

EXPOSURE TO DEFINED MIXTURES OF PERSISTENT ORGANIC POLLUTANTS (POPs) LEADS TO MITOCHONDRIAL INJURY IN THE RAT H4IIE HEPATOMA CELLS

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Introduction

Persistent organic pollutants (POPs) are regarded as a threat for humans and wildlife since they are toxic, resistant to degradation, bioaccumulate and biomagnify in animal bodies¹. Although, the exposure risk for chemical compounds (including POPs) is usually assessed for each compound individually, in real life, POPs occur as a complex mixture. Hence, understanding the effects resulting from these mixture exposures is required for evaluating their impact on the health of humans and wildlife. To this aim, a realistic POP mixture consisting of 29 POPs, each at individual concentrations corresponding to exposure levels reported in Scandinavian blood, has been constructed² and studied in several studies³⁻⁶. Among them, Wilson *et al.*, (2016)⁶ contributed to the understanding of the *in vitro* effects on human hepatoma cells of the total POP mixture and several POP sub-mixtures by applying High Content Analysis (HCA). The HCA as a high-throughput and quantitative technique⁶ has been used to examine the sublethal chemical induced toxicity based on multiple morphological and functional cell parameters⁷. The rat hepatoma cell lines with luciferase genetical modification, responsive to dioxins (named here after DR-H4IIE) from Biodetection system (BDS, the Netherlands), have been used widely for screening the aryl hydrocarbon receptor (AhR) transactivity of POPs in reporter gene assays⁸⁻¹¹. Hence, it is important to understand POP induced sub-lethal toxicity in this cell line in order to better interpret the cell response of the reporter gene assays. In this study, the effects regarding several cellular responses of seven complex POPs mixtures were investigated in the rat DR-H4IIE by HCA.

Materials and methods

A realistic mixture of 29 POPs (called the total POP mixture) was premade by the Norwegian University of Life Sciences, Oslo, based on the POP abundance in human blood from Scandinavians, as described in Berntsen *et al.* (2017)². The POPs belong to three groups: six perfluorinated compounds (PFAAs), seven brominated compounds (Br), and 16 chlorinated compounds (Cl) including seven polychlorinated biphenyls (PCBs) and nine organochlorine pesticides (OCPs). Six sub-mixtures consisting of one single class of compounds (PFAA, Br, Cl) or combined two classes (Br+Cl, PFAA+Cl, PFAA+Br) were also examined for their toxicity. They were tested at concentrations ranging from 0.1 to 1000-fold blood level.

Hoechst 33342 Nuclear Stain supplied by Perbio (Northumberland, England) and MitoTrackerTM Orange CMTM Ros dyes purchased from Life Technologies (Paisley, UK) were used to investigate nuclear and mitochondrial cytotoxicity, respectively, in the rat DR-H4IIE exposed to the seven mixtures. Valinomycin purchased from Sigma Aldrich (USA) was used as a positive control. The seeding density of the cells was 1×10^5 cells/ml to allow single cell visualization. Procedures closely followed the manufacturer's instructions. Briefly, after live cells were stained with MitoTracker (100 nM) and incubated for 30 min at 37 °C, they were fixed with 10% formalin solution for 20 min at room temperature, then washed with PBS. The fixed cells were then stained with Hoechst 33342 dye (1.6 μM) for 10 minutes, washed and the wells were filled with PBS. The plates were then sealed with a plate sealer and read using a CellInsightTMNXT High Content Screening (HCS) platform (Thermo Fisher Scientific, UK) connected to an automated microplate reader analyzer interfaced with a PC (Dell precision 136 T5600 workstation). Hoechst dye (Ex/Em 350/461 nm) was used to measure nuclear responses (cell number (CN), nuclear area (NA), and nuclear intensity (NI)), while MitoTracker dye (Ex/Em 554/ 576 nm) revealed mitochondrial characteristics (mitochondrial area (MA) and mitochondrial intensity (MI)). 25 field view images were collected for each condition with numerical outputs by x10 objective magnification. The results are presented as responses relative to that of DMSO. ANOVA was performed for statistical significance.

Results and discussion

High content analysis (HCA) was applied to evaluate several morphological and functional cellular parameters in the rat DR-H4IIE cells for the total POP mixture and six POP sub-mixtures. Hoechst 33342 dye is specific for DNA stain and can reveal the nuclear conditions of the cells, for example, the cell number (CN), nuclear intensity (NI) and areas (NA). From these the health status of the cells can be examined. MitoTracker dye can be used to observe mitochondrial functioning. The dye accumulates in active mitochondria, giving two parameters of mitochondrial membrane potential: mitochondrial area (MA) and mitochondrial intensity (MI). Figure 1 illustrates the visual images of DR-H4IIE obtained by HCA, directly comparing the nuclear and mitochondrial parameters of a negative control (A: DMSO), a positive control (B: 100 nM Valinomycin) and a sample (C: the total POP mixture at 1000-fold blood level).

Of the five cellular parameters targeted, cell viability (CN) (Figure 2A) and mitochondrial membrane intensity (MI) (Figure 2B) were observed as good markers of cytotoxicity of the rat DR-H4IIE cell line when exposed to the POP mixtures. Both NI and NA, as well as MA did not change over the entire POP concentration range (data not shown). Only Cl containing mixtures (the total POP mixture, and Cl, Br+Cl and PFAA+Cl sub-mixtures) were able to induce a significant decrease of CN at 1000-fold blood level, and MI (except the Br+Cl) at already 100-fold blood level (Figure 2). PFAA and Br or their combination PFAA+Br did not exhibit significant effects on these parameters.

The cell viability (CN) of cells exposed to 1000-fold blood level somehow decreased by about 10% to 20% for all seven mixtures, except the PFAA mixture (Figure 2A). The Cl containing mixtures decreased the cell number by about 20%, and in particular, PFAA+Cl and Br+Cl mixtures induced the maximum decrease by 21% and 26%, respectively, while the total POP mixture and the Cl mixture induced a decrease of only 13% and 14%, respectively.

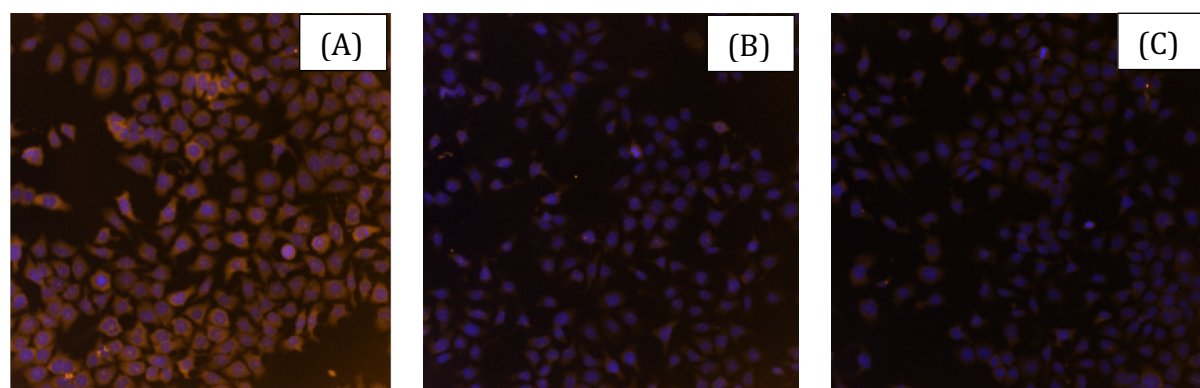
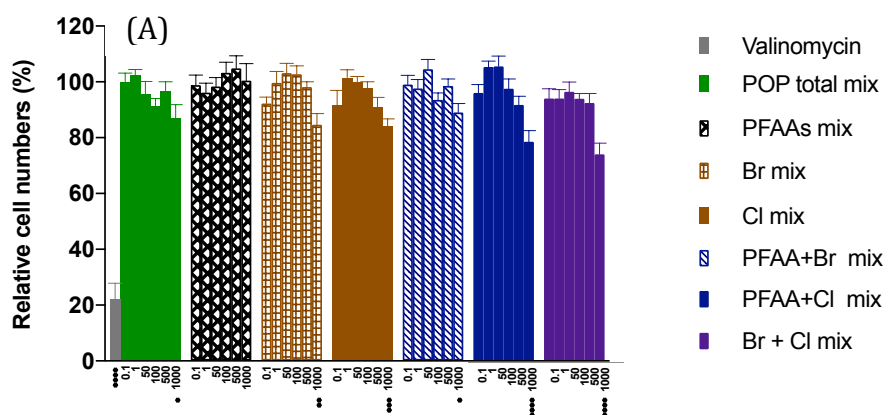


Figure 1: HCA images for (A) negative control (DMSO), (B) positive control (100 nM Valinomycin), (C) an example of DR-H4IIE exposed to the total POP mixture at 1000-fold blood level, after 24h exposure. The image was acquired at 10 \times objective magnification using Hoechst dye (blue; nuclear staining) and MitoTracker dye (orange; mitochondrial staining). Dimension 200 μ m x 200 μ m.



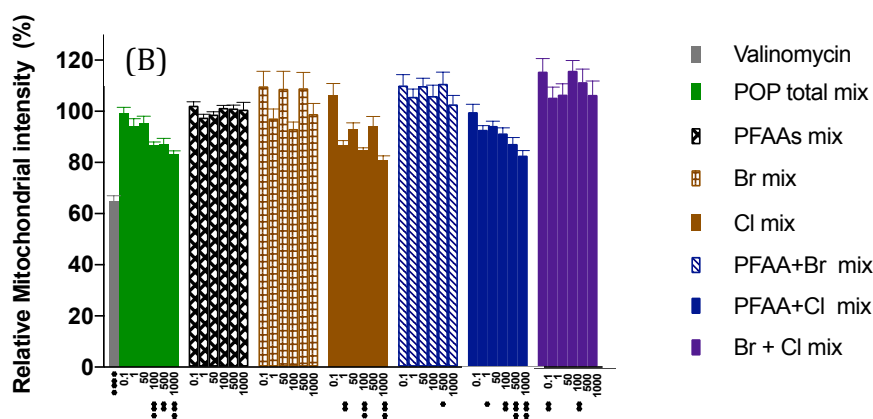


Figure 2: Cell viability in cell number (CN, Figure 1A), and mitochondrial intensity (MI, Figure 1B) of the total POP mixture (green, solid), PFAAs (black, triangle), Br (brown, square), Cl (brown, solid), PFAA+Br (blue, parallel), PFAA+Cl (blue, solid), and Br+Cl mixtures (purple, solid) in DR-H4IIE cells exposed to six different concentrations (1/10, 1, 50, 100, 500 and 1000-fold blood level) for 24h. Data expressed relative to the control DMSO treatment, Mean \pm SE, n=3, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****) expressing statistical significance.

The mitochondrial membrane intensity (MI) was significantly decreased in cells exposed to the POPs at 100-fold blood level only, for three out of the four Cl containing mixtures, which makes it the most sensitive parameter for detection of cytotoxicity. The total POP mixture and the PFAA+Cl and Cl mixtures decreased the MI of DR-H4IIE by about 20%, while the Br+Cl mixture did not induce MI reduction, indicating interactions among the compound in these mixtures. The decrease in MI parameter reflects the fact that cells are experiencing the loss of their mitochondrial potential, but no changes were observed for MA.

Reduction in MI is considered an early marker for an apoptosis-induction pathway¹², accompanied by an increase in MA due to mitochondrial swelling from the mitochondrial permeability transition pore opening¹³, followed by nuclear shrinkage. When the DR-H4IIE cells were exposed to the total POP, the Cl, and the PFAA+Cl mixtures, MI, comparable with the CN, was the earliest observable marker for apoptosis, before MA or NI and NA, at a concentration of 100-fold blood level. This may indicate that the DR-H4IIE cells are entering apoptosis, but the other markers namely, MA or NI and NA, have not occurred yet due to insufficient exposure time and/or concentrations. In comparison, Wilson *et al.*, (2016)⁶ found the increase in mitochondrial mass (area) when the human liver cancerous cell Hep G2 was exposed to Cl and Br mixtures at a concentration of more than 100 (at 48 hours) and 1000 (at 2 hours) fold blood level, respectively, along with the evaluation of the reactive oxygen species but not for membrane potential. Hence, they suspected that exposure to the Cl and Br mixtures did not lead to apoptosis in Hep G2.

This study shows that Cl containing mixtures at concentrations as low as 100-fold blood level induced mitochondrial dysfunction due to the reduction in mitochondrial membrane potential in DR-H4IIE. Park *et al.* (2013)¹⁴ saw *in vitro* in mouse myoblast cells the link between exposure to POPs such as AhR agonists and mitochondrial impairment of generating ATP, which, they suggested, leads to weight gain, glucose intolerance and metabolic syndrome. Furthermore, the group also found that plasma concentration of dioxin like-PCBs in Korean serum, was negatively correlated with ATP concentration but no relationship was seen for BDE-47, OCPs or PFAAs by studying on Hepa1c1c7 cells¹⁵. We found a relationship between mitochondrial injury and exposure to the Cl (both PCBs and OCPs) containing mixtures (but not to the Br+Cl mixture) at a low concentration corresponding to 100-fold blood level. Therefore, this exposure could lead to the insufficient production of ATP in mitochondria in the rat liver cells and to related diseases. No mitochondrial effect has been seen in cells exposed to Br and PFAA containing mixtures or even Br+Cl mixture, which is in agreement with the previous studies^{14,15}.

Using the same cell line (DR-H4IIE cells) in reporter gene assays, our group also showed an antagonistic effect on the aryl hydrocarbon receptor transactivity of the total POP mixture as well as the other three Cl containing mixtures (Cl, PFAA+Cl, and Br+Cl)¹¹. There could be a link between the antagonistic effect on the aryl hydrocarbon receptor, which is responsible for xenobiotic metabolism, and the impairment of the mitochondria of cells exposed to POPs. It may be that the reduction in ATP production of mitochondria could compromise the luciferase production in the reporter gene assays, leading to the observed antagonism of all Cl containing mixtures. However, this seems unlikely as cells exposed to high concentration of POPs alone did not show a decrease in AhR transactivity.

In conclusion, by using HCA, the MI was observed as the earliest marker for apoptosis in the rat DR-H4IIE exposed to the total POP and three other Cl containing sub-mixtures (Cl, PFAA+Cl, and Br+Cl), at a concentration of already 100-fold blood level, followed by the CN at 1000-fold blood level for all seven mixtures, except the PFAA mixture. The MA or NI and NA showed no changed within the tested concentration range.

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