



Tribromophenol induces the differentiation of SH-SY5Y human neuroblastoma cells in vitro

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Abstract

Tribromophenol is a pesticide with fungicide activity, presently used as a replacement of pentachlorophenol as a wood preservative, and as a flame retardant in electronic and electrotechnical devices. Retinoic acid differentiated and non-differentiated SH-SY5Y human neuroblastoma cell cultures were exposed to a range of concentrations of tribromophenol for 24, 48 and 72 h and the effects evaluated at morphological, basal cytotoxicity and biochemical levels. Neuroblastoma cell number, evaluated by quantification of total protein content, was increasingly inhibited in accordance with the concentration of tribromophenol and the exposure time period. According to the mean effective concentrations, differentiated cultures were nearly three times more sensitive than naive cells. Lysosomal function evaluated by the neutral red uptake was stimulated, particularly in non-differentiated cells. MTS metabolization was stimulated by all the treatments, with more potency at 24 h for differentiated cells. Acetylcholinesterase activity increased with the time of exposure in non-differentiated cells, while in differentiated cells the activity was doubled at 24 h. Morphological alterations were evident from 12.5 μM , showing hydropic degeneration and reduction in cell number, and from that concentration, piknosis and apoptotic bodies were observed. In conclusion, the main effects detected for tribromophenol were the induction of neuroblastoma cell differentiation, as expressed by the inhibition of cell growth and the increase in acetylcholinesterase activity with a critical cell concentration of 0.1 μM . Apoptosis was observed at high concentrations. The induction of cell differentiation and the special sensitivity of differentiated cells can explain some mechanisms involved in the embryotoxic and foetotoxic potential of tribromophenol.

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1. Introduction

Tribromophenol is a pesticide with fungicide activity, presently used as a replacement of pentachlorophenol as a wood preservative. It is also used as an antiseptic and a germicide, and as a chemical intermediate for its bismuth salt, as well as for pentachlorophenol and for

2,4,6-tribromophenoxy compounds. It is increasingly used as a flame retardant in thermoplastic polyester and epoxy resins, in acrylonitrile–butadiene–styrene resins, in phenolic resins and polystyrene, with enormous applications in electronic and electrotechnical devices such as mobile phones, television sets, computers, etc. (Tsunoda, 1989; EHC, 1997; Pullen, 2003). It is highly lipophilic and persistent, which causes bioaccumulation in the environment. Occupational exposure may occur through inhalation and dermal contact in its production, use and recycling. In the combustion of treated materials it is released into the environment through various waste streams (Lide, 1997). The general population may be exposed via ingestion of contaminated

Abbreviations: AChE, acetylcholinesterase; DTNB, 5,5'-dithio-bis (nitrobenzoic acid); EC₅₀, mean effective concentration; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H-tetrazolium salt.

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food and drinking water and by dermal contact. Tribromophenol is rapidly distributed in the body, and has been found to be present in relatively high concentrations in human blood samples (Thomsen, 2001).

The neurotoxic, embryotoxic and foetotoxic potential of tribromophenol has been suggested by Lyubimov et al. (1998). Toxicity signs described in rats included decreased motor activity, salivation, nasal discharge, tremors, prostration, clonic convulsions and death (EPA, 1990). The mechanisms of action and toxicity in human beings are unknown.

The aim of this study was to compare in vitro the effects of tribromophenol in undifferentiated and differentiated SH-SY5Y human neuroblastoma cells at morphological, basal cytotoxicity and biochemical levels. Upon differentiation, SH-SY5Y cells stop proliferating and increase process length (Pahlman et al., 1990). The differentiation is accompanied by increases in norepinephrine (NA) level, neuropeptide Y, neuron-specific enolase (NSE) activity, growth-associated protein-43, acetylcholinesterase and vesicle proteins such as synaptin/synaptophysin, secretogranin II, and SV2. Functionally, cell membrane potential is increased and the cells are more excitable following differentiation. For example, the cells begin to release NA following stimulation with acetylcholine. An increase in intracellular calcium, induction of voltage-gated calcium channel sensitivity, and functional changes in sodium and potassium conductances are also reported. These changes are indicative that differentiation induces a shift in the morphological, biochemical, and functional properties of SH-SY5Y cells so that they resemble the properties of mature neurons.

Toxicity indicators assessed were cell number by quantification of the total protein content of the cell culture; cytoplasmic membrane integrity by means of cytosolic lactate dehydrogenase (LDH) leakage, LDH activity and MTS metabolism; lysosomal function evaluated by the relative uptake of neutral red (RNRU) (Repetto and Sanz, 1993); and neuronal acetylcholinesterase (AChE) activity. Morphological effects were also investigated.

2. Materials and methods

2.1. Toxicant exposure

Stock solutions of tribromophenol (CAS: 118-79-6) (Merck, Darmstadt, Germany) were prepared in ethanol. A range of different concentrations of exposure solutions were prepared before use. After replacing the previous medium, the exposure solutions were added to the systems and incubated for the adequate exposure time period. Ethanol concentration in medium was 0.1%, including the control groups.

2.2. Model system

SH-SY5Y human neuroblastoma cell line was grown at 37 °C in 75-cm² flasks under a water-saturated sterile atmosphere containing 5% CO₂ in Ham's F-12 medium supplemented with 15% fetal calf serum (FCS). Cells were seeded at a density of 18,000 cells/well into 96-well tissue-culture plates for 48 h at 37 °C (seeding density was optimized at the beginning of the experiments; data not shown). Half of the cultures were differentiated by 3-day treatment with 10 μM of retinoic acid. The medium was replaced with 0.2 ml medium without FCS and with N1 Medium Supplement (Sigma, St Louis, USA) containing the test chemical in solution, and then incubated for 24, 48 or 72 h. The selected assays applied to this model system use colorimetric measurements for the quantification of the bioindicators after incubation with toxicants.

Cell number was quantified in situ, according to total cellular protein, using Coomassie Brilliant Blue G-250 (Repetto and Sanz, 1993) in the same 96-well tissue culture plates in which exposure originally took place (Repetto et al., 1994). Absorbency at 620 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland).

The neutral red (NR) assay is based on the uptake of neutral red, a supravital dye, and its accumulation in the lysosomes of viable uninjured cells. It was determined according to Borenfreund and Puerner (1984) and Repetto and Sanz (1993).

Cell viability was quantified by the MTS tetrazolium metabolism assay according to a procedure based on Baltrop et al. (1991). The MTS tetrazolium compound is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium.

LDH (EC 1.1.1.27) activity in cells and in culture medium was determined according to Duffy and Flint (1987) by following the production of NADH during the conversion of lactate to pyruvate.

AChE (Acetylcholine acetylhydrolase, EC 3.1.1.7) activity on intact cells was measured by adapting the method of Ellman et al. (1961), which follows the formation of the yellow 5-thio-2-nitrobenzoate anion (Repetto et al., 1994).

For the morphological study, SH-SY5Y cells were seeded in Lab-Tek[®] tissue culture chamber slides (Nunc, Inc., Naperville, IL). They were then exposed to tribromophenol for 24, 48 and 72 h, fixed in 70% methanol and stained with Mayer's hematoxylin and eosin.

2.3. Calculations and statistical analysis

Values for enzyme activities and relative neutral red uptake were corrected for cell culture total protein content to avoid misinterpretation due to the influence of the chemical tested on cell proliferation and cell

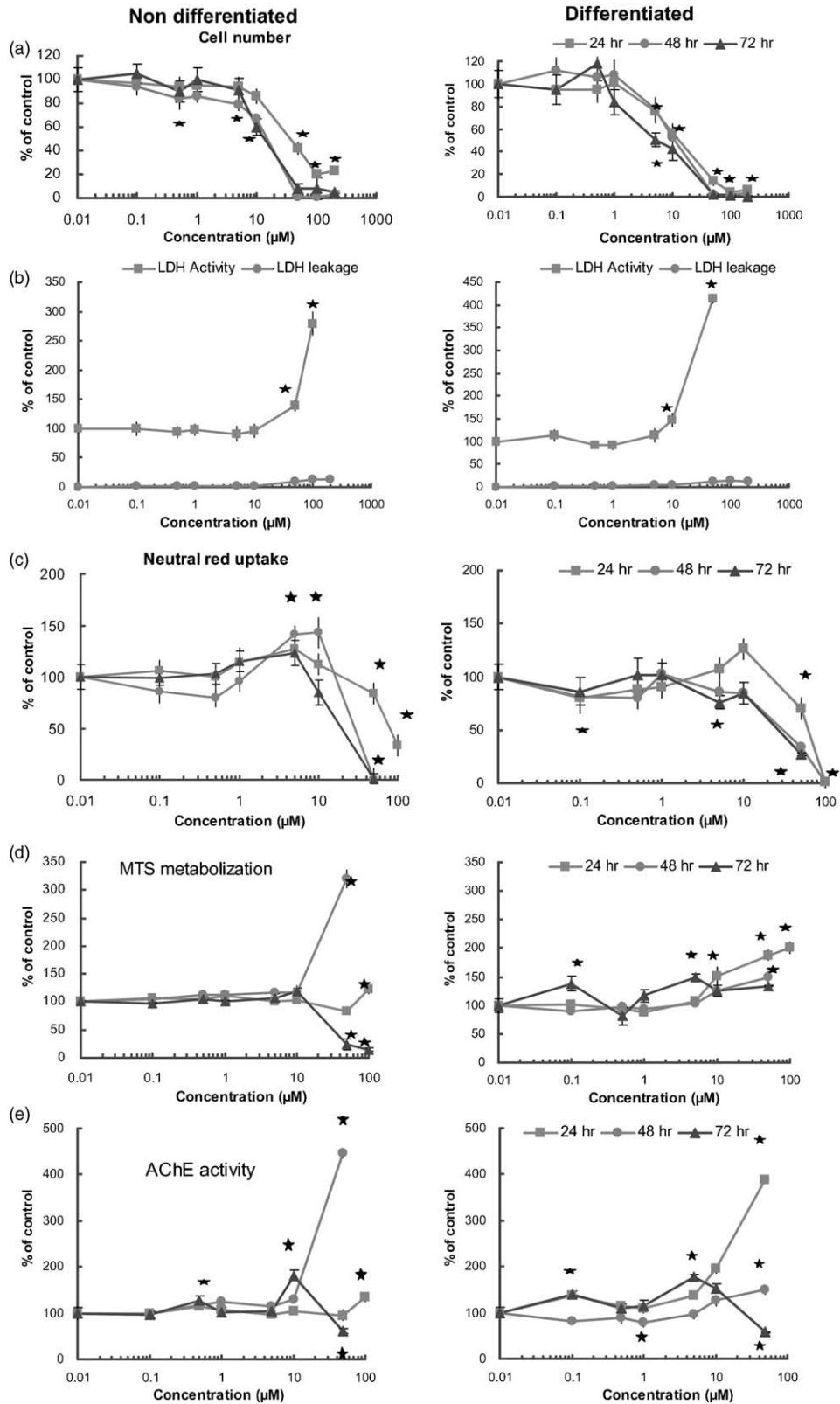


Fig. 1. In vitro effects of exposure to tribromophenol in non-differentiated and differentiated SH-SY5Y human neuroblastoma cells for 24 (■), 48 (●) and 72 h (▲) on (a) cell number, (b) lactate dehydrogenase leakage and activity, (c) relative neutral red uptake (d) MTS metabolization and (e) acetylcholinesterase activity. Each point represents the mean value of at least three experiments using six replicate cultures per concentration, expressed as the arithmetical mean percentage of unexposed controls \pm SEM. In order to avoid interference of chemicals in enzyme activities, the percentage of enzyme release of a culture was calculated as extracellular activity \times 100 divided by total activity (extracellular + intracellular activity) of the culture. * Indicates significant difference from control value ($P < 0.01$).

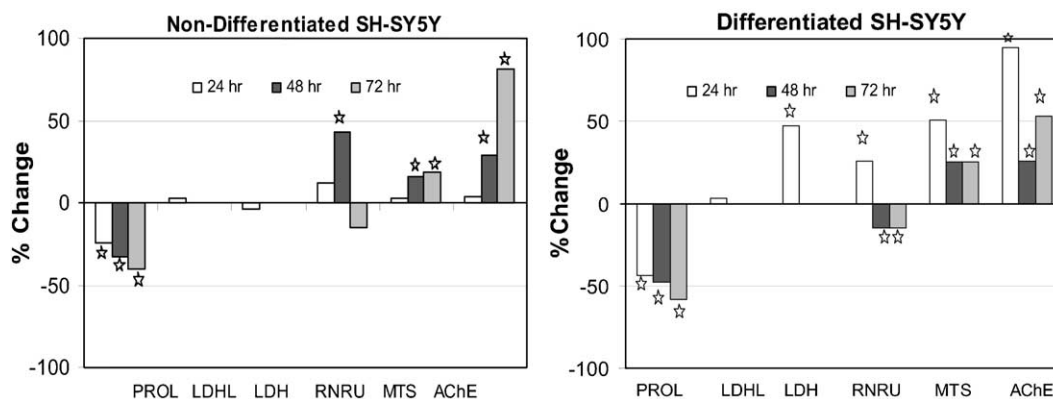


Fig. 2. Comparison of the extent of variation of each cytotoxicity and biochemical biomarker studied after exposure to 10 μM tribromophenol in non-differentiated and differentiated SH-SY5Y human neuroblastoma cells for up to 72 h. Toxicity indicators assessed in the in vitro test system were: cell proliferation (PROL), cytoplasmic membrane integrity to cytosolic lactate dehydrogenase leakage (LDHL), lactate dehydrogenase (LDH) intracellular activity, lysosomal function as relative neutral red uptake (RNRU), MTS dehydrogenase activity; and neuronal acetylcholinesterase (AChE) activity. Data expressed relative to mean value in respective unexposed controls. * Indicates significant difference from control value ($P < 0.01$).

detachment. All experiments were performed at least three times using 6 wells per concentration, and statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. EC_{50} and EC_{20} values were determined by linear interpolation.

3. Results and discussion

The in vitro effects of the neurotoxic pesticide tribromophenol were compared in undifferentiated and differentiated SH-SY5Y neuroblastoma cells at morphological, basal cytotoxicity and biochemical levels. The results of this study demonstrate a dose-dependent toxicity of tribromophenol on cultured SH-SY5Y human neuroblastoma cells, in which cell proliferation, evaluated by quantification of total protein content, was clearly inhibited. The effect was 2.6 times greater at 72 h of exposure than at 24 h, differentiated cells being nearly three times more sensitive than naive cells (Fig. 1a).

The stability of membranes was not markedly altered (Fig. 1b). Cytoplasmic membrane permeability, evaluated according to LDH leakage, a universally accepted marker of cell death (the intracellular enzyme is released when the membrane is disrupted causing cell death), was only significantly increased from 100 μM on, not exceeding 12% of the maximum possible—even at the highest assayed concentration—thus confirming the good viability of the cultures within the range of exposure concentrations.

Lysosomal function was evaluated according to the relative uptake of neutral red (Repetto and Sanz, 1993). In this modification of the standard neutral red cytotoxicity assay (Borenfreund et al., 1984), the results of cellular lysosomal uptake of the faintly cationic, supra-

vital dye are expressed relative to cell-culture protein content in order to avoid misinterpretation due to the influence of the chemical tested on cell proliferation and detachment. This marker of lysosomal function was less inhibited than cell growth, with some stimulation at intermediate concentrations at 48 h in non-differentiated cultures, and also at 24 and 72 h in differentiated cells (Fig. 1c).

Metabolic markers were altered by tribromophenol as well as the cytotoxicity indicators. LDH intracellular activity was stimulated at high concentrations in exposed cultures. MTS metabolism activity was increased in differentiated and non-differentiated cultures (Fig. 1d). LDH stimulation may occur as a result of the reported inhibition of the aerobic route; the increase in dehydrogenase activity may be a compensatory mechanism to the inhibitory effect on respiration due to the uncoupling of oxidative phosphorylation (Ratnikova and Yaguzhinskii, 1972). Both effects were previously described for the structurally related chemical pentachlorophenol, which is about two times more cytotoxic than tribromophenol (Jos et al., 2003).

Fig. 2 compares the extent of variation of each cytotoxicity and biochemical biomarker studied after exposure of SH-SY5Y human neuroblastoma cells for up to 72 h to 10 μM of tribromophenol. Non-statistically significant modifications were observed in cytoplasmic membrane permeability to LDH. The main modifications, particularly for differentiated cultures, were the inhibition of cell proliferation in parallel with increases in AChE activities and in MTS metabolism.

Fig. 3 shows that morphological changes were a sensitive indicator of the effects of tribromophenol. When SH-SY5Y human neuroblastoma cell cultures exposed to concentrations higher than 12.5 μM of tribromophenol for up to 72 h were compared with their respective controls (Fig. 3a), alterations, including reduction

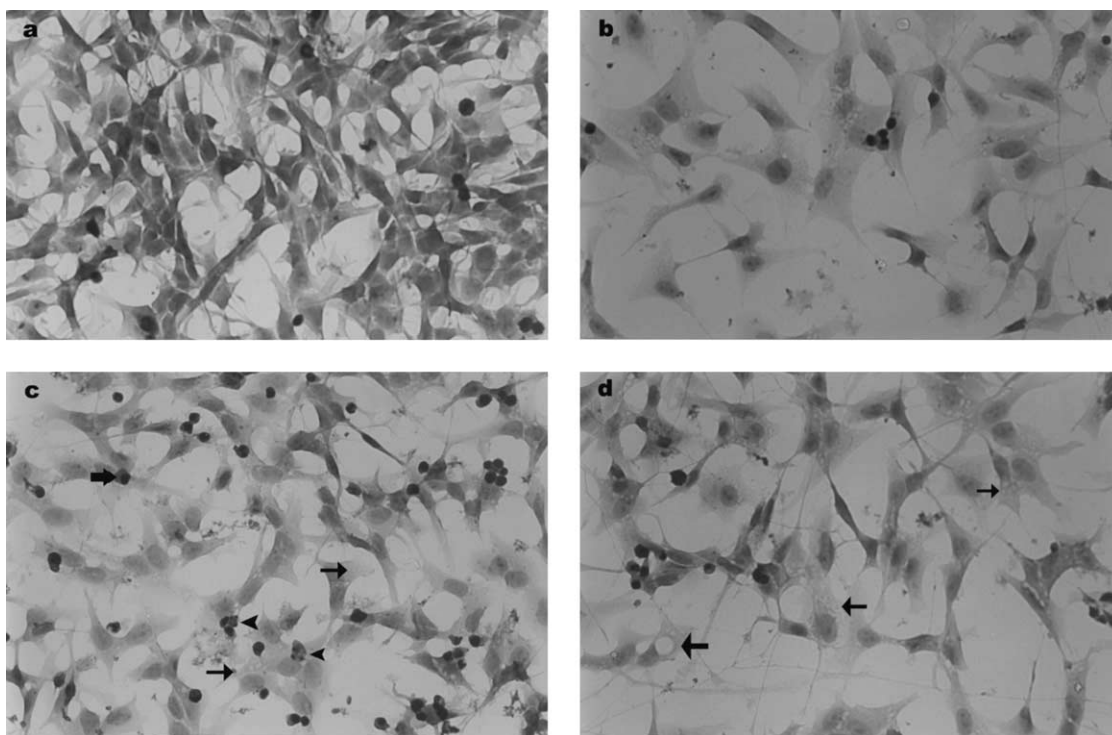


Fig. 3. Morphology of SH-SY5Y human neuroblastoma undifferentiated cell cultures stained with Maye's Hematoxylin and eosin: (a) control culture ($\times 400$) showing naïve cells, with short cytoplasmic prolongations interconnecting cells; (b) culture of undifferentiated cells exposed for 48 h to $12.5 \mu\text{M}$ of tribromophenol ($\times 400$), showing marked reduction of cell number, hydropic degeneration of the cytoplasm (cellular swelling), cells evidencing pyknotic nuclei and condensed cytoplasm; (c) culture exposed for 48 h to $25 \mu\text{M}$ of tribromophenol ($\times 400$) showing more pronounced injury, with many cells with pyknotic nuclei and condensed cytoplasm (\blacktriangleright), and general hydropic degeneration (\blackrightarrow), and presence of apoptotic bodies of cells developing apoptosis (\blacktriangleright); (d) culture exposed for 72 h to $12.5 \mu\text{M}$ tribromophenol ($\times 400$), showing differentiated cells with very long neurites interconnecting cells, and (\blackrightarrow) general hydropic degeneration.

of cell number, were increasingly observed. In addition to hydropic degeneration of the cytoplasm (cellular swelling), three-dimensional aggregated cell bodies were formed, with the presence of rounded cells. Increasing apoptosis induction by tribromophenol (from $25 \mu\text{M}$) was characterized in the morphological study by the presence of apoptotic bodies of neural cells developing apoptosis. At $75 \mu\text{M}$, more pronounced injury occurred, with many cells evidencing pyknotic nuclei and condensed cytoplasm. At higher concentrations of tribromophenol, cell detachment increased and the remaining cells were not viable.

The most prominent effect observed in human neuroblastoma cultures exposed to $6 \mu\text{M}$ of tribromophenol was the induction of a marked neuronal differentiation from 48 h exposure, with extension of very long neurites interconnecting cells. Neural AChE activity was also stimulated, particularly in non-differentiated cells at 48 h and at 24 h in differentiated cells. The increase in acetylcholinesterase and the reduction in cell proliferation induced by tribromophenol are both biochemical markers of neuronal differentiation, also demonstrated morphologically. The induction of neuronal differentiation by tribromophenol can contribute to its embryotoxic and foetotoxic potential (Lyubimov et al., 1998).

A more distinct pattern of cell differentiation with tribromophenol than with retinoic acid, a classical inducer of cell differentiation, occurred. The biochemical differentiation, quantified according to the increase in acetylcholinesterase activity, was less marked with tribromophenol than with retinoic acid. In addition, neurite outgrowth induced by tribromophenol developed slowly but more prominently than with retinoic acid, causing less damage to the cells. The neurites induced were longer and thinner than those observed with retinoic acid. Cells already differentiated with retinoic acid were also stimulated to a greater differentiation state by tribromophenol.

Table 1 includes the EC_{50} and EC_{20} concentrations of tribromophenol for the different bioindicators used. The lowest EC_{50} was obtained for acetylcholinesterase activity, $7.5 \mu\text{M}$ for non-differentiated cells and 2.5 for differentiated neuroblastoma cultures. The cytotoxicity according to the inhibition of cell proliferation at 24 h was $37 \mu\text{M}$, about two times less toxic than pentachlorophenol (Jos et al., 2003). The lowest EC_{20} obtained for non-differentiated treated cells was $0.36 \mu\text{M}$ for acetylcholinesterase activity, and $0.03 \mu\text{M}$ for acetylcholinesterase activity and MTS metabolism for differentiated cultures.

Table 1
Toxic effects of tribromophenol on the selected biomarkers according to EC₅₀ and EC₂₀ values (µM)^a

Indicator	Exposure time period (h)	Non-differentiated EC ₅₀	Cells EC ₂₀	Differentiated EC ₅₀	Cells EC ₂₀
Cell number	24	37±7	12.5±2	12.5±4	3.8±3
Cell number	48	15±1	4±2	10.5±2	4.5±3
Cell number	72	14±3	6.4±1	5±0.6	1.8±0.8
LDH leakage	24	> 200	> 200	> 200	> 200
LDH activity	24	80±7	24.5±3	10±1	5.8±1
Relative neutral red uptake	24	80±9	2.1±1	53±9	0.1±0.2
Relative neutral red uptake	48	29±4	0.5±1	30±4	0.5±0.2
Relative neutral red uptake	72	20±5	2.7±2	25±4	4±3
MTS metabolization	24	> 200	96±10	10±3	6±2
MTS metabolization	48	14±2	10.3±5	50±6	8.5±1
MTS metabolization	72	30±6	19±1	5±0.5	0.03±0.01
Acetylcholinesterase activity	24	>200	>200	6±1	0.04±0.01
Acetylcholinesterase activity	48	13±0.5	0.7±0.4	50±6	0.6±0.3
Acetylcholinesterase activity	72	7.5 ± 1	0.4±0.3	2.5±1	0.03±0.02

^a EC₅₀: Mean effective concentration, concentration of test chemical that modified each biomarker by 50% (positive or negative) in comparison with appropriate untreated controls; EC₂₀: Mean effective concentration, concentration of test chemical that modified each biomarker by 20% (positive or negative) in comparison with appropriate untreated controls.

The lowest statistically significant critical cellular toxic concentrations for human neuroblastoma cells were calculated according to Walum et al. (1993), being 0.5 µM for cell proliferation, neutral red uptake and acetylcholinesterase, for non-differentiated cells. In the case of differentiated cultures, the critical cellular toxic concentrations were 0.1 µM for acetylcholinesterase activity at 24 and 72 h, the last in coincidence with MTS metabolization and neutral red uptake at 48 h of exposure. The global critical cellular toxic concentration was 0.1 µM for increases in acetylcholinesterase activity.

Occupational exposure to tribromophenol is produced through inhalation and dermal contact, but the general population may be exposed via ingestion of food and drinking water. The neurotoxic, embryotoxic and foetotoxic potential of tribromophenol has been suggested by Lyubimov et al. (1998), with a NOAEL in rats for developmental neurotoxicity inferior to 0.03 mg/m³, and for maternal neurotoxicity of 0.3 mg/m³ (equivalent to 0.91 nM). Although the air concentration in a chronic in vivo study cannot be directly compared with the concentration used for the short term in vitro assay, it can be deduced that a similar sensibility was found.

In conclusion, differentiated cultures of human SH-SY5Y neuroblastoma cells were nearly three times more sensitive than naive cells for all the indicators considered. Lysosomal function evaluated by the neutral red uptake was stimulated, particularly in non-differentiated cells. MTS metabolization was stimulated by all the treatments, with more potency at 24 h for differentiated cells. Acetylcholinesterase activity increased with the time of exposure in non-differentiated cells, and in differentiated cells the activity was doubled at 24 h. Morphological alterations were evident from 12.5 µM,

showing hydropic degeneration and reduction in cell number. At higher concentrations than 12.5 µM more lesions were observed, including piknosis, as well as the presence of apoptotic bodies. In conclusion, the main effects detected for tribromophenol were the induction of neuroblastoma cell differentiation, as expressed by the inhibition of cell growth and the increase in acetylcholinesterase activity, and apoptosis at high concentrations. The induction of cell differentiation and the special sensitivity of differentiated cells can explain some mechanisms involved in the embryotoxic and foetotoxic potential of tribromophenol.

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