Cytotoxicity induced by cypermethrin in Human Neuroblastoma Cell Line SH-SY5Y

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Abstract

The purpose of this study was to evaluate the cytotoxic potential of Cypermethrin (CM) on cultured human Neuroblastoma SH-SY5Y cells. SH-SY5Y cells were treated with CM at 0–200µM for 24, 48, and 72 h, in vitro. It was found that CM induced the cell death of Neuroblastoma cells in a dose- and time-dependent manner, as shown by LDH assays. Next, some aspects of the process of cell death triggered by CM in the human SH-SY5Y cell line were investigated. It was revealed that the pan-caspase inhibitor Q-VD-OPh, sensitizes SH-SY5Y cells to necroptosis caused by CM. Furthermore, signal transduction inhibitors PD98059, SL-327, SB202190, SP600125 failed to attenuate the effect of the pesticide. Finally, it was shown that inhibition of TNF-a by Pomalidomide (PLD) caused statistically significant reduction in CM-induced cytotoxicity. Overall, the data obtained suggest that CM induces neurotoxicity in SH-SY5Y cells by necroptosis.

Key words
Cypermethrin, cell death, necroptosis, mechanism

INTRODUCTION

Cypermethrin (CM) [(RS)-a-cyano-3-phenoxybenzyl(1RS)-cis-trans-3-(2,2-dichloro-vinyl)-2,2-dimethylcyclopropa
carboxylate] is a synthetic type II pyrethroid which is extensively applied to control agricultural, as well as in consumer products for domestic purposes [1], due to their high insecticidal property and comparatively low toxicity. Some studies have demonstrated that CM crosses the blood–brain barrier (BBB) and induces neurotoxicity [2, 3]. The neurotoxic responses of CM are mainly mediated by the modulation of ion channels. As one of the primary targets for CM is the voltage-gated sodium channel, CM extends the opening of sodium channels in the central nervous system leading to hypo-polarization and hyper-excitation of the neurons [4]. Moreover, it is known that short-term neurotoxicity caused by CM is primarily mediated through hyper-excitation of the central nervous system [3]. Additionally, CM induces neurotoxicity by modulating the glutamate receptors, acetylcholine receptors and ATP-ases [2]. Furthermore, CM-mediated neurotoxicity is contributed by its ability to induce the generation of reactive oxygen species (ROS) and DNA damage [5]. Oral or intra-peritoneal administration of CM produces oxidative stress in the neuronal system [6]. ROS, especially superoxide anion and hydrogen peroxide, are important signaling molecules not only in developing and proliferating cells, but also in the induction of apoptosis [7]. A recent study by Maury et al. has shown that CM induces Ca2+ dependent activation of ROS, JNK1/2 and P38, causing disruption of BBB integrity in primary astrocytes [8]. A few reports have also shown the adverse effects of CM leading to nigrostriatal dopaminergic neurotoxicity [3, 9]. The potential developmental neurotoxicity of CM has also been studied in dopaminergic SH-SY5Y cells [10]. The decision taken by a cell to undergo apoptosis, autophagy and necroptosis is regulated by various factors, including the energy/ATP levels, the extent of damage or stress, and the presence of inhibitors of specific pathways. Our previous studies showed that the mixture of CPF and CM is more toxic to the SH-SY5Y cells than CPF used alone [11]. Hence, for the presented study, it was decided to examine the cytotoxicity of CM only, as well as some aspects of the process of cell death triggered by CM in the human SH-SY5Y cell line.

MATERIALS AND METHOD

Reagents. Cypermethrin [(RS)-a-cyano-3-phenoxybenzy
l(1RS)-cis-trans-3-(2,2-dichloro-vinyl)-2,2-dimethylcyclopropa
carboxylate] was purchased (Fluka, Sigma-Aldrich, St. Louis, MO, USA). Pomalidomide (3-amino-thalidomide), MAPK inhibitors: PD98059, SL-327, SB202190, SP600125 and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), were also obtained from Sigma, a Pan-caspase inhibitor Q-VD-OPh (N-(2-quinolyl)valyl-aspartyl
(2,6-difluoro- phenoxy) methylketone) was purchased (BioVision, Mountain View, CA, USA), and a lactate dehydrogenase (LDH) kit also purchased (Roche, Basel, Switzerland). Unless otherwise stated, all other reagents were purchased from the Sigma-Aldrich Chemical Company.

Reagents working solutions. Cypermethrin was dissolved in ethanol to concentration 100mM. Pan-caspase inhibitor, Q-VD-OPh (1 mM) and all MAPK inhibitors (10 mM) and Pomalidomide, PMD (10 mM) stock solutions were prepared in DMSO. Before performing the experiments, working solutions were prepared by dissolving an appropriate stock solution in culture medium. 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 SHSY5Y 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% CO2 at 37°C.

**Cytotoxicity studies.** As described previously [12], the cytotoxicity was estimated by measurement of Lactate Dehydrogenase (LDH) activity efflux from damaged cells into the medium. SH-SY5Y cells at 2×10⁵ cells/mL were treated with serial dilutions of CYP (1–200µM) for 24, 48 and 72h, at 37°C in 5% CO2. LDH activity released from SH-SY5Y cells, which represents cell death (necrosis), was determined with an LDH kit (In vitro Toxicology Assay Kit Lactate Dehydrogenase Based, Sigma) according to the manufacturer’s instructions.

Cytotoxicity was calculated by the following formula:

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\% \text{ Cytotoxicity} = 100 \times \left( \frac{E-S}{M-S} \right)
\]

where E is experimental LDH release from CM-treated cells; M is maximal LDH release caused by Triton X-100, and S is spontaneous LDH release from untreated cells [13].

Inhibitory studies were conducted by incubating SH-SY5Y cells (2×10⁵ cells/mL) with 50µM CM in the presence and absence of 5μM Q-VD-OPh, 20 μM PD98059 (ERK inhibitor), SL-327 (MEK inhibitor), SB202190 (p38 MAPK inhibitor), SP600125 (JNK inhibitor). After 48h of incubation in standard conditions, cytotoxicity of CM to SH-SY5Y cell after treatment using the LDH release were determined, as described above.

**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 software package, version 5.02, GraphPad Software Inc. USA). Significance was accepted at p < 0.05.

**RESULTS**

1. **Cypermethrin induces cytotoxicity in SH-SY5Y cell cultures.** To examine the toxic effects of CM, SH-SY5Y cells were treated with varying concentrations of CM and assayed for cell viability at various times after treatment using the LDH leakage from the SH-SY5Y cells into the incubation medium. Measuring LDH release is a useful method for detection of necrosis [12]. Treatment of cells with 1, 10, 25, 50, 100, 200 µM CM, at 24, 48 and 72 h, caused significant increases of cytotoxicity against SH-SY5Y cells (Tab. 1). The cytotoxic effects were dose- and time-dependent manner.

   Our subsequent experiments with CM were carried out at 50 µM, which caused 45.9±3.26% (p < 0.001) cytotoxicity to SH-SY5Y, after 48h treatment.

2. **Mechanisms of CM toxicity in SH-SY5Y cell cultures.** To investigate the mechanism by which CM induces cytotoxicity in SH-SY5Y cells, we examined the influence of inhibitors of proteins involved in cell death.

   To determine whether the major caspase pathways are involved in the regulation of CM-induced cytotoxicity, SH-SY5Y cells were exposed to 50 µM CM in the presence and absence of 5µM Q-VD-OPh, a pan-caspase inhibitor, for 48h. Q-VD-OPh slightly enhanced (p = 0.0329) CM-induced cytotoxicity in SH-SY5Y cell cultures (Fig. 2). In addition, the dose of 5µM Q-VD-OPh was not toxic against tested cells. Thus, these data suggest that Q-VD-OPh sensitizes SH-SY5Y cells to necrosis caused by CM.

   It is known that tumour necrosis factor (TNF) mediates apoptotic as well as necrotic forms of cell lysis; therefore, this study aimed to determine whether its activation mediates CM-induced cytotoxicity. For this purpose, CM-treated cells were exposed to Pomalidomide (PLD), pharmacological inhibitor of TNF-α production. The treatment with 25 µg/mL PLD cause slightly but statistically significant (p = 0.00345) reduction in CM-induced cytotoxicity (Fig. 3).

   To ascertain which MAP kinase signaling pathways mediated the toxic effects of CM, SH-SY5Y cells were exposed to 50 µM pesticide in the presence (20 µM) and absence of several specific inhibitors of these signaling pathways: PD98059 (ERK inhibitor), SL-327 (MEK inhibitor), SB202190 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor), for 24h.

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**Figure 1.** Cypermethrin (CM) causes dose-dependent SH-SY5Y cell cytotoxicity. Neuroblastoma cells were incubated for 24, 48 and 72h in serum-free media containing CM (0–200 µM), and Lactate dehydrogenase (LDH) ELISA kit was used to quantify LDH release and percentage of cytotoxicity was measured, as described above. Data are representative of three independent experiments. Statistical analysis was performed with one-way ANOVA. ***p < 0.001; **p < 0.01 when compared with controls.
As shown in fig. 3, PD98059, SL-327, SP600125 and SB202190 failed to alter the toxic effects of CM. However, SH-SYSY cells exposed to PD98059 exhibited enhanced cellular toxicity (Fig. 4).

DISCUSSION

Necrosis is a type of cell death that is morphologically characterized by swelling and rupture of intracellular organelles, eventually leading to the breakdown of the...
plasma membrane. The cytotoxicity assays for measuring necrosis are based on the leakage of intracellular molecules through impaired plasma membrane [13]. LDH is a soluble cytoplasmic enzyme that is present in almost all cells and is released into extracellular space when the plasma membrane is damaged by stress, injury, chemicals or intercellular signals. Thus, the quantification LDH from cells is one of the major methods to assess cell death by necrosis [14]. Necrosis has been defined as a type of cell death that lacks the features of apoptosis and is usually considered to be uncontrolled. However, several recent studies have clearly established that its occurrence and course might be tightly regulated [15].

The presented study examines whether exposure to CM results in cytotoxicity by assessing LDH leakage from the SH-SY5Y to the incubation medium. Treatment of cells with 0–200 µM of CM produced significant elevations in LDH leakage, and induced cytotoxicity in concentration- and time-dependent ways (Fig. 1).

The cytotoxic effect of Ripcort (a preparation contained an active compound cypermethrin) on SH-SY5Y cells was examined previously by Kokko et al. [16] in which exposure to 0.1–100 µM of CM showed dose-dependent 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. Unfortunately, in this work, CM was used with a mixture of xylene and petrol (820 g/L) and the toxic effects of these all substances could be additive. In earlier work [11], CM used in concentrations from 0.5–5 µM, for 24h, had no effect on SH-SY5Y cell viability as determined by MTT assay, and these observations are consistent with the data on cytotoxicity obtained by LDH leakage assay in the presented study. Additionally, CM had a significantly synergistic effect on CPF-induced cytotoxicity in studied cells [11]. Similarly, some studies showed that CM reduced the viability of astrocytes [8] and rat hepatocytes [17] in a dose-dependent manner.

It is known that necrosis can also function as an alternative programmed mode of cell death, triggered by the same death signals that induce apoptosis. The term ‘necroptosis’ was later defined as a type of cell death that lacks the features of apoptosis and is usually considered to be uncontrolled. The obtained results may have implications for estimating the risks of cypermethrin exposure.

CONCLUSION

The presented study provides insights concerning the molecular mechanisms of CM-induced cytotoxicity in human Neuroblastoma SH-SY5Y cells. CM was found to induce neurotoxicity in SH-SY5Y cells via necroptosis signaling pathways. The obtained results may have implications for estimating the risks of cypermethrin exposure.
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REFERENCES