Human blood-based exposure levels of persistent organic pollutant (POP) mixtures antagonise androgen receptor transactivation and translocation


A R T I C L E   I N F O

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A B S T R A C T

Introduction: Human exposure to persistent organic pollutants (POPs) has been linked to genitourinary health-related conditions such as decreased sperm quality, hypospadias, and prostate cancer (PCa). Conventional risk assessment of POPs focuses on individual compounds. However, in real life, individuals are exposed to many compounds simultaneously. This might lead to combinatorial effects whereby the global effect of the mixture is different from the effect of the single elements or subgroups. POP mixtures may act as endocrine disruptors via the androgen receptor (AR) and potentially contribute to PCa development.

Aim: To determine the endocrine disrupting activity of a POP mixture and sub-mixtures based upon exposure levels detected in a human Scandinavian population, on AR transactivation and translocation in vitro.

Materials and methods: The Total POP mixture combined 29 chemicals modelled on the exposure profile of a Scandinavian population and 6 sub-mixtures: brominated (Br), chlorinated (Cl), Cl + Br, perfluorinated (PFAA), PFAA + Br, PFAA + Cl, ranging from 1/10× to 500× relative to what is found in human blood. Transactivation was measured by reporter gene assay (RGA) and translocation activity was measured by high content analysis (HCA), each using stably transfected AR model cell lines.

Results: No agonist activity in terms of transactivation and translocation was detected for any POP mixtures. In the presence of testosterone the Cl + Br mixture at 100× and 500× blood level antagonised AR transactivation, whereas the PFAA mixture at blood level increased AR transactivation (P < 0.05). In the presence of testosterone the Cl and PFAA + Br mixtures at 1/10×, 1×, and 50× blood level antagonised AR translocation (P < 0.05).

Conclusion: Taken together, some combinations of POP mixtures can interfere with AR translocation. However, in the transactivation assay, these combinations did not affect gene transactivation. Other POP combinations were identified here as modulators of AR-induced gene transactivation without affecting AR translocation. Thus, to fully evaluate the effect of environmental toxins on AR signalling, both types of assays need to be applied.

1. Introduction

Man-made chemicals are a part of everyday life and some may mimic hormonal signalling in humans. These exogenous substances or mixtures, called endocrine disrupting chemicals (EDCs), alter the function of the endocrine system, subsequently causing adverse health effects (IPCS, 2012). Persistent organic pollutants (POPs) are a class of chemicals that remain in the environment for prolonged periods of time due to their structural resistance to conventional processes of degradations leading to bioaccumulation and bioamplification in those exposed. Consequently, elimination half-lives range from days to decades (Bu et al., 2015; Lee et al., 2014; Trudel et al., 2011; New Jersey...
Drinking Water Quality Institute Health Effects Subcommittee, 2015; times the estimated concentrations in human serum.

Br stock solutions where the estimated concentration of POPs are 10,000,000 Cl, PFAA + Cl and Cl + Br stock solutions are 1,000,000 times the estimated
The total in vitro mixture containing 29 different POP-based on human blood
Table 1
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and perfluorinated. In addition to the Total mixture which contains all
contains a total of 29 compounds which are chlorinated, brominated, 
compartmental POPs are investigated for their endocrine disrupting po-
number of cancers (Mamsen et al., 2017; Eke et al., 2017). Con-
potential as single compounds and may exert androgenic/anti-androgenic
substance mixtures representing sophisticated human-like exposure

Biologically, exposure to substances or mixtures which perturb the androgen signalling axis has previously been shown to induce geni-
obviously (Ge et al., 2018; Sengupta and Banerjee, 2014). Mechanistically, 
for the generation of high throughput data spe-
transactivation-based assays by analysing nuclear receptor transacti-
Typically, POPs are tested for their endocrine disrupting potential using 
due to their suspected endocrine disrupting potential via AR signalling. 
androgens (Jenster et al., 1993; Zhou et al., 1994).

In the current study, POP mixtures used are based on concentrations of relevant POPs measured in human blood, according to recent studies of the Scandinavian population (Haug et al., 2010; Knutsen et al., 2008; Polder et al., 2008; Polder et al., 2009; Van Oostdam et al., 2004) as described in Table 1 (Bernsten et al., 2017). This novel POP mixture contains a total of 29 compounds which are chlorinated, brominated, and perfluorinated. In addition to the Total mixture which contains all 29 compounds, there were a number of sub-mixtures synthesised: a chlorinated (Cl) mixture, brominated (Br) mixture, perfluorinated (PFAA) mixture, chlorinated + brominated (Cl + Br) mixture, per-
fluorinated + brominated (PFAA + Br) mixture perfluorinated + chlorinated (PFAA + Cl) mixture. The design of an environmentally-

For full table and description see reference (Bernsten et al., 2017).

a Average human blood levels of POPs based on a literature review of Scandinavian values, providing the basis for the in vitro mixture.

b Average human blood levels of POPs converted to ng/ml. A fat percentage of 0.6% was used. 1 ml blood was considered to have a weight of 1 g.

For the total in vitro mixture containing 29 different POP-based on human blood levels. For μM concentrations the estimated concentration of POPs in the Total, Cl, PFAA + Cl and Cl + Br stock solutions are 1,000,000 times the estimated concentrations in human blood, in comparison with the PFAA, Br and PFAA + Br stock solutions where the estimated concentration of POPs are 10,000,000 times the estimated concentrations in human serum.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average human blood levelsa ng/g lipid</th>
<th>Average human blood levelsb ng/ml</th>
<th>Total mixture stock measured concentrationμM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB 28</td>
<td>2.13</td>
<td>0.013</td>
<td>31.1</td>
</tr>
<tr>
<td>PCB 52</td>
<td>1.60</td>
<td>0.010</td>
<td>20.5</td>
</tr>
<tr>
<td>PCB 101</td>
<td>1.30</td>
<td>0.008</td>
<td>24.5</td>
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<td>PCB 118</td>
<td>10.67</td>
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<tr>
<td>PCB 138</td>
<td>37.00</td>
<td>0.222</td>
<td>428.5</td>
</tr>
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<td>PCB 153</td>
<td>60.33</td>
<td>0.362</td>
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<tr>
<td>PCB 180</td>
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<td>0.194</td>
<td>339.0</td>
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<td>0.873</td>
<td>1680.8</td>
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<td>p.o’.DDT</td>
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</tr>
<tr>
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<td>41,522.1</td>
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<td>0.560</td>
<td>336.8</td>
</tr>
</tbody>
</table>

For full table and description see reference (Berntsen et al., 2017).

* Average human blood levels of POPs based on a literature review of Scandinavian values, providing the basis for the in vitro mixture.

a Average human blood levels of POPs converted to ng/ml. A fat percentage of 0.6% was used. 1 ml blood was considered to have a weight of 1 g.

b Measured concentrations of the various compounds in the total mixture stock converted to μM.

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bovine serum, penicillin streptomycin, DMEM Glutamax™, L-glutamine.

2.1. Reagents

Cell culture reagents (fetal bovine serum, charcoal-stripped fetal bovine serum, penicillin streptomycin, DMEM Glutamax™, l-glutamine, and G418) were supplied by Life Technologies (Paisley, UK). Testosterone, phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), and MTT powder was purchased from Sigma-Aldrich (Poole, Dorset, UK). The luciferase reporter gene assay kit (containing luciferase buffer and lyophilised luciferase substrate) and a 5 × cell lysis reagent was purchased from Promega (Southampton, UK). Hoechst 33342 was purchased from Thermo Scientific (UK).

2.2. Chemical mixtures

The mixtures of POPs used in the current studies were obtained from the Norwegian University of Life Sciences, Oslo (Prof. E. Ropstad). Conceptually, the mixtures were designed to be chemically defined with regards to composition, and using concentrations ratios reflecting human exposure levels. A detailed description of the design can be found in the Berntsen et al. (2017) manuscript and supplementary material therein. In short, the total mixture contains 29 different POPs at concentrations of relevant POPs measured in human blood, as overviewed in Table 1. Additional information on sources and purity of the individual chemicals is given in Supplementary material 1. In addition to the total mixture, containing all the test compounds, sub-mixtures were constructed based on individual classes of POPs: a mixture containing i) the perfluorinated alkyl agents (PFAA mixture), ii) brominated flame retardants (Br mixture), iii) PCB and organochlorine pesticides (CI mixture). Combinations of these sub-mixtures were also created: PFAA + Br mixture, PFAA + CI mixture, Br + CI mixture. A full overview of these mixtures is given in Table S9 in Berntsen et al. (2016). All stocks were created in DMSO at concentrations equivalent to 1,000,000 × human blood concentrations for the total mixture, CI mixture, and all combinations with the CI mixture. The PFAA mixture, the Br mixture, and the PFAA + Br mixture stocks were 10 times more concentrated, and equivalent to 10,000,000 × human blood concentrations. Stocks were stored at −20 °C. For exposure studies in the cell models described below, stocks were serially diluted into medium, and tested at final concentrations equivalent to 1/10 ×, 1 ×, 50 ×, 100 ×, 500 × relative to blood levels. Final DMSO concentration was 0.2% and was kept constant in all dilution tested.

2.3. Cell culture

All cells were routinely cultured in 75 cm² tissue culture flasks (Nunc, Roskilde, Denmark) at 37 °C with 5% CO₂ and 95% humidity.

2.3.1. TARM-Luc cells

TARM-Luc cells were obtained from the University of Liege (Dr Marc Muller) and previously generated by Willemsen et al. (2004). Briefly, T47D (ATCC HTB-133) cells were obtained from ATCC (Manassas, VA, USA). T47D cells were grown in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin G (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) amphotericin B, and 1 μg ml⁻¹ bovine pancreas insulin.

For stable transfection T47D were seeded at approximately 70% confluency in a 60-mm culture dish. The next day cells were transfected with 2 μg MMTV-Luc and 0.2 μg pCDNA6/V5-His (Invitrogen), using Lipofectamine Plus (Life Technologies). The next day cells were inoculated into three 150-mm culture dishes for clone isolation. Cells were selected with 5 μg mL⁻¹ blasticidin (Invitrogen) and the medium was changed every 3–4 days. After 3 weeks visible clones were propagated into 24 well plates and tested for DHT (Sigma)-induced luciferase expression. To generate the TARM-Luc cell line, which is androgen responsive, the MMTV-Luc-containing TM-Luc line was transfected with the pSV-AR0 expression vector (provided by A. Brinkmann, Rotterdam, Netherlands) and the pcDNA3 selection vector for geneticin resistance (Invitrogen). Clone selection was performed using G418 (Life Technologies) in the medium. In the present study, TARM-Luc cells were cultured in DMEM Glutamax™ supplemented with 10% foetal bovine serum (FBS). For seeding and exposures TARM-Luc cells were cultured in DMEM Glutamax™ supplemented with 10% charcoal-stripped (CCS)-FBS.

2.3.2. Recombinant AR U-2 OS cells

Recombinant U-2 OS cells with an AR (GenBank Acc. NM_000044) cording sequence (AR U-2 OS) fused to the C-terminus of an enhanced green fluorescent protein (EGFP) stably integrated onto the human osteosarcoma U-2 OS line (ATCC® HTB-96™) were purchased from Thermo Scientific, USA (catalogue number R04-043-01). Cells were cultured in DMEM Glutamax™ supplemented with 10% FBS, 1% penicillin-streptomycin (P/S), 2 mM l-glutamine, and 0.5 mg/ml G418 sulphate Cells were seeded in DMEM Glutamax™ supplemented with 10% CCS-FBS, 1% P/S, 2 mM l-glutamine, and 0.5 mg/ml G418 sulphate. For exposure cells were cultured in DMEM Glutamax™ supplemented with 1% P/S, and 2 mM l-glutamine.

2.4. Reporter gene assay

The androgen receptor Reporter gene assay (RGA) was carried out as previously described (Frizzell et al., 2011). Briefly, TARM-Luc cells were seeded 40,000 cells per well in specialised white-walled, clear flat-bottomed 96 well plates (Greiner, Bio-One, Frickenhausen, Germany). Plates were incubated at 37 °C with 5% CO₂ overnight. Subsequently cells were exposed to POP mixtures in the presence of the solvent control (SC; 0.2% DMSO, v:v in media, uniformly maintained across all samples) at 1/10 ×, 1 ×, 50 ×, 100 ×, and 500 × relative to blood level for the agonist test. A standard curve of 0.1–1000 nM testosterone was generated (Supplementary material 2) and agonist test responses were analysed relatively. For the antagonist test, testosterone (50 nM) was used as the positive control (PC; a concentration which corresponds to EC50 on the standard curve) and each POP mixture was combined with the PC at 1/10 ×, 1 ×, 50 ×, 100 ×, and 500 × relative to blood level. Thus allowing for both synergistic and antagonist responses. Cells were exposed for 48 h. The supernatant was discarded and the cells washed twice with phosphate buffered saline (PBS) prior to lysis with 1 × cell lysis reagent (Promega, Southhamton, UK) used according to manufacturer's instructions, facilitated by agitation. Plates were then read using a Mithras Multimode Reader (Berthold, Other, Germany) which injected each well with 100 μl of luciferase substrate buffer from luciferase reporter gene assay kit (Promega, Southhamton, UK), used according to manufacturer's instructions, and measured the response of each well via detection of luminescence. TARM-Luc cells upon AR transactivation express a luciferase signalling protein allowing for the detection of both agonist and antagonist responses which were compared to the SC (0.2% DMSO in media) and PC (50 nM testosterone in media) respectively.

2.5. MTT assay

TARM-Luc cells were seeded in clear flat-bottomed 96-well plates (Nunc, Roskilde, Denmark) and followed the same seeding and exposure protocol as described for the RGA. After 48 h of exposure, the supernatant was then discarded and the cells washed once with PBS. Cells were then incubated for 3 h with 2 mg/ml thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO, USA) solution
diluted 1:6 in assay media. Healthy cells convert soluble yellow MTT solution to insoluble purple formazan crystals. The supernatant was removed and 200 μl solution of DMSO added to dissolve the formazan crystals during a 10 min incubation with agitation at 37 °C. Optical density was measured at 570 nm with a reference filter at 630 nm using a Sunrise spectrophotometer (TECAN, Switzerland). Metabolic activity was calculated as a percentage absorbance of the sample compared with the absorbance of the solvent control. Metabolic activity was used as an indirect measure of gross cytotoxicity.

2.6. High content analysis

Recombinant AR U-2 OS cells were prepared by seeding 6000 cells per well in black walled 96 well plates with clear flat bottoms (Greiner, Germany). Plates were incubated at 37 °C 5% CO₂ overnight. Subsequently cells were exposed as described above with the relevant SC (0.2% DMSO) and PC (50 nM testosterone). After 6 h of exposure cells were washed with 1 x PBS and fixed using 5% formalin. Cells were then washed twice with 1 x PBS and subsequently stained using 2 μM Hoechst 33342. Plates were read using CellInsight NXT High Content Analysis Platform (ThermoFisher Scientific, UK) using Spot Detector bioapplication which measures the translocation activity of the AR. For the agonist test responses were assessed against a testosterone standard curve (0.1–100 nM) and for antagonist testing responses were measured against the PC (50 nM testosterone) which was the same as that used for the RGA.

Recombinant AR U-2 OS cells express a stably-transfected androgen receptor fused with an enhanced GFP. A wavelength of 485 nm was used to detect EGFP-AR localisation upon successful ligand binding to androgen response elements (AREs, Fig. 1). The primary channel 2 output was spot count per object;

\[ \text{Spot count per object} = \frac{\text{total number of spots (EGFP - AR)}}{\text{nuclear (cell) number}} \]

Hoechst 33342 was used to counter stain cell nuclei in each well. A wavelength of 386 nm was used to detect binding of Hoechst 33342 to nuclei. Output parameters included; object count (cell number), nuclear area, and nuclear intensity. The HCA platform measures the binding of Hoechst 33342 to cell nuclei. The platform refers to one cell nucleus as an object. Cell nuclei and object count are interchangeable; object count is a measure of cell number. Cell nuclei are validated based upon circularity, size, and intensity of fluorescence. Therefore, a reduction in object count (cell number) reflects gross cellular toxicity induced by POP mixtures. A decreased nuclear (object) area combined with increased nuclear intensity is indicative of an apoptotic programme taking place in cells.

2.7. Statistical analysis

All experiments were carried out in triplicate and in three independent exposures. Data was analysed using Microsoft Excel in addition to Graphpad PRISM software, version 5.01 (San Diego, CA). Values are expressed as the mean of 9 triplicates from the 3 independent exposures ± standard error of the mean (SEM). Data is expressed as a percentage of the solvent or positive controls, where applicable. Analysis carried out includes i) one-way ANOVA followed by
Dunnett’s procedure for multiple comparisons; the mean concentrations were tested for significant difference at the 95% confidence level. Significant values were as follows; P ≤ 0.05 (*), P ≤ 0.01 (**), and P ≤ 0.001 (***)

3. Results

The potential endocrine disrupting activity of an environmentally-relevant POP mixture containing 29 compounds and sub-mixtures thereof on AR transactivation and translocation was investigated.

3.1. Cytotoxicity and cellular health

Gross cytotoxicity was evaluated in the AR RGA cell model (TARM-Luc) by quantifying metabolic activity using MTT conversion. Cellular health in the AR translocation assay (recombinant AR U-2 OS) was monitored using a HCA platform. Here, multiple parameters for pre-lethal cytotoxicity were measured, including cell number (object count) and nuclear morphology (nuclear area, nuclear intensity).

3.1.1. Metabolic activity of AR RGA cell model (TARM-Luc) exposed to POP mixtures as measured by MTT conversion

The MTT assay was carried out after 48 hour exposure to the POP mixtures as a measure of metabolic activity. No significant effects were induced by the Total mixture on metabolic activity compared to the SC (Fig. 2A). Non-monotonic responses for several of the sub-mixtures were observed (Fig. 2B–H), however, the Total mixture and sub-mixtures did not significantly increase or decrease metabolic activity compared to the SC (Fig. 2).

3.1.2. Evaluation of markers for pre-lethal cytotoxicity in the AR translocation cell model (recombinant AR U-2 OS) using HCA

HCA demonstrated a number of significant findings. Object count, a parameter for cell number, was only affected significantly by the Cl mixture (500× relative to blood level) which decreased cell number, when compared to the SC (Cl mixture 500× = 73.2% ± 7.9 SEM vs SC = 100% ± 7 SEM; P < 0.05). The other POP mixtures did not significantly impact cell number, as evaluated by object count. The Cl mixture and PFAA + Cl mixture (both at 1×) decreased nuclear area by 4.3% ± 1.6 SEM (P < 0.05) and 3.6% ± 0.7 SEM (P < 0.001) respectively, when compared to the SC. The Br mixture (500×), Cl mixture (1/10×, 1×, 50×, 100×, and 500×), PFAA mixture (100×, and 500×), and Total mixture (1×, 100×, and 500×) all increased nuclear intensity significantly (P < 0.05), when compared to the SC (Fig. 3). The PFAA + Br mixture at 1/10× decreased nuclear intensity by 4.8% ± 1 SEM (P < 0.05) when compared to the SC (Fig. 3).

3.2. Androgen receptor (AR) mediated reporter gene transactivation

At the concentrations tested (1/10×, 1×, 50×, 100×, and 500×) none of the POP mixtures tested showed any agonist activity on AR translocation when compared to the SC. When all the mixtures where tested in the presence of testosterone, only Cl and PFAA + Br mixtures significantly antagonised AR translocation activity at 1/10×, 1×, and 50×. The Cl mixture antagonised AR translocation by 28.5% ± 5.1 SEM (P < 0.01), 24.5% ± 2.4 SEM (P < 0.05), and 25.4% ± 6.1 SEM (P < 0.05), respectively for 1/10×, 1×, and 50× relative to blood level when compared to the PC (Fig. 5). The PFAA + Br mixture antagonised AR translocation by 34.8% ± 6.9 SEM (P < 0.001), 27.6% ± 7.1 SEM (P < 0.01), and 24.2% ± 6.5 SEM (P < 0.05) respectively for 1/10×, 1×, and 50× relative to blood level when compared to the PC (Fig. 5).

4. Discussion

This study evaluated human exposure-based POP mixtures with respect to their potential effect on AR-mediated gene transactivation and AR receptor translocation using in vitro cell models. Cytotoxicity and cellular health data is important to consider for cellular assays, as the functional AR-related read-outs (transactivation and translocation) used in this study, depend on normal physiological and molecular functioning of the cells. A combined interpretation of the observations, whether by MTT (Fig. 2) or HCA (Fig. 3), indicates that none of the mixtures is grossly cytotoxic at any of the concentrations tested. However, assessing antagonism in the RGA by a single gross cytotoxic or cell viability endpoint such as MTT is a limitation of this study as even subtle effects in cellular health (not detected by MTT) can influence antagonism measurements, more so than agonism. For pre-lethal cytotoxicity markers, although significant changes in nuclear parameters were observed for many of the mixtures at given concentrations, the absolute changes are small, and the biological significance with regards to cell viability might be limited, as no clear dose-response relationships are seen.

Transactivation based assays are widely utilised for establishing endocrine disrupting activity of potential EDCs (Frizzell et al., 2011). In recent papers, it has been reported that individual compounds within the current mixture are agonists or antagonists of the AR (Table 2). However, the issue with these studies is the test concentrations used to exert an effect. For example, PCB 52 was shown to antagonise AR transactivation activity at 3.2 μM (Takeuchi et al., 2017), yet in the current mixture, based upon human blood levels, PCB 52 is only found at 20.5 pM. AR disrupting effects can thus only result from the presence of many individual compounds within (sub-)mixtures reported to exert an effect on AR transactivation. The current study demonstrates that, in the presence of testosterone, the Cl + Br mixture (both 100× and 500×) antagonise AR transactivation, whereas the PFAA mixture (1×) increases AR transactivation (Fig. 4). To validate these results, cytotoxicity analysis, as determined by MTT, carried out concomitantly with the RGA demonstrated no changes in metabolic activity for these mixtures. The results of this study are unique from previous studies as the concentration range tested was based upon a realistic mixture observed in an actual human population (1×) and cover a range relevant to differing population exposure levels (i.e. 1/10× for those rarely exposed to 500× for occupational exposure). Therefore, the current study provides an exposure model relevant to human health.

In addition to measuring AR-mediated transactivation in response to POP mixtures, the present study utilised a HCA platform for analysing the endocrine disrupting effect of these mixtures via AR translocation using stably transfected AR model cell lines (recombinant AR U-2 OS). The HCA platform is a powerful tool allowing for the identification of translocation changes of the AR. Our HCA results in recombinant AR U-2 OS cells revealed that the CI and PFAA + Br mixtures (both at 1/10×, 1×, and 50×) significantly antagonised AR translocation activity. In a recent study the same POP mixtures were used (Wilson et al., 2016b), however, exposure levels ranged from 500× to 10,000× relative to human blood level, consistent with the current findings that no effect is observed at concentrations higher than 50×. This is the first
study to report findings based upon actual human exposure levels in the context of the AR.

Results observed in this study (Fig. 6) are important as the data suggests that some mixtures may not exert an agonist or antagonist effect yet when combined with another mixture may in fact potentiate endocrine disrupting effects in terms of AR transactivation. This was the case for the Br and Cl individual mixtures. Individually, these mixtures did not significantly increase (agonist) or decrease (antagonist) AR transactivation activity, however, when combined to form the Cl + Br mixture an antagonist effect was seen. Similarly, this was the case for the PFAA and Br mixtures as determined by HCA. The PFAA and Br mixtures did not impact AR translocation activity, yet when combined together to form the PFAA + Br mix there was an antagonist response seen in AR translocation (Fig. 5A). Conversely, results also demonstrate that while individual mixtures exert an antagonist effect, the effect may be lost when combining the mixture with another mixture. This was observed by HCA as the Cl mixture antagonised AR translocation (Fig. 5A), yet when combined with the PFAA mix to form the PFAA + Cl mix no effect was seen. Recent studies have similarly demonstrated evidence of the cocktail effect and indeed lack of an effect of an overall mixture where the parts have known endocrine disrupting properties. At low nanomolar concentrations HCB, p,p′-DDE, PCB, PFOA, and PFOS (all of which are contained in the current total mixture) exerted a mitogenic effect in vitro; while the total mixture did still exert a proliferative effect the observed effect was lower than predicted (Gogola et al., 2019). Furthermore, a 1/10× and 1× mixture induced a greater
mitogenic effect than the 10× mixture (Gogola et al., 2019). This pattern of low level exposure is similarly displayed in our results with 1/10×, 1×, and 50× mixtures antagonising AR translocation, yet the same mixtures at 100× and 500× did not. In another study, a mixture of PFAAs (the same as those used in the current study—but at much higher concentrations) demonstrated a higher than additive effect by antagonising AR transactivation (Kjeldsen and Bonefeld-Jorgensen, 2013).

Receptor transactivation assays are well established within endocrine disruptor testing regimes (Frizzell et al., 2011; Takeuchi et al., 2017; Schrader and Cooke, 2000; Schrader and Cooke, 2003). Translocation is a sub-component of AR gene regulation and is taken for granted in transactivation-based assays. However, the monitoring of translocation is not a widely used assay in the area of endocrine disruptor testing.

We hypothesised that compounds which elicit androgenic effects in vivo but do not disrupt receptor transactivation may be acting as endocrine disruptors via another pathway, specifically disruption of receptor translocation. Therefore, we tested the effects of the POP mixtures on both receptor transactivation using RGA and translocation using HCA. Our results demonstrate that CI and PFAA + Br mixtures at multiple concentrations (1/10×, 1×, and 50×) can disrupt androgen receptor signalling at the level of receptor translocation but not impact upon AR-mediated gene transactivation as determined by RGA. This is important as the AR is responsible for the transcriptional regulation of many different genes across the genome (which cannot be assessed by RGA). As such, the AR translocates and binds to specific DNA regions (androgen response elements) in the promoter region of target genes. While our HCA assay cannot identify specific changes in these binding sites, it does in fact serve as a valuable screening tool that will indicate whether the total number of DNA-bound AR is increased (agonist) or decreased (antagonised). To our knowledge we are the first group to investigate AR disruption to this level of scrutiny, including transactivation and translocation.

A recent in vivo study demonstrated anti-androgenic effects (decreased testicular weight) following exposure to PCBs, while another associated exposure to p,p'-DDE with decreased ano-genital distance in first-trimester boys (Persson and Magnusson, 2015; Torres-Sanchez et al., 2008). In the current study the Cl mixture (including PCBs) at 1/10×, 1×, and 50× antagonised AR translocation but not transactivation. Therefore, it is possible that the anti-androgenic effect is elicited via AR translocation but this would be missed in transactivation assays. Furthermore, there are conflicting reports as to whether PFAAs antagonise AR transactivation (Kjeldsen and Bonefeld-Jorgensen, 2013; Du et al., 2013). This addresses the need for more cutting edge molecular biology assays to elucidate the mechanism of action of potential endocrine disrupting chemicals that do not act via the classical AR transactivation pathway and our HCA system is capable of screening...
such chemicals.

The discrepancy between the RGA and HCA analysis requires further investigation. While it is conceivable that a mixture would have no effect on AR translocation, but interfere with transactivation at a later step (as seen for the PFAA and Cl + Br mixtures, Fig. 4), it is indeed more difficult to understand how a specific mixture could inhibit AR translocation without affecting transactivation mediated by this receptor. It is important to note that the two tests are performed in different cell lines, one a human mammary gland tumour cells, the other a human osteosarcoma cell. The two cell types may present different sets of intracellular protein kinases, translocation complexes, or other receptors. According to the ThermoFisher manual (043-01.03_AR_RAP_LC06613402), translocation of EGFP-AR in recombinant AR U-2 OS cells in the present of agonists leads to the formation of nuclear foci, while antagonists leads to a more uniform nuclear distribution (Marcelli et al., 2006), presumably due to the absence of successful AR-DNA binding. Also, recombinant AR U-2 OS cells express the endogenous human androgen receptor (hAR) and, furthermore it was shown that incubation with testosterone further induces hAR transcription in these cells (Grad et al., 2001). In contrast, the T47D parent line for the TARM-Luc cells did not express a functional endogenous hAR (Willemsen et al., 2004), therefore a functional AR placed under the control of a SV40 constitutive enhancer was integrated in the TARM-Luc cell genome. It is thus conceivable that, in the conditions of our translocation antagonist tests, the testosterone present induces expression of the endogenous hAR in the recombinant AR U-2 OS cells that would

![Figure 4](image)

**Table 2**

Reported AR effects of individual POPs contained in the human exposure-based mixture.

<table>
<thead>
<tr>
<th>Compound</th>
<th>AR agonist (μM)</th>
<th>AR antagonist (μM)</th>
<th>POP mixture(^a) (pM)</th>
<th>Reference(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 PCB 28</td>
<td>0.76</td>
<td>31.1</td>
<td>(Hamers et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>2 PCB 52</td>
<td>3.2</td>
<td>20.5</td>
<td>(Takeuchi et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>3 PCB 101</td>
<td>1.4</td>
<td>24.5</td>
<td>(Takeuchi et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>4 PCB 118</td>
<td>0.47</td>
<td>137.9</td>
<td>(Hamers et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>5 PCB 138</td>
<td>1</td>
<td>429.5</td>
<td>(Hamers et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>6 PCB 153</td>
<td>1/8.5</td>
<td>698.3</td>
<td>(Takeuchi et al., 2017; Hamers et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>7 PCB 180</td>
<td>2.1</td>
<td>339</td>
<td>(Hamers et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>8 p,p-DDE</td>
<td>100</td>
<td>1065.9</td>
<td>(Schrader and Cooke, 2000; Xu et al., 2006)</td>
<td></td>
</tr>
<tr>
<td>9 HCB 0.0005-0.005</td>
<td>0.613</td>
<td>228.2</td>
<td>(Ralph et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>10 a-Chlordane</td>
<td>No</td>
<td>23.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 Oxychlordane</td>
<td>No</td>
<td>33</td>
<td>(Schrader and Cooke, 2000)</td>
<td></td>
</tr>
<tr>
<td>12 Trans-nonachlor</td>
<td>No</td>
<td>99.1</td>
<td>(Schrader and Cooke, 2000)</td>
<td></td>
</tr>
<tr>
<td>13 α-HCH</td>
<td>10</td>
<td>16.8</td>
<td>(Schrader and Cooke, 2000)</td>
<td></td>
</tr>
<tr>
<td>14 β-HCH</td>
<td>No</td>
<td>75.6</td>
<td>(Schrader and Cooke, 2000)</td>
<td></td>
</tr>
<tr>
<td>15 γ-HCH (Lindane)</td>
<td>No</td>
<td>16.8</td>
<td>(Schrader and Cooke, 2000)</td>
<td></td>
</tr>
<tr>
<td>16 Dieldrin</td>
<td>20</td>
<td>56.2</td>
<td>(Andersen et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>17 PBDE 47</td>
<td>Yes</td>
<td>1.78</td>
<td>(Ren and Guo, 2013)</td>
<td></td>
</tr>
<tr>
<td>18 PBDE 99</td>
<td>Yes</td>
<td>0.75</td>
<td>(Ren and Guo, 2013)</td>
<td></td>
</tr>
<tr>
<td>19 PBDE 100</td>
<td>1</td>
<td>0.38</td>
<td>(Ren and Guo, 2013; Stoker et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>20 PBDE 153</td>
<td>Yes</td>
<td>0.21</td>
<td>(Ren and Guo, 2013)</td>
<td></td>
</tr>
<tr>
<td>21 PBDE 154</td>
<td>No</td>
<td>0.3</td>
<td>(Stoker et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>22 PBDE 209</td>
<td>No</td>
<td>0.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23 HBCD 0.1–10 + DHT</td>
<td>No</td>
<td>5.45</td>
<td>(Christen et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>24 PFHxS 19</td>
<td>780.9</td>
<td>4152.21</td>
<td>(Jørgensen and Bonefeld-Jorgensen, 2013)</td>
<td></td>
</tr>
<tr>
<td>25 PFOS 3.2</td>
<td>420.9</td>
<td>429.5</td>
<td>(Jørgensen and Bonefeld-Jorgensen, 2013)</td>
<td></td>
</tr>
<tr>
<td>26 PFOA 9.6</td>
<td>109.25</td>
<td>109.25</td>
<td>(Jørgensen and Bonefeld-Jorgensen, 2013)</td>
<td></td>
</tr>
<tr>
<td>27 PFNA 44</td>
<td>37.54</td>
<td>37.54</td>
<td>(Jørgensen and Bonefeld-Jorgensen, 2013)</td>
<td></td>
</tr>
<tr>
<td>28 PFUnDA</td>
<td>No</td>
<td>33.68</td>
<td>(Jørgensen and Bonefeld-Jorgensen, 2013)</td>
<td></td>
</tr>
<tr>
<td>29 PFDA 24</td>
<td>24</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 PFUnDA</td>
<td>No</td>
<td>0.94</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Concentrations (picomolar) listed in this column are the individual compound concentrations contain within the mixture (1× blood level) based upon a Scandinavian population (Berntsen et al., 2017).

\(b\) References correlate to AR agonist and AR antagonist columns which contain the micromolar concentrations of individual POPs within known AR effects as reported in the literature.

*Fig. 4.* Transactivation effects of 48 hour exposure to POP mixtures in the TARM-Luc androgen receptor reporter gene assay cell line. Analysing AR transactivation following exposure to POP mixtures + PC compared to the PC alone. Results are representative of 3 independent experiments \((n = 3, \text{ mean } \pm \text{ SEM}). \) * = \(P < 0.05; \) ** = \(P < 0.01; \) *** = \(P < 0.001. \) Only significant results are presented. Full list of results are provided in Supplementary material 2.
compete with the EGFP-AR for the translocation machinery, thereby rendering the test more sensitive for disruption of translocation. This effect would not be observed in the TARM-Luc cell line, as in those cells the AR is constitutively expressed.

Test duration, exposure time, and different timing of the primary read-outs after beginning exposure, are additional differences between the RGA and HCA tests. The RGA test exposure time was 48 h whereas the HCA test was 6 h. Possibly the inhibition of translocation observed in the HCA test is actually a retardation, which may be caught up with in the longer exposure of TARM-Luc cells and therefore prove irrelevant for transactivation.

Further investigations will be required to understand the molecular basis underlying these different reactions in the two cell lines, concerning both AR nuclear translocation and transactivation mechanisms, which may allow for the identification of novel molecular pathways underpinning specific EDCs mechanism of action that may correlate with phenotypic changes at the cellular and tissue level.

5. Conclusion

In conclusion, the data generated in the current study raises a number of key concerns. Firstly, transactivation-based assays, although very useful in endocrine disrupting research, have their limitations and as such should be used initially as a screening tool when investigating potential endocrine disrupting chemicals. After this, more in-depth molecular biology assays should be conducted on compounds at specific concentrations which warrant further investigation. This is supported by the data which shows POP mixtures which antagonised AR translocation activity (Cl and PFAA + Br mixtures at 1/10×, 1×, and 50× relative to blood level) but did not mediate any AR-specific transactivation antagonism. This concept is supported by recent in vivo studies which demonstrate anti-androgenic effects following PCB exposure yet

Fig. 5. Translocation effects of 6 hour exposure to POP mixtures on the androgen receptor in HCA model cell lines. A. Analysing AR translocation activity following exposure to POP mixtures + PC compared to the PC alone. B–G. Representative images of the antagonist test; SC (B) channel 2 filter for EGFP (i.e. AR spots), SC (C) composite, PC (D) channel 2 filter for EGFP (i.e. AR spots), PC (E) composite, Cl mixture 1/10× (F) channel 2 filter for EGFP, and PFAA + Br mixture 1/10× (G) channel 2 filter for EGFP. Results are representative of 3 independent exposures (n = 3, mean ± SEM). * = P < 0.05; ** = P < 0.01; *** = P < 0.001. Only significant results are presented. Scale bars = 100 μm. Full list of results are provided in Supplementary material 3. Additional images are provided in Supplementary material 4.
in the current study did not antagonise AR transactivation. Therefore, without the use of HCA the Cl and PFAA + Br mixtures at 1/10 ×, 1 ×, and 50 × would have not been detected as endocrine disruptors of AR signalling. Secondly, the data demonstrates that where POP mixtures exert an endocrine disrupting effect (antagonism), the effect may be lost in a Total mixture or sub-mixtures; for example, this was the case for the Cl mixture which antagonised AR translocation activity at 1/10 ×, 1 ×, and 50 × yet in the Total, Cl + Br, and PFAA + Cl mixtures no antagonism was detected, thus the individual effect of the Cl mixture was lost. Thirdly, the data provided evidence of the cocktail effect; individual compounds and/or mixtures may not exert an endocrine disrupting effect, yet the combinatorial effect of specific mixtures may result in an endocrine disrupting effect. This was the case for the Br and Cl mixtures in the RGA which individually did not antagonise AR transactivation, yet together the Cl + Br mixture antagonised AR transactivation significantly. Similarly, in the HCA the PFAA and Br mixtures did not antagonise AR translocation activity, yet together the PFAA + Br mixture antagonised AR translocation activity significantly.

Lastly, interestingly, the data shows that new emerging POPs, such as those within the PFAA mixture, have the ability to disrupt AR signalling. This was evident in the RGA whereby in the presence of testosterone the PFAA mixture at blood level increased AR transactivation to levels higher than testosterone alone. Assessing individual compounds of this mixture at the relevant concentration would be a worthwhile investigation in future studies. In conclusion the current study uses human exposed based POP mixtures at blood-based concentrations and demonstrates evidence of endocrine disruption via AR transactivation and translocation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105083.

Declaration of competing interest

The authors declare they have no competing financial interests.

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