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In vitro neurotoxic hazard characterisation of dinitrophenolic herbicides

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ABSTRACT

Dinitrophenolic compounds are powerful toxicants with a long history of use in agriculture and industry. While (high) human exposure levels are not uncommon, in particular for agricultural workers during the spraying season, the neurotoxic mechanism(s) that underlie the human health effects are largely unknown. We therefore investigated the *in vitro* effects of two dinitrophenolic herbicides (DNOC and dinoseb) on a battery of neurotoxicity endpoints in (dopaminergic) rat PC12 cells.

Cell viability, mitochondrial activity, oxidative stress and caspase activation were assessed using fluorescence-based bioassays (CFDA, alamar Blue, H_2DCFDA and Ac-DEVD-AMC, respectively), whereas changes in intracellular $[Ca^{2+}]_i$ were assessed using single-cell fluorescence microscopy with Fura-2AM. The combined results demonstrate that exposure to both DNOC and dinoseb is linked to calcium release from the endoplasmic reticulum and activation of caspase-mediated apoptotic pathways. In subsequent experiments, immunofluorescent labelling with specific antibodies was used to determine changes in intracellular α-synuclein levels, demonstrating that both DNOC and dinoseb increase levels of intracellular α-synuclein. The combined results indicate that *in vitro* exposure to DNOC and dinoseb activates pathways that are not only involved in acute neurotoxicity but also in long-term effects as seen in neurodegeneration.

1. Introduction

Humans are exposed to a plethora of chemicals that are potentially neurotoxic. While different in their presumed primary modes of action, many classes of chemicals disturb intracellular calcium homeostasis as a common mode of action. Our previous research has demonstrated that *e.g.*, PCBs (Langeveld et al., 2012; Westerink, 2014), PBDEs (Dingemans et al., 2010; Westerink, 2014) and different classes of pesticides, including conazole fungicides (Heusinkveld et al., 2013) and organochlorine, pyrethroid and organophosphate insecticides (Heusinkveld and Westerink, 2012; Meijer et al., 2014) all target intracellular calcium homeostasis. In several instances, disturbance of the intracellular calcium concentration ($[Ca^{2+}]_i$) already occurs at concentrations that do not yet affect the presumed primary mode of action, highlighting the importance of this common endpoint. Despite the presence of a large number of pesticides in the list of compounds that can affect $[Ca^{2+}]_i$, there is currently relative limited data on the potential disturbance of calcium homeostasis by herbicides.

The dinitrophenolic compounds 4,6-dinitro-*o*-cresol (DNOC; CAS No. 534-52-1; Table 1) and 2-(1-methylpropyl)-4,6-dinitrophenol (dinoseb; CAS No. 88-85-7; Table 1) are derivatives of 2,4-dinitrophenol (2,4-DNP; CAS No. 51-28-7). Human exposure to DNOC and dinoseb is mainly occupational due to the use as defoliating agents in potato culture, as herbicide targeting grass and broadleaf weeds and as insecticide in grape production (Whitacre et al., 2004). Furthermore, dinitrophenolics have a long history of use in polymer industry and in the manufacture of dyes and explosives. These compounds are easily absorbed via various routes of exposure due to their lipophilicity (DNOC log K_{ow}: 2.56; dinoseb log K_{ow}: 3.56; Parker et al., 1951). Despite a ban on the use of DNOC and dinoseb as pesticide in Europe in the late 1990's, both

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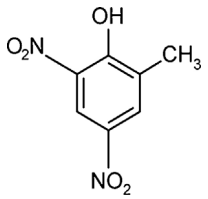
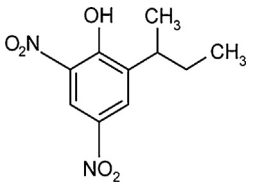
Table 1
Molecular structure and chemico-physical properties of DNOC and dinoseb.

		
Chemical name	2-Methyl-4,6-Dinitrophenol (DNOC)	2-(1-Methylpropyl)-4,6-Dinitrophenol (Dinoseb)
CAS number	534–52-1	88–85-7
Mol. weight (g/mol)	198.1	240.2
Log K_{OW}	2.56	3.56

compounds have been found consistently in the environment (see *e.g.* Duyzer, 2003; Quaghebeur et al., 2004; Schummer et al., 2009), which is at least partly due to their continuing use in (plastic) industry but also to their slow environmental degradation (Schummer et al., 2009). Continued use of these compounds in other parts of the world also results in local detection in surface and drinking water (Li et al., 2012).

Dinitrophenolic herbicides are powerful toxicants in plants, animals and humans with high acute toxicity (*e.g.* mammalian LD_{50} dinoseb: oral = 37 mg/kg body weight, dermal = 200 mg/kg body weight; Puls, 1988). Toxicity is thought to be related primarily to uncoupling of mitochondrial phosphorylation (uncoupling in mouse-brain mitochondrial preparation EC_{100}: DNOC 20 μM, dinoseb 0.5 μM; Ilivicky and Casida, 1969), leading to increased oxidative metabolism and depletion of cellular ATP, subsequently resulting in cell and tissue damage (Ilivicky and Casida, 1969; Palmeira et al., 1994). Considering the dependence of the nervous system on ATP homeostasis, neuronal cells may thus be particularly vulnerable to the toxic effects of dinitrophenolic herbicides, especially since *in vivo* experiments indicated that dinitrophenolic compounds cross biological membranes, such as the blood-brain and the placental barrier (Gibson and Rao, 1973).

Acute toxicity of dinitrophenolic compounds is characterized by delirium, dizziness, loss of consciousness and dysregulation of body temperature that often leads to fatal hyperthermia (Estuardo et al., 2006; Grundlingh et al., 2011). Although there is no known remedy, successful administration of dantrolene has been reported (Siegmueller and Narasimhaiah, 2010). Since dantrolene is an antagonist of ryanodine receptors that prevents calcium release from the endoplasmic reticulum (Van Winkle, 1976), this indicates that changes in calcium homeostasis may comprise part of the mode of action of dinitrophenolic compounds. In the central nervous system, calcium plays a pivotal role in many inter- and intraneuronal processes ranging from normal neuronal functioning to differentiation and degeneration (Spitzer et al., 2004; Westerink, 2006; Mattson, 2007).

Dopaminergic rat pheochromocytoma cells PC12; (Greene and Tischler, 1976) are a well-known, extensively characterized model for neurotoxicity studies (Westerink and Ewing, 2008; Westerink, 2014). These cells express different types of voltage-gated calcium- and sodium channels and display several characteristics of mature dopaminergic neurons such as synthesis, storage and vesicular release of dopamine (DA) (Shafer and Atchison, 1991; Westerink and Ewing, 2008). PC12 cells have thus proven suitable for the functional study of calcium-related neurotoxicity of compounds *in vitro* (Heusinkveld et al., 2013; Westerink, 2014). In the current study, we therefore used PC12 cells to study the effects of acute exposure to two common dinitrophenolic herbicides (DNOC and dinoseb) on intracellular calcium homeostasis and *in vitro* neurotoxicity.

2. Materials and methods

2.1. Chemicals

Fura-2-AM, 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM) and 2,7-dichlorodihydrofluorescein diacetate (H_2-DCFDA) were obtained from Molecular Probes (Invitrogen, Breda, The Netherlands); DNOC and dinoseb were obtained Pestanal® grade, 99.8% purity (Riedel de Haën, Seelze, Germany); all other chemicals were obtained from Sigma (Zwijndrecht, The Netherlands), unless otherwise noted. Saline solutions, containing (in mM) 125 NaCl, 5.5 KCl, 2$CaCl_2$, 0.8 $MgCl_2$, 10HEPES, 24 glucose and 36.5 sucrose (pH 7.3), were prepared with de-ionized water (Milli-Q®; resistivity >18 MΩ·cm). Stock solutions of 2 mM ionomycin in DMSO were kept at -20 °C. Stock solutions of 0.1–100 mM DNOC and dinoseb (Pestanal® grade, 99.8% purity, Riedel de Haën, Seelze, Germany) were prepared in DMSO and diluted in saline to obtain the desired concentrations just prior to the experiments (all solutions used in experiments, including control experiments, contained 0.1% DMSO).

2.2. Cell culture

PC12 cells (Greene and Tischler, 1976) were grown for up to 10 passages in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 5% fetal calf serum and 10% horse serum (ICN Biomedicals, Zoetermeer, The Netherlands) in a humidified incubator at 37 °C and 5% CO_2 as described previously (Heusinkveld et al., 2013). For Ca^{2+} imaging experiments, cells were subcultured in poly-L-lysine coated glass-bottom dishes (MatTek, Ashland, MA, USA) as described previously (Heusinkveld et al., 2013). For cell viability and caspase experiments cells were sub-cultured in poly-L-lysine coated 24-wells plates (Greiner Bio-one, Solingen, Germany) at a density of 5×10^5 cells/well. For experiments assessing oxidative stress, *i.e.* enhanced production of reactive oxygen species (ROS), cells were seeded in poly-L-lysine coated 48-wells plates at a density of 2.5×10^5 cells/well. For alpha-synuclein immunostaining cells were subcultured on poly-L-lysine coated coverslips at a density of 5×10^5 cells per coverslip.

2.3. Absorbance spectra dinitrophenolics

To assess potential interference of DNOC and dinoseb with the excitation and emission wavelengths used in different fluorescence assays, absorbance spectra (300–600 nm) of