A realistic mixture of Persistent Organic Pollutants (POPs) reveals possible synergism to inhibit the transactivation activity of the Aryl hydrocarbon Receptor (AhR) *in vitro*

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Introduction

Persistent organic pollutants (POPs) are defined as organic chemicals resistant to environmental degradation. Therefore, they tend to bioaccumulate and biomagnify in human bodies, which potentially causes harm to human health. In real life, people are exposed to POPs not as individual compounds but as mixtures of chemicals. This exposure could lead to an overall adverse effect that may not be seen while studying one chemical at a time (Kortenkamp 2007; Thrupp et al. 2018). Aryl hydrocarbon receptor (AhR) was recently shown to be involved in normal development, homeostasis and immunology, but it has also been known as a key receptor in mediating toxicity of xenobiotics. Dysfunction of AhR is usually associated with phenotypic abnormalities, impaired immune system, endocrine disruption and cancers. This study aims to determine, *in vitro,* the impact of a realistic mixture of POPs on mammalian health at the level of AhR function, considering tissue and species specificity.

Materials and methods

To study both the AhR agonistic and antagonistic activities of mixtures of POPs, three different AhR luciferase reporter cell lines (rat hepatoma DR-H4IIE, human hepatoma DR-Hep G2 and human mammary gland carcinoma DR-T47-D cells) were used. A mixture of 29 POPs (POP mixture) including six perfluorinated (PFAA), seven brominated (Br), and 16 chlorinated (Cl) compounds (seven polychlorinated biphenyls (PCBs) and nine organochlorine pesticides), listed in the 2001 Stockholm Convention on Persistent Organic Pollutants, was prepared based on their concentrations found in Scandinavian human blood. In addition, six sub-mixtures consisting of one single class of compounds (PFAA, Br and Cl) or two combined classes (Cl + Br, Cl + PFAA, Br + PFAA) were also made to evaluate possible interactions among these groups. The mixtures were designed and premade by the Norwegian University of Life Sciences, Oslo (Berntsen et al. 2017).

DR-H4IIE cells were purchased from BioDetection System (The Netherlands) while the two human cell lines were home-made in our lab (ULiege) (Van Der Heiden et al. 2008). All the exposure experiments were repeated independently in triplicates, with final concentrations of the POP mixture and sub-mixtures up to 2000 times higher than the mean blood level, in 0.3% and 0.4% DMSO in culture medium, for agonistic and antagonistic tests, respectively. The cells were exposed to a series of several dilutions of the test mixtures in agonistic tests, while for antagonistic, they were exposed to the same dilution series but co-exposed with a constant TCDD concentration of 15 pM, 150 pM or 650 pM, corresponding to the TCDD EC_{50} in DR-H4IIE, DR-T47-D and DR-Hep G2, respectively. A TCDD dose-response curve was also performed on the same plate for quality check. Cytotoxicity was monitored by visual inspection of cells under the microscope and by LDH and MTT assays.

Results and Discussions

No cytotoxicity was recorded for the tested concentrations of the mixtures of interest.

POP mixture effects. Exposure to the POP mixture did not induce any significant (> 10% response relative to the maximal TCDD-response) AhR agonistic responses; however, it triggered an AhR antagonistic response in all cell lines. In general, the rat cell line was more sensitive than the two human cell lines. At a concentration in the culture medium corresponding to the mean blood level, the POP mixture did not interfere with the cell's response to TCDD. Although the 29 compounds were in the total POP mixture (even at the highest dose of 2000 times higher than the mean blood levels) with individual concentrations not expected to have any effect on the AhR transactivation

activity (data not shown), dose-dependent antagonistic responses were already observed at concentrations of above 75 and 250 times the blood level in DR-H4IIE cells and in the two human cell lines, respectively (Figure 1). These concentrations in blood could be plausibly reached in humans after a food contamination incident or in highly exposed sub-populations. The results indicated a significant combined effect of the 29 compounds in the total POP mixture and also a difference in sensibility between the rat and human cell lines.

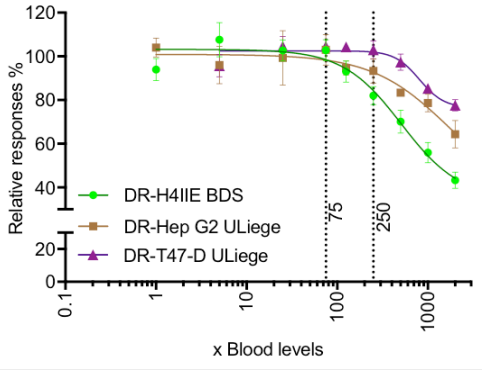

Figure 1: Dose-response curves of the POP mixtures in DR-H4IIE BDS (green, circle), DR-Hep G2 ULiege (brown, square), and DR-T47-D ULiege (purple, triangle) cells co-exposed to 15 pM, 150 pM and 650 pM TCDD, respectively. Dashed lines indicate 75 and 250 times the blood level.

Sub-mixtures. In parallel with the complete POP mixture, six complementary sub-mixtures (PFAA, Br, Cl, Cl + Br, Cl + PFAA, Br + PFAA) were also tested in order to study the possible interactions among these groups. Similar to the complete POP mixture, only antagonism was seen for some of the sub-mixtures (Table 1) but no agonism was observed.

In DR-H4IIE, the results showed that neither PFAA nor Br sub-mixtures or their PFAA + Br mixture presented AhR antagonism. On the other hand, all sub-mixtures containing Cl (Cl, Cl + Br, Cl + PFAA sub-mixtures) exerted AhR antagonistic responses. DR-Hep G2 gave similar results but with a lower activity. In contrast, in DR-T47-D, the antagonisms of the mixtures were not clearly grouped; for example, PFAA containing mixtures (PFAA, PFAA + Br and PFAA + Cl) also revealed AhR antagonistic activities along with the others (Table 1). That indicates a tissue, hepatic versus mammary gland, and species, rat versus human -specific for AhR transactivation activities of these sub-mixtures.

Table 1: AhR antagonism efficiencies of the POP mixture and six sub-mixtures (PFAA, Br, Cl, Cl + Br, Cl + PFAA, Br + PFAA) at a concentration 2000 times higher than in blood in three different cell lines, DR-H4IIE BDS, DR-Hep G2 ULiege and DR-T47-D ULiege. Percentages represent the level of cell response relative to the co-exposing 15 pM, 150 pM and 650 pM TCDD response (respectively for DR-H4IIE BDS, DR-Hep G2 ULiege and DR-T47-D ULiege cell lines), 100 % of response meaning thus no AhR antagonism.

Submixtures	DR-H4IIE BDS	DR- Hep G2 ULiege	DR-T47-D ULiege
PFAAs	90%	99%	85%
Br	108%	99%	109%
Cl	53%	69%	76%
Br + Cl	50%	72%	70%
PFAAs + Cl	37%	62%	65%
PFAAs + Br	102%	118%	69%
POPs	40%	64%	76%

Focusing on DR-H4IIE cells, the Cl sub-mixture was responsible for the antagonism of the POP mixture, contributing to 80% of the POP response. Also, the response to the Cl sub-mixture was similar to the one of the Cl + Br mixture ($p = 0.168$), but different from that of the Cl + PFAA mixture ($p < 0.0001$), the latter being very similar to the POP mixture response ($p = 0.31$). Indeed, when DR-H4IIE cells were exposed to the Cl + PFAA mixture, the antagonistic response was the same as the response of the POP mixture. Their dose-response curves completely

overlapped (Figure 2). The fact that the PFAA sub-mixture alone displayed only very weak antagonistic activity (inhibiting only 10% of the TCDD co-exposure) suggests that its effect in combination may be indirect, somehow enhancing the antagonism exerted by the Cl sub-mixture (Table 1).

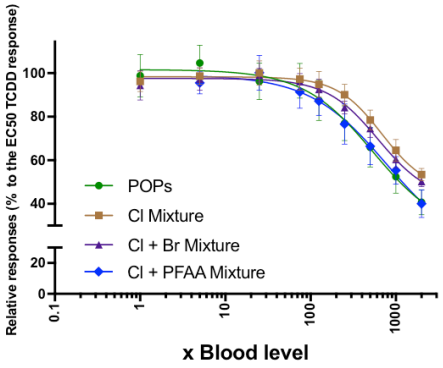

Figure 2: Dose-response curves of the POP (green, circle), Cl (brown, square), Cl + Br (purple, triangle) and Cl + PFAA (blue, diamond) mixtures in DR-H4IIE BDS cells co-exposed to 15 pM TCDD.

The difference in AhR transactivation response between the hepatoma cells (DR-H4IIE and DR-Hep G2) and the mammary gland cells (DR-T47-D) could be due to the high levels of estrogen receptors in DR-T47-D (Van der Heiden et al. 2009), while the two tissues also have different AhR down-stream gene batteries available. This complex situation can also explain the indirect AhR antagonism of the PFAA mixture. More data on gene expression (qPCR) would be needed, addressing the AhR, ER, AR and PPAR pathways (Fang et al. 2012).

Measured versus predicted POP mixture effects in DR-H4IIE BDS. We used the concentration addition method (Payne et al. 2000) for predicting the AhR antagonistic responses of DR-H4IIE cells for the POP mixture and the three active sub-mixtures, Cl, Cl + Br and Cl + PFAA (Figure 3) from the IC_{50} of 16 AhR antagonistics compounds, three from Br and 13 from Cl sub-mixtures (data not shown).

By using the concentration addition model, it was shown that the calculated IC_{50} of the Cl sub-mixture from the observed IC_{50} of all 13 active chlorinated compounds was about the same as the measured IC_{50}, 1.88 ± 0.15 μM and 2.5 μM, respectively. In contrast, the measured IC_{50} = 21.89 ± 3.095 for the total POP mixture was half of the calculated IC_{50} = 43.2 μM, indicating a possible synergistic effect. Figure 3B shows an overlap of the Cl predicted sub-mixture effect within the 95% confidence interval of the observed one, while the POP mixture revealed a 2-fold shift (Figure 3A). In the same way, the predicted IC_{50} were 2.53 μM for the Cl + Br mixture (Figure 3C) and 44.9 μM for the Cl + PFAA mixture (Figure 3D), compared to their observed 1.5 ± 0.12 μM and 27 ± 5 μM, respectively. Nevertheless, there were no statistically significant difference between the measured and predicted effects (t-test, unpaired, parametric, $p > 0.05$) for the total POP mixture and the three sub-mixtures.

Conclusions

We tested the AhR agonistic and antagonistic activities of a realistic mixture of 29 POPs, in which each individual compound was present at a concentration which did not produce any interference with the AhR transactivation activity. However, the POP mixture induced a significant AhR antagonistic activity at a concentration 75 and 250 times higher than the human blood level, in the rat and the human cell lines, respectively, illustrating the principle of "something from nothing" (Thrupp et al. 2018). These concentrations could realistically occur in food contaminant incidents or in a highly exposed sub-population. AhR transactivation activities in the three cell lines exposed to six sub-mixtures were different due to the species and tissue-specific responses. Cl containing mixtures were AhR antagonistic in hepatoma cells (DR-H4IIE and DR-Hep G2) but not PFAA containing mixtures. PFAA sub-mixtures were suspected to indirectly enhance the AhR antagonistic activity of Cl sub-mixture in rat cells. Finally, the 2-fold higher predicted IC_{50} of the total POP mixture compared to the measured one revealed a possible synergic antagonism between the individual compounds of the POP mixture in the DR-H4IIE cells.

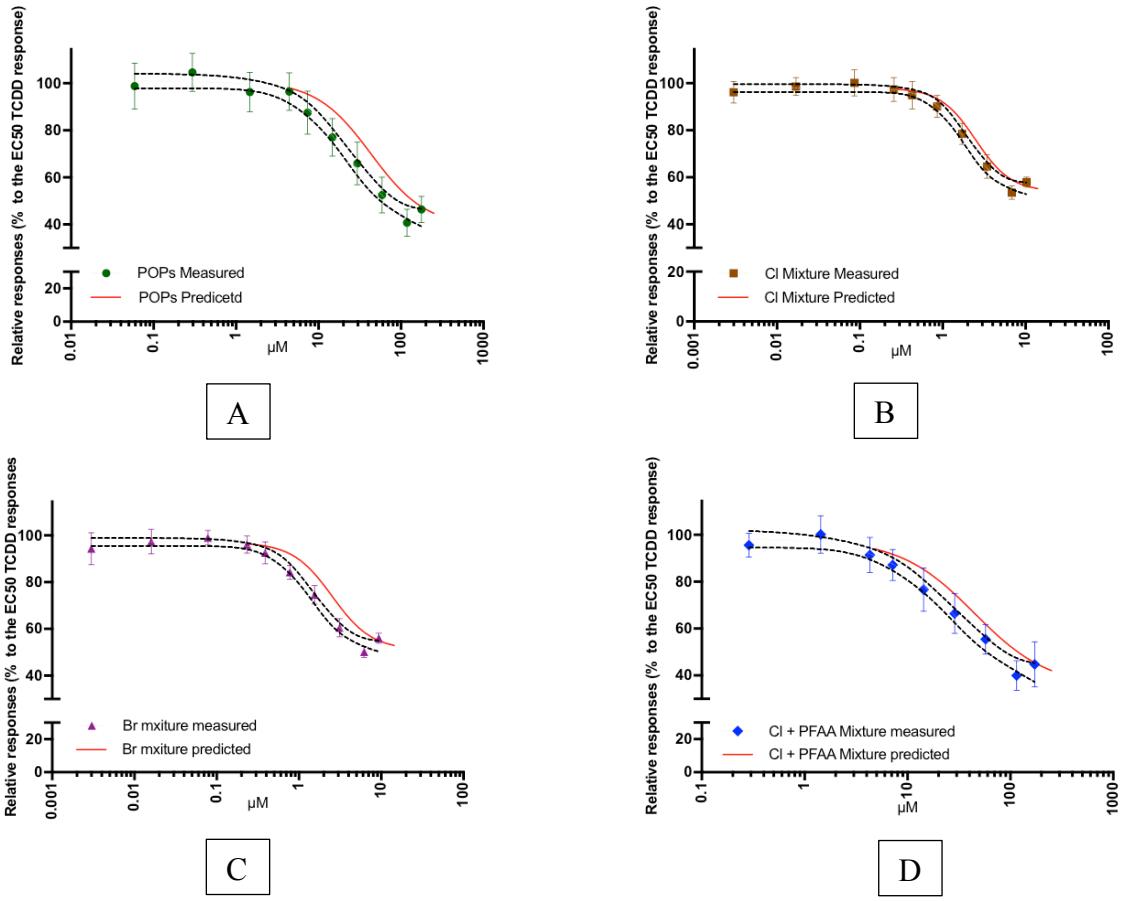

Figure 3: Measured (with 95% confidence interval in dashed black lines) and predicted dose-response curves (red lines) of the POP (green, circle, 3A), Cl (brown, square, 3B), Cl + Br (purple, triangle, 3C) and Cl + PFAA (blue, diamond, 3D) mixtures according to the concentration addition model (Payne et al., 2000).

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