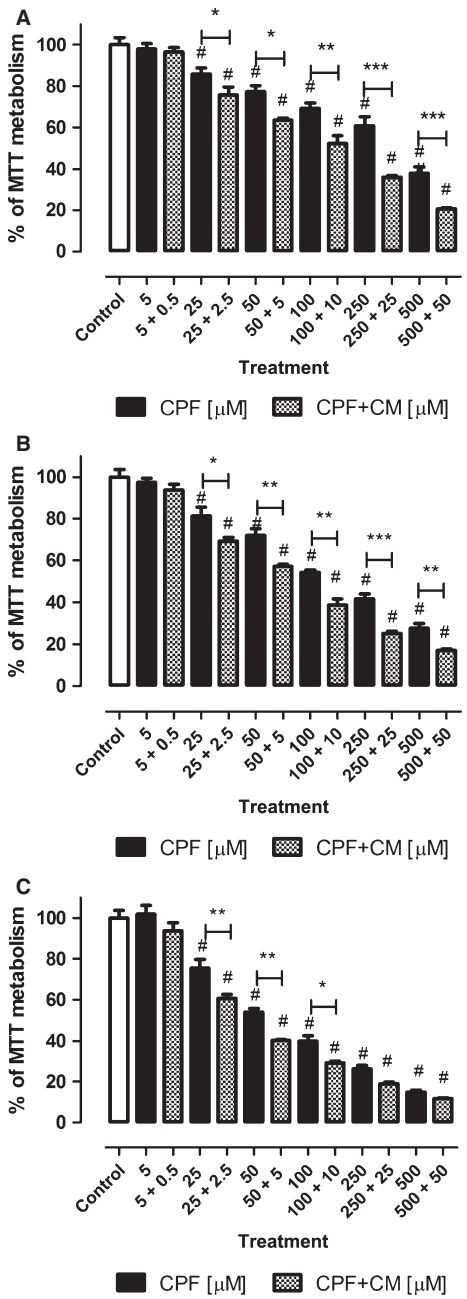

Fig. 1. Chlorpyrifos (CPF) or mixture of chlorpyrifos and cypermethrin (CPF+CM) decreases MTT metabolism in SH-SY5Y cells. Neuroblastoma cells were incubated with various concentrations of CPF and CPF+CM, for 24, 48 and 72 hr. Panel A represents the toxic effect of pesticides at 24 hr, Panel B at 48 hr and Panel C at 72 hr. Data are representative of at least three independent experiments. Statistical analysis was performed with one-way ANOVA. '#' Statistically significant difference ($p \leq 0.05$) when compared with control.

Effects of Pesticides on DNA fragmentation. To further confirm the effects of pesticides on the induction of apoptosis in SH-SY5Y cells, fragmentation of DNA (a hallmark of apoptosis) was examined by Cell Death Detection ELISA^{plus} kit. As shown in fig. 2, CPF-induced apoptosis of *SH-SY5Y* cells in a concentration-dependent manner.

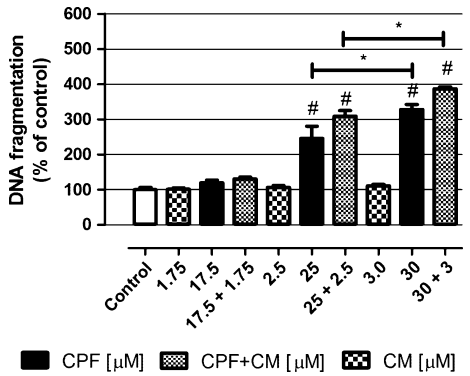

Fig. 2. Chlorpyrifos (CPF), cypermethrin (CM) or mixture of chlorpyrifos and cypermethrin (CPF+CM) induces apoptosis in SH-SY5Y cells. SH-SY5Y cells were incubated with various concentrations of CPF and CPF+CM for 24 hr. The Cell Death Detection ELISA kit was used to quantify DNA fragmentation cells undergoing apoptosis. Data are representative of at least three independent experiments. Statistical analysis was performed with one-way ANOVA. '#' Statistically significant difference ($p < 0.001$) when compared with control *$p < 0.05$.

Statistically significant changes were noted after cell treatment with CPF at concentration 25 μM and its mixture with 2.5 μM CM ($p < 0.001$). The increase in DNA fragmentation in SH-SY5Y cells was observed during the use of the mixture of pesticides as compared to the use of CPF alone, but there were no statistically significant differences.

This study also found that CM, at the concentrations of 1.75, 2.5 and 3 μM, does not induce apoptosis in SH-SY5Y cells (fig. 2).

Mechanism of pesticide-induced apoptosis in neuroblastoma SH-SY5Y cells.

In this study, the concentration of CPF (25 μM) and its mixture with 2.5 μM CM was chosen based on the minimum concentrations of pesticides that caused a significant apoptosis in SH-SY5Y cells.

To investigate the mechanism by which CPF and CPF+CM induced apoptosis in SH-SY5Y cells, we examined the influence of tested compounds on the expression of proteins involved in cell death. We performed Western blot analysis to detect the activation of caspase-3, caspase-3-cleaved, Bcl-xL and Bcl-2 in pesticide-treated cells. Activation of caspases was evident within 1 and 3 hr of CPF and CPF+CM treatment (fig. 3A–B). Above-mentioned changes correlated with expression of antiapoptotic proteins: Bcl-xL and Bcl-2 (fig. 3C–D). The decrease in their expression was observed in the mentioned period of time.

This study also found that Q-VD-OPh, a pan-caspase inhibitor, significantly inhibited pesticide-induced apoptosis in SH-SY5Y cells (fig. 4). These findings suggest that CPF and CPF+CM induced apoptosis, at least partially, via the caspase-3 pathway.

To determine whether various MAP kinase signalling pathways are involved in the regulation of pesticide-induced apoptosis, SH-SY5Y cells were exposed to pesticides in the presence and absence of several specific inhibitors of these signalling pathways: PD98059 (ERK inhibitor), SL-327 (MEK inhibitor), SB202190 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor), for 24 hr. As shown in fig. 5, none of the signal transduction inhibitors reversed pesticide-induced apoptosis as determined by DNA fragmentation.

As SH-SY5Y cells possess functional cholinergic system consisting of cholinergic receptors and cholinesterase [22], a possible role of this system activation during CPF- and CPF+CM-induced toxicity was examined. Thus, SH-SY5Y cells were exposed to CPF and CPF+CM in the presence of either atropine (muscarinic receptor antagonist) or mecamylamine (nicotinic receptor antagonist). As presented in fig. 6, atropine administration effectively reduced pro-apoptotic properties of tested pesticides. On the contrary, mecamylamine was not able to attenuate pesticide-induced apoptosis. Therefore, our data suggest that stimulation of muscarinic receptors may contribute to CPF- and CPF+CM-induced toxicity in SH-SY5Y cells.

As TNF-α is pro-apoptotic mediator [23], we decided to determine whether its activation mediated pesticide-induced apoptosis. CPF- and CPF+CM-treated cells were exposed to pomalidomide, a pharmacological inhibitor of TNF receptor. The treatment with 25 μg/mL pomalidomide caused statistically significant reduction in both CPF- and CPF+CM-induced apoptosis (fig. 7).

Apoptosis is induced by a subgroup of the tumour necrosis factor (TNF) receptor superfamily. These so-called death receptors include CD95 (Fas/APO-1), DR3, TNF-R1 and two TRAIL receptors. To further analyse whether tested compounds could be related to modulation of CD95 receptor, we examined the expression of CD95 receptor in cells subjected to CPF and CPF+CM. If the CD95 pathway is involved in pesticide-induced apoptosis, the effective activation of this pathway using an anti-CD95 antibody should enhance the drug-induced death. The co-incubation of SH-SY5Y cells with anti-CD95 antibody significantly increased apoptosis induced by CPF and CPF+CM in these cells, as shown in fig. 8A. On the contrary, to see whether blockage of CD95 receptor could mediate increased resistance to pesticide-induced apoptosis in neuroblastoma cells, we used SAB4700005, anti-CD95 (clone LT95) antibodies. The antibody LT95 does not induce CD95-mediated apoptosis. As shown in fig. 8B, treatment of SH-SY5Y cells with SAB4700005 prior to addition of CPF and CPF+CM did not reduce pesticide-induced apoptotic cell death. These results indicate that CD95 receptors may be involved in apoptosis caused by tested compounds.

Cellular response to TNF-α is mediated through interaction with receptors TNF-R1 and TNF-R2 and results in activation of pathways that favour both cell survival and apoptosis depending on the cell type and biological context [23]. As there is substantial evidence showing that TNF-α can promote neuronal cell survival through TNFR2 receptor [23], we examined the ability of anti-TNF-R2 antibody to inhibit apoptosis of SH-SY5Y cells induced by tested pesticides.

As shown in fig. 9, treatment of SH-SY5Y cells with SAB4502989 and anti-TNF-R2 antibody, prior to addition of

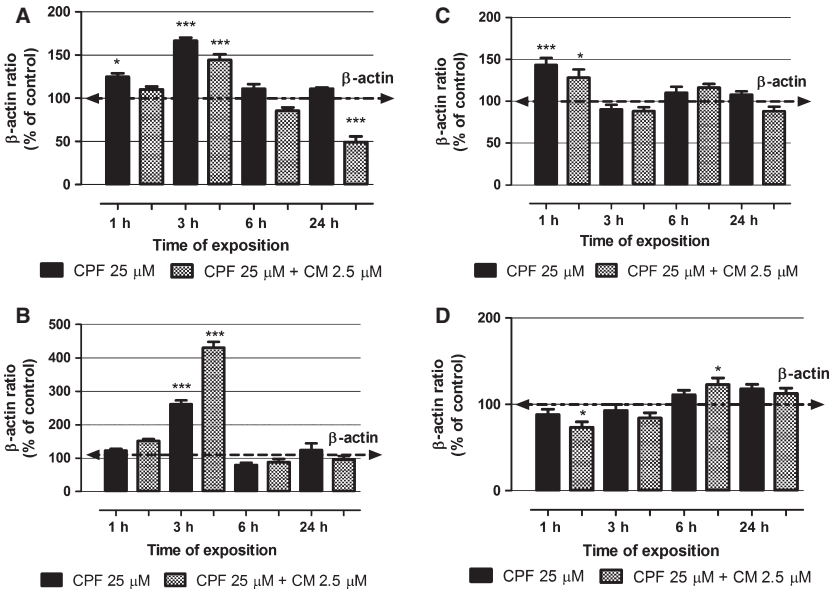

Fig. 3. The effect of chlorpyrifos (CPF) or mixture of chlorpyrifos and cypermethrin (CPF+CM) on the expression of caspases: (A) – caspase-3; (B) – caspase-3-cleaved and anti-apoptotic proteins: (C) – Bcl-2; (D) – Bcl-xL in neuroblastoma cells SH-SY5Y. Protein level was analysed in cell lysates by means of Western blotting using target-specific antibodies. The equivalent amount of protein was verified by reprobing the blot with anti-ß-actin antibody (internal control). Data are representative of three independent densitometric analyses (ImageJ) of immunoblots for individual proteins. Statistical analysis was performed with one-way ANOVA $*p < 0.05$; $***p < 0.001$ *versus* the control.

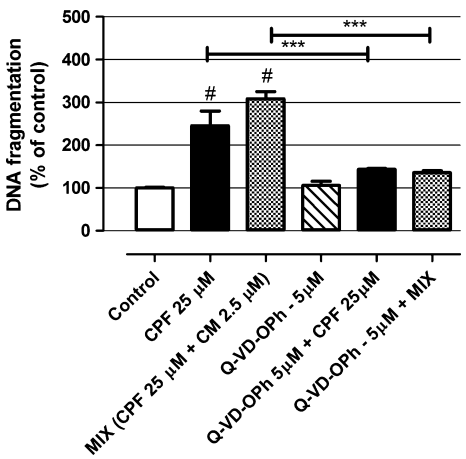

Fig. 4. The effect of Q-VD-OPh (pan-caspase inhibitor) on chlorpyrifos (CPF) or mixture of chlorpyrifos and cypermethrin (CPF+CM)-induced SH-SY5Y cell apoptosis. SH-SY5Y cells were pre-incubated with 5 μM Q-VD-OPh for 1 hr before treatment with CPF or CPF+CM for 24 hr. The Cell Death Detection ELISA kit was used to quantify DNA fragmentation cells undergoing apoptosis. Data are representative of three independent experiments. Statistical analysis was performed with one-way ANOVA. '#' Statistically significant difference ($p < 0.001$) when compared with control $***p < 0.001$.

CPF and CPF+CM, markedly reduced pesticide-induced apoptotic cell death.

Discussion

In this study, we have characterized some aspects of the process of cell death triggered by chlorpyrifos (CPF) in the human SH-SY5Y cell line derived from neuroblastoma. Furthermore, it was extended to investigate the ability of cypermethrin (CM), used at a fixed concentration of 1 out of 10 of the concentration of CPF, to modulate the neurotoxic effect of this organophosphate compound. Cypermethrin at this concentration is often used in a mixture with chlorpyrifos as insecticides for plant protection.

The cellular toxicity of the pesticides on SH-SY5Y cells was assessed by the MTT metabolism assay. Our data showed that CPF and its mixture with CM (CPF+CM) induced cytotoxicity in concentration- and time-dependent manners (fig. 1). In contrast, CM (except for the concentration of 50 μM) had no effect on MTT metabolism in SH-SY5Y cells.

Chlorpyrifos induced toxicity at concentrations as low as 25 μM. The mixture of CPF+CM caused significantly higher cytotoxicity than that caused by CPF, indicating that CM had an additional cytotoxic effect on SH-SY5Y cells (fig. 1). Also, the IC50 values for the mixture of pesticides were about three times lower than for CPF.

Chlorpyrifos is used as a model pesticide to investigate the role of pesticide exposure in the induction of neuronal cell death. Similarly, a recent study showed that CPF significantly reduced cell viability and cytotoxicity in SH-SY5Y cells in a concentration-dependent manner [10]. Additionally, Caughlan *et al.* [11] reported that CPF caused a dose-dependent reduction in viability of rat cortical neurons and CPF was cytotoxic to dopaminergic neuronal cell line, PC-12 [24].

The cytotoxic effect of CM (the preparation ripcord contained an active compound cypermethrin) on SH-SY5Y cells was examined previously by Kokko *et al.* [25]. In this work, exposure to 0.1–100 μM of CM showed dose-dependent

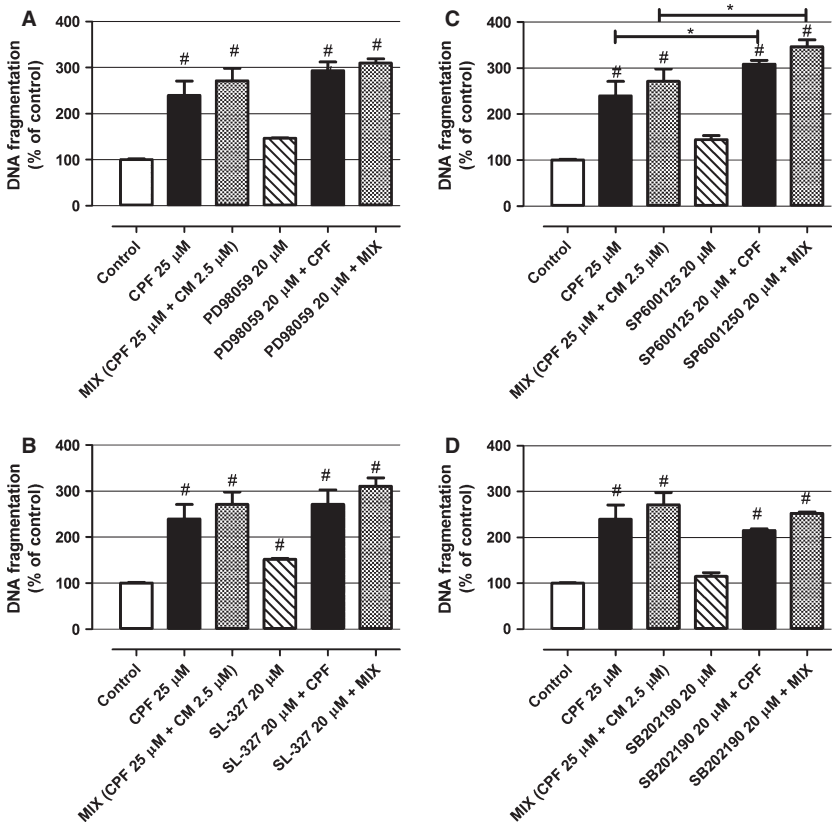

Fig. 5. The effects of signal transduction inhibitors on chlorpyrifos (CPF) or mixture of chlorpyrifos and cypermethrin (CPF+CM)-induced SH-SY5Y cell apoptosis. SH-SY5Y cells were pre-incubated in the presence and absence of 20 μM PD98059 (ERK inhibitor) – Panel A; 20 μM SL-327 (MEK inhibitor) – Panel B; 20 μM SP600125 (JNK inhibitor) – Panel C; 20 μM SB202190 (p38 MAPK inhibitor) – Panel D, for 1 hr before treatment with CPF or CPF+CM for 24 hr. The Cell Death Detection ELISA kit was used to quantify DNA fragmentation cells undergoing apoptosis. Data are representative of three independent experiments. Statistical analysis was performed with one-way ANOVA. '#' Statistically significant difference when compared with control $*p < 0.05$.

cytotoxicity. The detectable toxicity of CM started at 1 μM concentration and at a concentration of 15 μM the cell viability (WST-1) was about 72% of control, while at 25 μM decreased to 15% of control.

Our results showed much lower cytotoxicity of CM on SH-SY5Y cells. CM used in concentrations from 0.5 to 25 μM had no effect on cell viability (MTT) in studied cells. The effect of cytotoxiciy of CM appeared at the concentration of 50 μM.

Differences in the cytotoxicity of CM on SH-SY5Y cells may be due to the application of a pyrethroid with different degrees of purity. In a cited work [25], Ripcord was used, which contained an active compound CM and a mixture of xylene and petrol 820 g/L. It should be noted that xylene and petrol may be also toxic to SH-SY5Y cells and when used with CM, the toxic effects of these all substances could be additive.

The toxicity of complex chemical mixtures may differ from the toxicity observed when individual components are tested as pure chemicals. Previously, our *in vivo* studies showed that CPF and CM in a mixture dermally administered, strongly inhibited cholinesterase activity in plasma and brain and were very toxic against rat central nervous system [26]. In the current study, we observed that CM at concentration starting from 2.5 μM had significantly synergistic effect on CPF-induced cytotoxicity in SH-SY5Y cells, as determined by MTT assays (fig. 1).

The results clearly indicate that the mixture of CPF and CM is more toxic to the SH-SY5Y cells than CPF used alone. This finding could be important because in common use for plant protection are insecticides containing CPF and CM in proportions used in this study, for example Nurella 500EC, CYMAX or Spine, from which aqueous emulsions containing pesticides at micromolar concentrations are prepared.

Chlorpyrifos and its mixture with CM apparently triggered cell death by apoptosis, as indicated by the characteristic fragmentation of genomic DNA, one of the hallmarks of apoptosis. The observed effect was concentration-dependent, and SH-SY5Y cells were similarly sensitive to combination of CPF+CM or CPF used alone (fig. 2). It is noteworthy that CM at the concentration of 1.75, 2.5 and 3 μM does not induce apoptosis in SH-SY5Y cells (fig. 2).

So, the study of the molecular mechanisms of apoptosis was analysed using CPF and its mixture with CM.

These observations are consistent with the data of other authors and support earlier findings which indicated that CPF induces neurotoxicity through apoptotic mechanisms [11,24].

Because of that and to confirm pro-apoptotic features of tested pesticides, their influence on caspases 3, Bcl-2 and

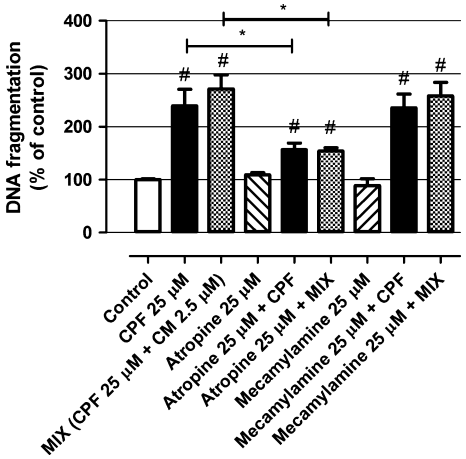

Fig. 6. The effects of cholinergic antagonists on chlorpyrifos (CPF) or mixture of chlorpyrifos and cypermethrin (CPF+CM)-induced SH-SY5Y cell apoptosis. SH-SY5Y cells were pre-incubated in the presence and absence of 25 μM atropine (muscarinic cholinergic receptor antagonist) or 25 μM mecamylamine (nicotinic cholinergic receptor antagonist), for 1 hr before treatment with CPF or CPF+CM for 24 hr. The Cell Death Detection ELISA kit was used to quantify DNA fragmentation cells undergoing apoptosis. Data are representative of three independent experiments. Statistical analysis was performed with one-way ANOVA. '#' Statistically significant difference when compared with control $*p < 0.05$.

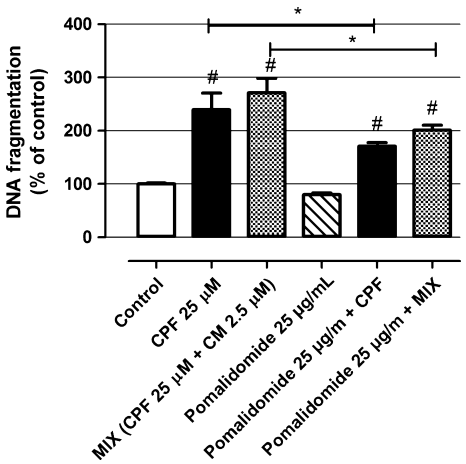

Fig. 7. The effects of TNF-α inhibitor on chlorpyrifos (CPF) or mixture of chlorpyrifos and cypermethrin (CPF+CM)-induced SH-SY5Y cell apoptosis. SH-SY5Y cells were pre-incubated in the presence and absence of 25 μg/mL pomalidomide (TNF receptor inhibitor) for 1 hr before treatment with CPF or CPF+CM for 24 hr. The Cell Death Detection ELISA kit was used to quantify DNA fragmentation cells undergoing apoptosis. Data are representative of three independent experiments. Statistical analysis was performed with one-way ANOVA. '#' Statistically significant difference when compared with control $*p < 0.05$.

Bcl-xL expression was examined by Western blotting (fig. 3). Caspase-3 is a critical executor of apoptosis being responsible for the proteolytic cleavage of many key proteins, which damage initiates the cell death programme [17]. CPF and CPF+CM were shown to increase the expression of cleaved caspase 3, which indicates its activation and the beginning of the changes leading to cell death. The addition of caspase inhibitor Q-VD-OPh significantly attenuated pesticide-induced apoptosis in SH-SY5Y cells (fig. 4), confirming the importance of this pathway in terms of assessed process. Notwithstanding, Q-VD-OPh use did not lead to a complete inhibition of initiation-programmed cell death by tested compounds, suggesting that other pathways are also involved.

Besides the activation of caspases 3, simultaneously, Western blot analysis has shown a decrease in mitochondrial survival proteins Bcl-2 and Bcl-xL. The observed changes may suggest that tested compounds disrupted mitochondrial function (intrinsic pathways), the consequence of which is apoptosis.

Similarly, Park *et al.* [10] demonstrated that CPF induced apoptosis in SH-SY5Y cells by the activation of caspase-3 and nuclear condensation, and another study showed that CFP-induced apoptosis in dopaminergic neuronal components of PC12 cells is partially mediated by the activation of this caspase [24]. Further, it has been shown that major caspase pathways are integral to pesticide-induced apoptosis [27].

As activation of MAPKs, including ERK1/2, p38 MAPK, MEK 1/2 and JNK, has been implicated in the activity of neurotoxins, SH-SY5Y cells were exposed to CPF and CPF+CM in the presence and absence of MAPKs inhibitors [4,11,28].

Our study showed that none of the signal transduction inhibitors reversed the toxic effects of pesticides on studied cells, at 24-hr exposure. After 24 hr, 25 μM CPF and its mixture with 2.5 μM CM were slightly toxic to SH-SY5Y cells, while the addition of SP 600125 (JNK inhibitor) significantly enhanced DNA fragmentation in SH-SY5Y cells. SB202190 (p38 MAPK inhibitor), PD 980059 (ERK inhibitor) and SL-327 (MEK inhibitor) did not cause this effect (fig. 5). It should be noted that SP600125 and SL-327 are also toxic to SH-SY5Y cells and thus enhanced toxicity exhibited in the presence of pesticide may constitute an additive effect. On the other hand, we have not examined the expression of MAPKs in SH-SY5Y cells.

In other studies, CPF treatment (100 μM, for 24 hr) activated MAPK pathways, including ERK 1/2, the JNK, and the p38 MAP kinase and MAPK inhibitors (10 μM U0126, 5 μM SP600125 or 5 μM SB203580, respectively), and inhibited CPF-induced apoptosis in the dopaminergic cell line SH-SY5Y [4] and PC-12 [24]. In these studies, all the MAPK inhibitors affected cell survival by reducing ROS generation and caspase activation, suggesting that JNK, ERK1/2 and p38 MAPK activation occurs upstream of caspase-3 activation and that oxidative stress activates these signalling molecules [4,24]. In other *in vitro* models, Saulsbury *et al.* [29] reported that CPF-induced apoptosis was not related to MAPK signalling pathways in placental cells except p38 MAPK when pesticide was applied at a concentration of 60 μM, at 12 hr. However, none of the signal transduction inhibitors tested attenuated the toxic effects of CPF at 24 hr [29]. Furthermore, Caughlan *et al.* [11] demonstrated that CPF (60 μM, at 12 hr) activated ERK1/2, p38 and JNK signalling pathways in rat cortical neurons. This apoptosis was attenuated by SL-327, an

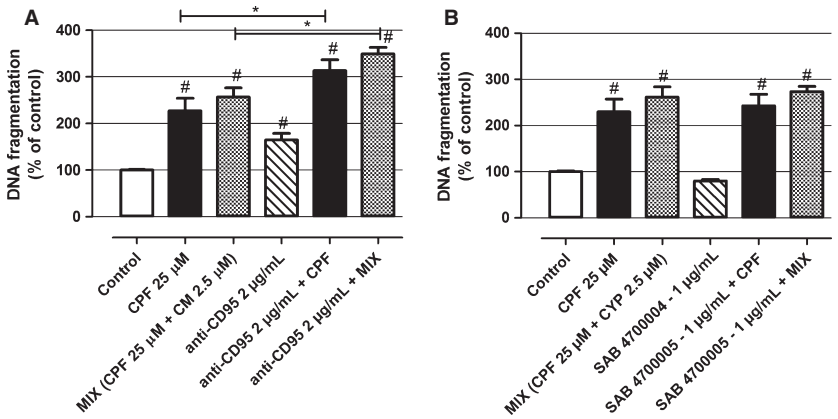

Fig. 8. The effects of anti-CD95 antibodies on chlorpyrifos (CPF) or mixture of chlorpyrifos and cypermethrin (CPF+CM)-induced SH-SY5Y cell apoptosis SH-SY5Y cells were pre-incubated in the presence and absence of 2 µg/mL anti-CD95 and clone Dx2 antibodies or 1 µg/mL SAB4700005, anti-CD95 and clone LT95 antibodies, for 1 hr before treatment with CPF or CPF+CM for 24 hr. Panel A represents anti-CD95 and clone Dx2 antibodies which may induce CD95-mediated apoptosis. Panel B represents SAB4700005, anti-CD95 and clone LT95, which does not induce CD95-mediated apoptosis. The Cell Death Detection ELISA kit was used to quantify DNA fragmentation cells undergoing apoptosis. Data are representative of three independent experiments. Statistical analysis was performed with one-way ANOVA. '#' Statistically significant difference when compared with control $*p < 0.05$.

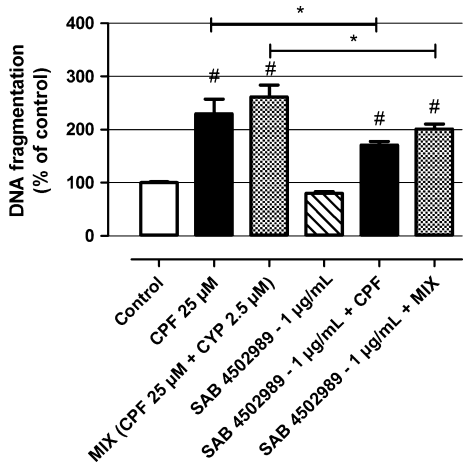

Fig. 9. The effects of anti-TNF-R2 antibody on chlorpyrifos (CPF) or mixture of chlorpyrifos and cypermethrin (CPF+CM)-induced SH-SY5Y cell apoptosis. SH-SY5Y cells were pre-incubated in the presence and absence of 1 µg/mL SAB 4502989 (TNF-R2 antibody) for 1 hr before treatment with CPF or CPF+CM for 24 hr. The Cell Death Detection ELISA kit was used to quantify DNA fragmentation cells undergoing apoptosis. Data are representative of three independent experiments. Statistical analysis was performed with one-way ANOVA. '#' Statistically significant difference when compared with control $*p < 0.05$.

inhibitor of ERK1/2, and by SB202190, an inhibitor of JNK activation. In contrast, blocking p38 signalling potentiated chlorpyrifos-induced apoptosis [11].

Considering our findings, as well as studies of a number of authors who investigated the toxic effects of CPF on other cells, this result is quite predictable. CPF activated ERK1/2, p38 and JNK signalling pathways in the dopaminergic cell line (PC-13, SH-SY5Y) at concentrations (100 µM) much higher than in the present study (25 µM). In other experimental models, the lower concentration of CPF (60 µM) was not related to MAPK signalling pathways at 24 hr incubation, while at 12 hr, CPF activated only p38 MAPK. Moreover, some MAPK inhibitors had different effects on cell survival [30].

Here, we report also that the CD95 (Fas/Apo1)-receptor was activated by tested compounds and mediated apoptosis in neuroblastoma cells via DNA fragmentation.

Pre-incubation of SH-SY5Y cells with anti-CD95 antibody in combination with pesticides resulted in an increase of apoptosis in SH-SY5Y cells. As the activation of the CD95 (Fas/Apo1)-receptor produced augmentation of the toxic effects of pesticide tested, it remains a possibility that CPF and CPF+CM induced neurotoxicity via CD95-mediated apoptotic cell death. These observations have been supported by the fact that inhibition of CD95 receptor by SAB4700005 did not reduce pesticide-triggered apoptosis.

In the present study, we demonstrated for the first time that pomalidomide (TNF-α inhibitor) attenuated the pesticide-induced apoptosis via reducing the DNA fragmentation in SH-SY5Y cells. These data indicate that the TNF-α receptors contribute to the induction of SH-SY5Y cells apoptosis after insecticide treatment and participate in the pro-apoptotic pathways to mediate pesticide-induced apoptosis.

The mechanism of the protective effect of inhibition of TNF-α can be mediated by TNF-α signalling via the TNF-α type 1 receptor (TNF-R1, p55TNFR) and TNF-α type 2 receptor (TNF-R2, p75TNFR). We also noted that inhibition of TNF signalling through TNFR2 induced by SAB4502989 caused inhibition of CPF- and CPF + CM-induced toxicity. Overall, our data suggest that TNF-α may participate in CPF and CPF+CM toxicity and corroborate earlier findings stating that TNF-α levels are increased in animals exposed to CPF [31].

Pomalidomide is the newest immunomodulatory drug and was created by a chemical modification of thalidomide. Its

mechanism of action is incompletely understood but involves anti-angiogenic effects and immunomodulation [32]. Apart from the immunomodulatory effects showed by pomalidomide on the inhibition of TNF-a, IL-1b, IL-6 and IL-12 and the enhancement of IL-10 production, pomalidomide may have other unknown anti-inflammatory effects [33]. Direct cytotoxic effects have been shown by pomalidomide, including the inhibition of nuclear factor kappa-B (NF- κB) and apoptosis induction via the caspase 8/death receptor pathway [34].

Tumour necrosis factor-alpha (TNF- α) has been shown to trigger many signalling pathways. It is known that TNF-α signalling is mediated via two distinct receptors, TNFR1 and TNFR2, which showed partially overlapping signalling mechanisms and biological roles depending on cell type [35]. Contrary to the cytotoxic effects of TNF-α through TNFR1, there is substantial evidence showing that TNF-α can promote neural cell survival through another TNF-α receptor, TNFR2 via the activation of NFκB [34]. As shown by Saulsbury *et al.* [29], chlorpyrifos-induced toxicity was characterized by the loss of mitochondrial potential, the appearance of nuclear condensation and fragmentation, down-regulation of Bcl-2 as well as up-regulation of TNF-α and FAS mRNA. However, in this study, pharmacological inhibition of FAS and TNF-α receptors did not attenuate CPF-induced toxicity in placental cells [29].

In conclusion, the current study provides insights concerning the molecular mechanisms of pesticide-induced apoptosis in neuronal cells. Both CPF and its mixture with CM were found to induce neurotoxicity in SH-SY5Y cells via apoptotic pathways. The obtained results may have implications for pesticide exposure in human beings, especially agricultural workers. Consequently, the exposure to investigated pesticides should be reduced.

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References

1 Påhlman S. Human neuroblastoma cells in culture: a model for neuronal cell differentiation and function. Acta Physiol Scand 1990;**592**:25–37.
2 Eaton DL, Daroff RB, Autrup H, Bridges J, Buffler P, Costa LG *et al.* Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. Crit Rev Toxicol 2008;**38**:1–125.
3 Abou-Donia MB, Lapadulla DM. Mechanisms of organophosphorus ester-induced delayed neurotoxicity: type I and Type II. Annu Rev Pharmacol Toxicol 1990;**30**:405–40.
4 Ki YW, Park JH, Lee JE, Shin IC, Koh HC. JNK and p38 MAPK regulate oxidative stress and the inflammatory response in chlorpyrifos-induced apoptosis. Toxicol Lett 2013;**218**:235–45.
5 Slotkin TA, Seidler FJ. Prenatal chlorpyrifos exposure elicits presynaptic serotonergic and dopaminergic hyperactivity at adolescence: critical periods for regional and sex-selective effects. Reprod Toxicol 2007;**23**:421–7.
6 Qiao D, Seidler FJ, Slotkin TA. Developmental neurotoxicity of chlorpyrifos modeled in vitro: comparative effects of metabolites and other cholinesterase inhibitors on DNA synthesis in PC-12 and C-6 glioma cells. Environ Health Perspect 2001;**109**:909–91.
7 Yang D, Howard A, Bruun D, Ajua-Alemanj M, Pickart C, Lein PJ. Chlorpyrifos and chlorpyrifos-oxon inhibit axonal growth by interfering with the morphogenic activity of acetylcholinesterase. Toxicol Appl Pharmacol 2008;**228**:32–41.
8 Yu F, Wang Z, Ju B, Wang Y, Bai D. Apoptotic effect of organophosphorus insecticide chlorpyrifos on mouse retina in vivo via oxidative stress and protection of combination of vitamins C and E. Exp Toxicol Pathol 2008;**59**:415–23.
9 Li Q, Kobayashi M, Kawada T. Chlorpyrifos induces apoptosis in human T cells. Toxicology 2009;**255**:53–7.
10 Park JH, Lee JE, Shin IC, Koh HC. Autophagy regulates chlorpyrifos-induced apoptosis in SH-SY5Y cells. Toxicol Appl Pharmacol 2013;**268**:55–67.
11 Caughlan A, Newhouse K, Namgung U, Xia Z. Chlorpyrifos induces apoptosis in rat cortical neurons that is regulated by a balance between p38 and ERK/JNK MAP kinases. Toxicol Sci 2004; **78**:125–34.
12 Igbedioh SO. Effects of agricultural pesticides on humans, animals, and higher plants in developing countries. Arch Environ Health 1991;**46**:218–24.
13 Maurya SK, Rai A, Rai NK, Deshpande S, Jain R, Mudiam MK *et al.* Cypermethrin induces astrocyte apoptosis by the disruption of the autocrine/paracrine mode of epidermal growth factor receptor signaling. Toxicol Sci 2012;**125**:473–87.
14 Wang XZ, Liu SS, Sun Y, Wu JY, Zhou YL, Zhang JH. Beta-cypermethrin impairs reproductive function in male mice by inducing oxidative stress. Theriogenology 2009;**72**:599–611.
15 Flaskos J, Harris W, Sachana M, Muñoz D, Tack J, Hargreaves AJ. The effects of diazinon and cypermethrin on the differentiation of neuronal and glial cell lines. Toxicol Appl Pharmacol 2007;**219**:172–80.
16 Singh AK, Tiwari MN, Prakash O, Singh MP. A current review of cypermethrin-induced neurotoxicity and nigrostriatal dopaminergic neurodegeneration. Curr Neuropharmacol 2012;**10**:64–71.
17 Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;**281**:1309–12.
18 Ashkenazi A, Dixit VM. Death receptors: signalling and modulation. Science 1998;**5381**:1305–8.
19 Whitmarsh AJ. Regulation of gene transcription by mitogen-activated protein kinase signaling pathways. Biochim Biophys Acta 2007;**1773**:1285–98.
20 Hetman M, Kanning K, Smith-Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol-3 kinase. J Biol Chem 1999;**274**:22569–80.
21 Litchfield JT, Wilcoxon FA. A simplified method of evaluating dose-effect experiments. J Pharmacol Exp Ther 1949;**96**:99–113.
22 Kovalevich J, Langford D. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. Methods Mol Biol 2013; **1078**:9–21.
23 Takei Y, Laskey R. Tumor necrosis factor alpha regulates responses to nerve growth factor, promoting neural cell survival but suppressing differentiation of neuroblastoma cells. Mol Biol Cell 2008;**19**:855–64.
24 Lee JE, Park JH, Shin IC, Koh HC. Reactive oxygen species regulated 626 mitochondria-mediated apoptosis in PC12 cells exposed to chlorpyrifos. Toxicol Appl Pharmacol 2012;**263**:148–62.
25 Kakko I, Toimela T, Tähti H. The toxicity of pyrethroid compounds in neural cell cultures studied with total ATP, mitochondrial enzyme activity and microscopic photographing. Environ Toxicol Pharmacol 2004;**15**:95–102.
26 Latuszyńska J, Luty S, Raszewski G, Przebirowska D, Tokarska-Rodak M. Neurotoxic effect of dermally applied chlorpyrifos and cypermethrin. Reversibility of changes. Ann Agric Environ Med 2003;**10**:197–201.

27 Carlson K, Jortner BS, Ehrich M. Organophosphorus compound-induced apoptosis in SH-SY5Y human neuroblastoma cells. Toxicol Appl Pharmacol 2000;**168**:102–13.
28 Newhouse K, Hsuan SL, Chang SH, Cai B, Wang Y, Xia Z. Rotenone-induced apoptosis is mediated by p38 and JNK MAP kinases in human dopaminergic SH-SY5Y cells. Toxicol Sci 2004;**79**:137–46.
29 Saulsbury MD, Heyliger SO, Wang K, Round D. Characterization of chlorpyrifos-induced apoptosis in placental cells. Toxicology 2008;**244**:98–110.
30 Ku BM, Lee YK, Jeong JY, Mun J, Han JY, Roh GS *et al.* Ethanol-induced oxidative stress is mediated by p38 MAPK pathway in mouse hippocampal cells. Neurosci Lett 2007;**419**:64–7.
31 Rowsey PJ, Gordon CJ. Tumor necrosis factor is involved in chlorpyrifos-induced changes in core temperature in the female rat. Toxicol Lett 1999;**109**:51–9.
32 McCurdy AR, Lacy MQ. Pomalidomide and its clinical potential for relapsed or refractory multiple myeloma: an update for the hematologist. Ther Adv Hematol 2013;**4**:211–6.
33 Tsai MJ, Chen C, Chen SH, Huang YT, Chiu TH. Pomalidomide suppresses cerulein-induced acute pancreatitis in mice. J Gastroenterol 2011;**46**:822–33.
34 Mitsiades N, Mitsiades C, Poulaki V, Chauhan D, Richardson P, Hideshima T *et al.* Apoptotic signaling induced by immunomodulatory thalidomide analogs in human multiple myeloma cells: therapeutic implications. Blood 2002;**99**:4525–30.
35 Thommesen L, Lægreid A. Distinct differences between TNF receptor 1- and TNF Receptor 2-mediated activation of NFκB. J Biochem Mol Biol 2005;**38**:281–8.