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Perfluorinated compounds affect the function of sex hormone receptors

Lisbeth Stigaard Kjeldsen · Eva Cecilie Bonefeld-Jørgensen

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Abstract Perfluorinated compounds (PFCs) are a large group of chemicals used in different industrial and commercial applications. Studies have suggested the potential of some PFCs to disrupt endocrine homeostasis, increasing the risk of adverse health effects. This study aimed to elucidate mechanisms behind PFC interference with steroid hormone receptor functions. Seven PFCs [perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnA), and perfluorododecanoate (PFDoA)] were analyzed in vitro for their potential to affect estrogen receptor (ER) and androgen receptor (AR) transactivity as well as aromatase enzyme activity. The PFCs were assessed as single compounds and in an equimolar mixture. PFHxS, PFOS and PFOA significantly induced the ER transactivity, whereas PFHxS, PFOS, PFOA, PFNA and PFDA significantly antagonized the AR activity in a concentration-dependent manner. Moreover, PFDA weakly decreased the aromatase activity at a high test concentration. A mixture effect more than additive was observed on AR function. We conclude that five of the seven PFCs possess the potential in vitro to interfere with the function of the ER and/or the AR. The observed mixture effect emphasizes the importance of considering the combined action of PFCs in future studies to assess related health risks.

Keywords Androgen receptor · Estrogen receptor · Luciferase reporter gene assay · Aromatase · Perfluorinated compound · Perfluoroalkyl acid · Endocrine disruptor

Abbreviations

4-AOD	4-Androsten-4-ol-3,17-dione
AR	Androgen receptor
CA	Concentration addition
CV	Coefficient of variation
CYP	Cytochrome P450
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
E2	17β-Estradiol
ER	Estrogen receptor
HF	Hydroxyflutamide
LDH	Lactate dehydrogenase
LOEC	Lowest observed effect concentration
MOEC	Maximum observed effect concentration
PFAA	Perfluoroalkyl acid
PFC	Perfluorinated compound
PFDA	Perfluorodecanoate
PFDoA	Perfluorododecanoate
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoate
PFOA	Perfluorooctanoate
PFOS	Perfluorooctane sulfonate
PFUnA	Perfluoroundecanoate
POP	Persistent organic pollutant
SC	Solvent control

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L. S. Kjeldsen · E. C. Bonefeld-Jørgensen (✉)
Centre for Arctic Health and Unit of Cellular and Molecular Toxicology, Department of Public Health, Aarhus University, Building 1260, Bartholins Allé 2,
8000 Aarhus, Denmark
e-mail: ebj@mil.au.dk

Introduction

Perfluorinated compounds (PFCs) comprise a large group of synthetic chemicals widely spread throughout the environment, in wildlife and human populations. These compounds

have been produced over the last 60 years for many industrial purposes and in consumer-related products such as foodstuff packaging, nonstick cookware (e.g., Teflon), waxes, paints, cosmetics and as water and oil repellents for leather, paper, and textiles. The extensive application of PFCs is due to their unique physicochemical characteristics such as chemical and thermal stability as well as surface-active properties which account for their ability to make materials stain, oil, and water resistant (Parsons et al. 2008; Suja et al. 2009). However, the stability, that makes PFCs desirable for commercial use, also entails that they are environmental contaminants due to their resistance to various modes of degradation (Giesy and Kannan 2002).

Human exposure to PFCs is ubiquitous and numerous studies have reported high levels of several PFCs in human samples such as blood, tissues and breast milk (Lau et al. 2007; Fromme et al. 2009; Lau 2012). Dietary intake is believed to be the major exposure route in the general adult population (Haug et al. 2011; Fromme et al. 2009). Foodstuffs may become contaminated directly from food packaging coated with grease and water repellent coatings or by bioaccumulation into plant-based or animal foods. Other routes of exposure include drinking water, inhalation of indoor air and household dust, and to a lesser extent, dermal contact with consumer products containing PFCs (Trudel et al. 2008; Fromme et al. 2009; Haug et al. 2011).

The most common and well-studied PFCs are the perfluoroalkyl acids (PFAAs), including perfluorooctanoate (PFOA), perfluorooctane sulfonate (PFOS), and perfluorohexane sulfonate (PFHxS). In addition to commercially synthesized PFOS and PFOA, these compounds are also formed in the environment from abiotic and biotic transformation of precursor perfluorinated chemicals (Fromme et al. 2009). Governmental regulations in USA and Europe on use and production of PFOS and PFOA have been made. In addition, PFOS has recently been found to fulfill the criteria for being considered as a persistent organic pollutant (POP) under the Stockholm Convention (Stockholm convention Listing of POPs in the Stockholm Convention http://chm.pops.int/Convention/ThePOPs/ListingofPOPs/tabid/2509/Default.aspx. Accessed Jan. 17th 2013).

Unlike the legacy POPs (e.g., dioxins, polychlorinated biphenyls, organochlorine pesticides), which accumulate in lipid rich tissues, PFCs bind to blood proteins and are primarily detected in human liver and kidney (Maestri et al. 2006; D'Eon et al. 2010). In addition, some PFCs have been detected in tissues of the lung, thyroid, gonads, adipose tissue, pancreas, skeletal muscles, and brain as well as in breast milk and umbilical cord blood (Maestri et al. 2006; Tao et al. 2008; Haug et al. 2011; Apelberg et al. 2007b; Needham et al. 2011). Most PFCs are very slowly eliminated from the human body; however, toxicokinetic profiles and underlying mechanisms are not yet fully understood (Andersen et al. 2008). Elimination half-lives have been estimated to be 3.8, 5.4, and 8.5 years for PFOA, PFOS and PFHxS, respectively (Olsen et al. 2007).

Most PFC toxicity studies have been done in animals, mainly in rodents. General toxicological findings associated with laboratory animals exposed to PFCs include hepatomegaly, hepatocellular adenomas, testicular and pancreatic tumors (Andersen et al. 2008), reproductive (Butenhoff et al. 2004) and developmental (Lau et al. 2004) deficits, neurotoxicity (Johansson et al. 2009), immunotoxicity (Keil et al. 2008; Fair et al. 2011; Peden-Adams et al. 2008; DeWitt et al. 2011) and thyroid hormone alterations (Lau et al. 2003; Yu et al. 2009a; Yu et al. 2009b). Vast species differences concerning toxicokinetic profiles of PFCs have been demonstrated, and humans are thought to be very slow eliminators compared to other species such as rodents (Kudo and Kawashima 2003; Kennedy et al. 2004). Thus, an understanding of body burden is crucial for an interspecies extrapolation of toxicological effects.

Data on human health effects of PFCs are relatively sparse. A recent study have shown for the very first time a relation between serum levels of PFCs and the risk of breast cancer in Greenlandic Inuit (Bonefeld-Jorgensen et al. 2011). The PFCs are transported across the human placenta (Needham et al. 2011; Apelberg et al. 2007a; Fei et al. 2007), and exposure to environmental contaminants during periods of embryonic, fetal, and infant development are of great concern because chemical exposures sustained during early development and programming of organ functions may potentially lead to functional deficits and increased risks of disease later in life (Grandjean et al. 2008). Epidemiological studies have indicated that PFCs have the potential to affect fetal growth and child development (Fei et al. 2007; Apelberg et al. 2007b; Fei and Olsen 2011; Hoffman et al. 2010; Fei et al. 2008; Washino et al. 2009). Recently, an association between PFC exposure at commonly prevalent serum concentrations and reduced humoral immune response in children at ages 5 and 7 years was reported (Grandjean et al. 2012). In addition, several studies have indicated that human fecundity (Fei et al. 2009; Joensen et al. 2009) and onset of puberty (Lopez-Espinosa et al. 2011) may be affected by PFCs.

Little is known about the underlying cellular mechanisms of action of PFCs that account for the outcomes observed in toxicological studies. The ability of several PFCs to induce peroxisome proliferation in rodents is well documented (Ikeda et al. 1985; Just et al. 1989; Intrasuksri et al. 1998; Kudo et al. 2005; Sohlenius et al. 1992). The key event in this mode of action is believed to be activation of the peroxisome proliferator-activated receptor alpha (PPARα) (Lee et al. 1995; Issemann and Green 1990; Dreyer et al. 1992; Vanden Heuvel et al. 2006). However, the human relevance for this mechanism of action has been questioned, as there are marked differences in PPARα levels in human liver compared to rodents (Klaunig et al. 2003).

Animal and in vitro studies have indicated that PFCs may have the potential to disrupt endocrine homeostasis. Estrogen-like properties have been reported for some PFAAs and fluorotelomer alcohols in human cell lines (Maras et al. 2006; Henry and Fair 2011; Benninghoff et al. 2011), in monkey kidney cells (Du et al. 2012), and in yeast cells modified by incorporation of human estrogen receptors (ERs) (Ishibashi et al. 2007). Studies have demonstrated that PFAAs can induce expression of estrogen-responsive genes (Benninghoff et al. 2011; Wei et al. 2007; Tilton et al. 2008). Estrogen hormones are of great importance for the female reproductive system. Moreover, they play key roles in both male and female fetal development and are essential for growth, differentiation and function of a broad range of tissues, e.g., the central nervous system, the musculoskeletal system, the immune system, and the cardiovascular system in both men and women (Heldring et al. 2007; Bjornstrom and Sjoberg 2005). Changes in sex steroid hormone biosynthesis upon PFAA exposure have been reported in vitro and in animal studies as well (Kraugerud et al. 2011; Biegel et al. 1995; Cook et al. 1992; Liu et al. 1996; Du et al. 2012; Rosenmai et al. 2012). Furthermore, mounting evidence from animal and in vitro studies suggests that PFAAs may disrupt thyroid hormone signaling (Thibodeaux et al. 2003; Luebker et al. 2005; Lau et al. 2003; Yu et al. 2009a; Yu et al. 2009b; Du et al. 2012) (Long et al. 2013).

The present study aimed to elucidate the mechanisms by which PFAAs can interfere with sex hormone function and cause an increased risk for health effects in humans. The specific objectives of our study were to assess in vitro the potential of seven PFAAs [PFHxS, PFOS, PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnA), and perfluorododecanoate (PFDoA)] (Table 1) to affect ER- and androgen receptor (AR) transactivity as well as aromatase activity.

The aromatase (encoded by the *CYP19* gene) is the key enzyme in the biosynthesis of estrogens from cholesterol, since it catalyzes the final rate-limiting step in which androgens are converted to estrogens. Consequently, the aromatase enzyme is crucial for the maintenance of the homeostatic balance between the traditionally considered male and female sex hormones. Thus, aromatase activity (i.e., regulation of estrogen synthesis from androgens) is thought to be a critical endpoint concerning sexual development and differentiation (Jones et al. 2006).

Materials and methods

Chemicals

The high-affinity ER ligand 17β-estradiol (E2) was obtained from Sigma-Aldrich (Denmark) and used as dose–response control in the ER transactivation assay. E2 was dissolved in extra pure 96 % EtOH from Merck (Darmstadt, Germany) to produce a stock solution of 100 nM.

The AR agonist dihydrotestosterone (DHT) was obtained from Sigma-Aldrich (Denmark) and the AR antagonist hydroxyflutamide (HF) was from MicroMol GmbH (Luckenwalde, Germany). DHT and HF were used as dose–response controls in the AR transactivation assay. DHT was dissolved in dimethyl sulfoxide (DMSO) from Thermo Scientific (Denmark) to produce a stock solution of 10 mM and HF was dissolved in extra pure 96 % EtOH to produce a stock solution of 20 mM.

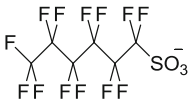
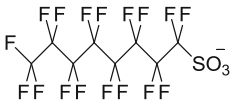
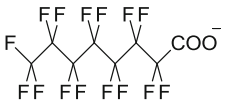
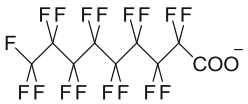
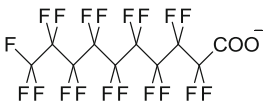
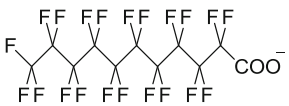
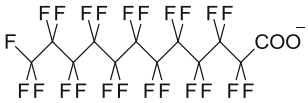
The aromatase inhibitor 4-androsten-4-ol-3,17-dione (4-AOD) was obtained from Sigma-Aldrich (Steinheim, Germany) and dissolved in DMSO to a stock concentration of 50 mM. The aromatase substrates [1β-^{3}H] 4-androstene-3,17-dione (250 mCi, 9.25 MBq) and unlabelled 4-androstene-3,17-dione were obtained from Perkin Elmer (Hvidovre, Denmark) and Riedel-de Haën (Seelze, Germany), respectively. The unlabelled 4-androstene-3,17-dione was dissolved in extra pure 96 % EtOH to a stock concentration of 35 mM.

PFHxS, PFOS, PFOA, PFNA, PFDA, and PFUnA were all purchased from ABCR (Germany). PFDoA was purchased from Sigma-Aldrich (Denmark). The purity of the test compounds was above 95 % (specific purities and CAS no. are presented in Table 1). PFHxS, PFOS, PFOA, PFNA, PFDA, and PFUnA were dissolved in DMSO into stock solutions of 500 mM. PFDoA was dissolved in EtOH to give a stock solution of 50 mM. All PFAAs stock solutions were stored in the dark at room temperature. Most compounds were harmful and appropriate personal protective methods and materials were used throughout all experiments. All compounds were handled avoiding light. The PFAAs were diluted with appropriate culture medium immediately before use to give less than 0.1 % (v/v) solvent not affecting the cell viability of the MVLN, CHO-K1 or JEG-3 cells (not shown).

ER transactivation assay

The estrogenic and antiestrogenic activities of the PFAAs were assessed using the stably transfected MVLN cell line (kindly provided by M. Pons, INSERM, Montpellier, France), derived from the human breast adenocarcinoma MCF-7 cell line carrying an estrogen response element-luciferase reporter vector (Demirpence et al. 1993; Pons et al. 1990). The ER transactivation assay was performed as previously described (Bonefeld-Jorgensen et al. 2005) with minor modifications. MVLN cells were seeded in white 96-well microtiter plates (Perkin Elmer) with a density of approximately 7×10^{4} cells per well. Each PFAA was tested in at least three independent assays alone and upon co-treatment with 25 pM E2 (E2-EC_{20}, corresponding to the concentration inducing approximately 20 % of the maximum

Table 1 Chemical structures, CAS no. and purity of the PFAAs

Compound	Chemical structure	CAS no. *	Purity
PFHxS (perfluorohexane sulfonate)		355-46-4	98%
PFOS (perfluorooctanesulfonate)		1763-23-1	98%
PFOA (perfluorooctanoate)		335-67-1	95 %
PFNA (perfluorononanoate)		375-95-1	97%
PFDA (perfluorodecanoate)		335-76-2	98%
PFUnA (perfluoroundecanoate)		2058-94-8	95%
PFDoA (perfluorododecanoate)		307-55-1	96%

The CAS no. is for the protonated acid form of the perfluoroalkylates

effect of E2 in this study). Protein content was determined by adding 50 μL of fluorescamine diluted in acetonitrile (500 mg/L) to each well followed by fluorometric measurements in the Wallac VICTOR2 (Perkin Elmer, USA) at

355/460 nm wavelength, according to a standard curve of bovine serum albumin (BSA, Promega). The measured luciferase data were then corrected for cell density using the protein measurements. Within each assay, the PFAAs were tested in triplicate in various concentrations within the range of 1×10^{-9}–1×10^{-4} M. An E2 concentration-response control (3.1–300 pM) was performed in parallel in each assay. Additionally, E2-EC_{20} (25 pM) and E2-EC_{100} (100 pM) served as positive controls at each 96-well microtiter plate. The ER-mediated transactivation in MVLN cells has previously been documented in our laboratory using the ER antagonist ICI 182,780 as described (Bonefeld-Jorgensen et al. 2005). The average intra-assay coefficient of variation (CV) of the solvent controls and the positive controls (E2-EC_{20} and E2-EC_{100}) was below 9 % and the inter-assay CV of the positive control (E2-EC_{20}) was below 12 %.

AR transactivation assay

The androgenic and antiandrogenic activities of the PFAAs were assessed using the Chinese hamster ovary cell line CHO-K1 (ATCC no. CCL-61; Manassas, VA, USA). The CHO-K1 cells were transiently co-transfected with an MMTV-LUC reporter vector (kindly provided by R. M. Evans, Howard Hughes Medical Institute, CA, USA) and an AR expression plasmid pSVAR0 (kindly provided by A. O. Brinkmann, Erasmus University, Rotterdam, The Netherlands). The AR transactivation assay was performed as previously described (Andersen et al. 2002) with minor modifications. Briefly, 24 h before transfection, CHO-K1 cells were seeded in white 96-well microtiter plates (Perkin Elmer) with a density of approximately 8,000 cells per well. The transfection was carried out for 5 h using 0.3 μL/well of the transfection reagent FuGene (Roche, Hvidovre, Denmark) and 150 ng cDNA per well of the AR expression plasmid pSVAR0 and the MMTV-LUC reporter vector in a ratio of 1:100. Protein content in each well was determined by fluorometric measurements as described for the ER transactivation assay.

Each PFAA was tested in at least three independent assays alone and upon co-treatment with 25 pM DHT (DHT-EC_{80}, corresponding to the concentration inducing approximately 80 % of the maximum effect of DHT in this study). Within each assay the PFAAs were tested in triplicate in various concentrations within the range of 1×10^{-9}–1×10^{-4} M, without removal of the transfection reagent and cDNA. A DHT concentration-response control (0.002–1 nM) was performed in parallel in each assay, and additionally, DHT-EC_{80} (25 pM) served as a control at each 96-well microtiter plate. A HF concentration-response control (0.5–500 nM) was included in each assay as well to serve as inhibitor concentration-response control. The average intra-assay CV of the solvent controls and the positive control (DHT-EC_{80}) was below 10 % and the inter-assay CV of the positive control (DHT-EC_{80}) was below 11 %.

Aromatase activity

Effects on aromatase activity were assessed using the human choriocarcinoma JEG-3 cell line (ATCC no. HTB-36; Manassas, VA, USA). The aromatase activity assay was performed as previously described (Bonefeld-Jorgensen et al. 2007) with minor modifications. After termination of the 2 h aromatization process, we extracted 300 μL of the culture medium with 750 μL of $CHCl_3$ (CHROMASOLV® ≥99.8 %, Sigma-Aldrich), and treated 150 μL of the aqueous phase with 150 μL of dextran-charcoal (Sigma-Aldrich) in PBS (5 %). Finally, an aliquot of 150 μL of the water phase was mixed with 4 mL of Hionic Fluor (Perkin Elmer) in a 6-mL vial for scintillation (Perkin Elmer), and the samples were assayed for radioactivity (Wallac liquid scintillation counter, Perkin Elmer). The measured aromatase activities were subtracted background level, corrected to cell protein concentration, and related to the solvent control (set to 100 %).

For determination of protein concentrations, the leftover culture medium was removed and cells were lyzed with 500 μL/well lysis buffer. Subsequently, a 100-μL aliquot (in replicate) from each well was transferred to a white 96-well microtiter plate (Perkin Elmer) and added 50 μL/well fluorescamine diluted in acetonitrile (500 mg/L). Finally, fluorometric measurements (Wallac VICTOR2, Perkin Elmer) at 355/460 nm wavelength were performed according to a standard curve of bovine serum albumin (BSA, Promega).

The PFAAs were tested in triplicate in various concentrations within the range of 1×10^{-8}–1×10^{-4} M in at least three independent assays. In each assay the aromatase inhibitor 4-AOD was analyzed in parallel at two concentrations, 1×10^{-8} M and 1×10^{-5} M, corresponding to 4-AOD-IC_{50} and 4-AOD-IC_{100}, respectively. The average intra-assay CV of the solvent controls and the inhibitor controls (4-AOD-EC_{50} and 4-AOD-EC_{100}) was below 8 % and the inter-assay CV of the inhibitor control (4-AOD-EC_{50}) was below 22 %.

Cytotoxicity

The cytotoxicity of the PFAAs was measured within the assays using the Cytotoxicity Detection Kit (LDH) from Roche (Denmark) as described (Ghisari and Bonefeld-Jorgensen 2005). As a positive control, cells in triplicate were lyzed by Triton-X (final concentration of 1 %), corresponding to a maximal release of LDH. As a negative control, culture medium from cells exposed to solvent control was used.

Mixture analyses

The seven PFAAs were combined in an equimolar fashion, and the mixture was assayed for effects on the ER and AR transactivity. MVLN and CHO-K1 cells were exposed to the PFAA mixture in the absence and presence of appropriate hormone (25 pM of E2 and DHT, respectively) in parallel with the single PFAAs which served as controls. The mixture was assayed in the ER and AR transactivation assays in triplicate in at least three independent experiments at mixture concentrations within the range of 7×10^{-8}–3.5×10^{-4} M and 7×10^{-9}–7×10^{-4} M, respectively.

After completing concentration-response analyses of the single PFAAs, mixture effect concentrations were predicted as described (Kruger et al. 2008; Birkhoj et al. 2004) by applying the principle of concentration addition (CA). Briefly, this model relies on the assumption that mixture components, which do not interact, differ only in potency and consequently, they can be considered as dilutions of one another. Therefore, each component in the mixture is assumed to contribute to the overall effect by acting in proportion to its concentration (Kortenkamp and Altenburger 1998; Cedergreen et al. 2008). Thus, this model may predict the mixture concentration that produces a predetermined effect, given that the ratio of each compound in the mixture, and data on the concentration of each mixture component that individually produces the same effect as the mixture are known.

Under assumption of additivity, mixture concentrations corresponding to selected effect levels were predicted using the expression $EC_{mix}=1/\Sigma(p_i/EC_i)$, where EC_{mix} is the concentration of the mixture that is required to produce the predetermined effect E, p_i corresponds to the fraction of compound i present in the given mixture, and EC_i is the concentration of compound i alone causing the same E (determined from the concentration-response curves of the single PFAAs). These predicted mixture effect concentrations were compared to the actual observed effects to determine possible mixture effects.

Additionally, we evaluated the combined effects of the PFAAs in the mixture by estimation of isobole coefficients (Kortenkamp and Altenburger 1998) at given effect levels, using the expression $\Sigma(c_i/EC_i)$, where c_i is the concentration of compound i in the mixture that produces the same effect E. The isobole coefficients were calculated by replacing $c_i=p_i \times EC_{mix}$. Isobole coefficients equal to 1 indicate an additive mixture effect. Values below 1 indicate synergistic mixture effects and values above 1 indicate antagonistic mixture effects.

Statistical analysis

The PFAAs were tested in at least three independent experiments in triplicate with appropriate solvent and medium controls in parallel, ensuring standardization of the assays. If one of the triplicate values deviated more than 30 % from the other two values, the mean was calculated from the two wells only. All experimental data was related to the respective solvent controls. In the transactivation assays, the relative transcriptional activity was expressed as "fold of induction" above the corresponding solvent control (set to 100 %).

Statistical analyses were performed on mean values from each independent experiment given as mean ± standard deviation (SD) of triplicate. For each test compound, only results obtained at non-cytotoxic concentrations were included in the statistical analysis performed in SPSS 20.0 (SPSS Inc., Chicago, IL). Due to relatively few data points per concentration and non-normality of the data, nonparametric statistics were used. The Kruskal–Wallis test was used to compare differences between concentrations and the Jonckheere–Terpstra test (two-tailed) was used to analyze for a linear trend between concentration and response. If one or both tests showed a significant difference ($p<0.05$), the Mann–Whitney test was used to compare each concentration with the control.

Concentration–response curves and calculations of EC_{50} (the concentration that induces half of the maximum response) and IC_{50} (the concentration that induces half of the maximum inhibitory response) were performed in SigmaPlot 11.0 (SPSS Inc., Chicago, IL) by fitting the data to the sigmoidal 4-parameter Hill equation.

Results

ER transactivation

Cytotoxicity

Agonistic and antagonistic effects of the PFAAs on the ER were analyzed in MVLN cells in absence and presence of 25 pM E2, respectively (Table 2 and Fig. 1). PFHxS, PFOA, PFNA, PFDA, PFUnA, and PFDoA were found to be toxic to the MVLN cells at concentrations $\geq1\times10^{-4}$ M, whereas PFOS was cytotoxic at concentrations $\geq6\times10^{-5}$ M. The mixture of the seven PFAAs was toxic at concentrations $\geq1.4\times10^{-4}$ M, corresponding to a concentration of 2×10^{-5} M of each PFAA in the mixture. The results given refer only to effects observed at concentrations not being toxic to the MVLN cells.

PFAAs tested as single compounds

Three of the seven tested PFAAs significantly ($p<0.05$) affected the agonistic ER transactivity in a concentration-dependent manner. PFHxS, PFOS, and PFOA induced the luciferase activity, causing maximum responses in the range of 158 to 229 % compared with the solvent control (set to 100 %) with PFOS displaying the highest fold of induction

Table 2 Summary of effects on ER transactivation

	Compounds tested alone					Compounds tested with 25 pM E2		
	LOEC (M)	MOEC (M)	% of SC at MOEC[a] ± SD	EC_{50} (M)	Cytotoxicity (M)	LOEC (M)	MOEC (M)	% of SC+25 pM E2 ± SD
Solvent	–	–	100	–	–			100[b]
E2	3.1×10^{-12}	3×10^{-10}	454±92	4.8×10^{-11}	–			
PFHxS	2×10^{-5}	9×10^{-5}	158±1	5.8×10^{-5}	$\geq1\times10^{-4}$	4×10^{-5}	9×10^{-5}	187±24
PFOS	1×10^{-5}	5×10^{-5}	229±23	2.9×10^{-5}	$\geq6\times10^{-5}$	1×10^{-5}	5×10^{-5}	210±13
PFOA	3×10^{-5}	9×10^{-5}	168±13	6.5×10^{-5}	$\geq1\times10^{-4}$	3×10^{-5}	9×10^{-5}	145±15
Mix	3.5×10^{-5}	3.5×10^{-5}	125±22	ND	$\geq1.4\times10^{-4}$	3.5×10^{-5}	3.5×10^{-5}	150±25

The individual PFAAs and the equimolar mixture were analyzed in the ER transactivation assay alone and upon co-exposure with 25 pM E2. No effects were observed for PFNA, PFDA, PFUnA, and PFDoA (data not shown)

LOEC the lowest tested concentration at which a significant effect (p <0.05) was detected, *MOEC* the lowest tested concentration causing the maximum effect (non-toxic), *SC* solvent control (maximum 0.1 %), *SD* standard deviation, EC_{50} concentration that induces half of the maximum response, *ND* could not be determined, – no effect observed

[a] Percent of response observed for compound at MOEC relative to the response observed for solvent control

[b] 25 pM E2 set to 100 %

(Table 2 and Fig. 1). EC_{50} values of the three ER active test compounds were estimated to be in the range of 2.9×10^{-5} to 6.5×10^{-5} M, indicating similar potencies of PFHxS, PFOS, and PFOA. However, the relative potencies of the three PFAAs were approximately 10^6–fold lower than the positive control 17β-estradiol (E2, Table 2). Thus, the observed estrogenic effects of PFHxS, PFOS and PFOA were relatively weak compared to the natural estrogen ligand.

Upon co-exposure with 25 pM E2, the PFHxS, PFOS, and PFOA significantly (p <0.05) further enhanced the E2-induced ER response (set to 100 %), with observed effects in the range of 145 to 210 % at the highest tested non-cytotoxic concentration. None of the seven tested PFAAs exerted ER antagonistic effects (data not shown).

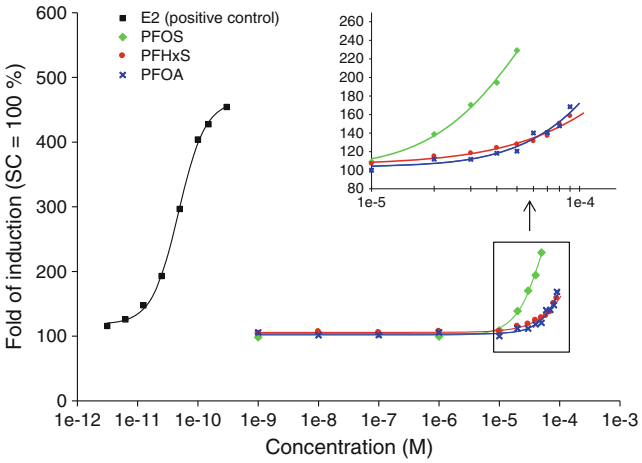


Fig. 1 ER transactivity in MVLN cells. Concentration-response curves for PFOS, PFHxS, PFOA and the positive control 17β-estradiol (E2). Data represent mean of at least three independent experiments each performed in triplicate. *SC* solvent control (maximum 0.1 %). In the *upper right corner* of the figure is shown an enlargement of the concentration-response curves for PFOS, PFHxS, and PFOA (1×10^{-5}–1×10^{-4} M)

PFAAs tested in mixture

The PFAA mixture significantly induced the ER transactivity to an effect level of 125 % relative to the solvent control (set to 100 %) at mixture concentrations of 3.5×10^{-5} M and 7×10^{-5} M (p=0.037 and p=0.004, respectively); however, only 3.5×10^{-5} M appears in Table 2 since this concentration makes up both the lowest observed effect concentration (LOEC) and the lowest tested concentration causing the maximum effect (i.e., the maximum observed effect concentration (MOEC)).

Also, upon co-exposure with 25 pM E2, the mixture significantly enhanced the E2-induced ER response (set to 100 %) at mixture concentrations of 3.5×10^{-5} M and 7×10^{-5} M (p=0.004 and p=0.010, respectively), with a maximum effect of 150 % observed at 3.5×10^{-5} M (LOEC and MOEC, Table 2), and a similar enhancing effect of 140 % observed at 7×10^{-5} M. Since effects of the mixture on the ER were observed at only two test concentrations (3.5×10^{-5} M and 7×10^{-5} M), it was not possible neither to construct a concentration-response curve nor to make any predictions concerning combination effects of the PFAA mixture on the ER.

AR transactivation

Cytotoxicity

Agonistic and antagonistic effects of the PFAAs on the AR were analyzed in CHO-K1 cells in absence and presence of 25 pM DHT, respectively (Table 3 and Fig. 2). PFOS and

Table 3 Summary of effects on AR transactivation

	Compounds tested alone					Compounds tested with 25 pM DHT			
	LOEC (M)	MOEC (M)	% of SC at MOEC[a] ± SD	EC_{50} (M)	Cytotoxicity (M)	LOEC (M)	MOEC (M)	% of SC+25 pM DHT ± SD	IC_{50} (M)
Solvent	–	–	100	–	–	–	–	100[b]	–
DHT	2×10^{-12}	1.0×10^{-10}	512±80	1.1×10^{-11}	–				
HF					–	5×10^{-9}	5×10^{-7}	48±7	2.0×10^{-8}
PFHxS	1×10^{-4}	1×10^{-4}	74±16	–	–	5×10^{-5}	1×10^{-4}	58±3	3.0×10^{-5}
PFOS	5×10^{-5}	5×10^{-5}	76±8	–	$\geq1\times10^{-4}$	5×10^{-6} [c]	5×10^{-5}	60±9	4.7×10^{-6}
PFOA	1×10^{-4}	1×10^{-4}	74±13	–	–	1×10^{-5}	1×10^{-4}	70±11	1.1×10^{-5}
PFNA	–	–	–	–	–	5×10^{-5}	1×10^{-4}	60±3	5.2×10^{-5}
PFDA	–	–	–	–	$\geq1\times10^{-4}$	1×10^{-5}	5×10^{-5}	84±2	6.0×10^{-6}
Mix	–	–	–	–	$\geq2.1\times10^{-4}$	7×10^{-6}	7×10^{-5}	53±5	6.8×10^{-6}

The individual PFAAs and the equimolar mixture were analyzed in the AR transactivation assay alone and upon co-exposure with 25 pM DHT. No effects were observed for PFUnA and PFDoA (data not shown). LOEC, MOEC, and EC_{50} are defined in the legend to Table 2

IC_{50} concentration that induces half of the maximum inhibitory response, *SC*: solvent control (maximum 0.1 %), *SD* standard deviation, – no effect observed

[a] Percent of response observed for compound at MOEC relative to the response observed for solvent control

[b] 25 pM DHT set to 100 %

[c] Borderline significant

PFDA were found to be toxic to the CHO-K1 cells at concentrations $\geq1\times10^{-4}$ M (Table 3), whereas PFUnA and PFDoA were toxic at concentrations $\geq5\times10^{-5}$ M (data not shown). The mixture of the seven PFAAs was toxic at concentrations $\geq2.1\times10^{-4}$ M, corresponding to a concentration of 3×10^{-5} M of each PFAA in the mixture. The results given refer only to effects observed at concentrations not being toxic to the CHO-K1 cells.

PFAAs tested as single compounds

None of the tested chemicals acted as agonists in the AR transactivation assay. However, PFHxS, PFOS, and PFOA alone elicited a significant (p <0.05) inhibiting effect (74, 76, and 74 %, respectively) on AR function at a relative high test concentration (Table 3). The concentration-response relationship of the AR agonist DHT is presented in Fig. 2a.

Upon co-treatment with 25 pM DHT, five of the seven PFAAs (PFOS, PFHxS, PFOA, PFNA, and PFDA) elicited significant (p <0.05) concentration-dependent antagonistic effects on DHT-induced AR transactivity. At the MOEC, these compounds antagonized the DHT-induced response (set to 100 %) down to effect levels in the range of 58 to 84 % (Table 3 and Fig. 2b). Moreover, the AR-antagonizing PFAAs showed similar potencies (IC_{50} values ranging from 4.7×10^{-6} M to 5.2×10^{-5} M) with PFOS being the most potent AR inhibitor. The relative potencies of the PFAAs were approximately 10^2–10^3-fold lower than the inhibitor control hydroxyflutamide (HF, Table 3).

PFAAs tested in mixture

The PFAA mixture alone did not elicit any effect on AR transactivity. However, upon co-exposure with 25 pM DHT, the mixture significantly (p <0.05) antagonized the DHT-induced AR transactivity with an IC_{50} of 6.8×10^{-6} M (Table 3). At the MOEC, the mixture antagonized the DHT-induced response (set to 100 %) down to 53 %, giving rise to a slightly higher percentage-wise maximum inhibitory effect than observed for the single antiandrogenic PFAAs (Table 3). Moreover, the lowest tested concentration of the mixture, at which a significant (p <0.05) effect was detected (LOEC), corresponded to a concentration of 1×10^{-6} M of each single component in the mixture (i.e., a mixture concentration of 7×10^{-6} M, Table 3). Thus, antagonizing effects on the AR were observed for the mixture at concentrations below LOECs for the single antiandrogenic PFAAs (PFHxS, PFOS, PFOA, PFNA, and PFDA).

The five antiandrogenic PFAAs formed the basis of the evaluation of mixture effects as described in the "Materials and methods" section. The predicted effect concentrations for the mixture were calculated at three effect levels, causing inhibition of DHT-induced AR activity (set to 100 %) down to 95, 90, and 85 %, using information on the mixture ratio and the concentration-response curves of the individual mixture components (Table 4). At the 95 % inhibition level (IC_{95}), the predicted mixture effect concentration was within the 95 % confidence band for the observed effects, suggesting an additive mixture effect (Fig. 3). The corresponding isobole coefficient was estimated to give a value

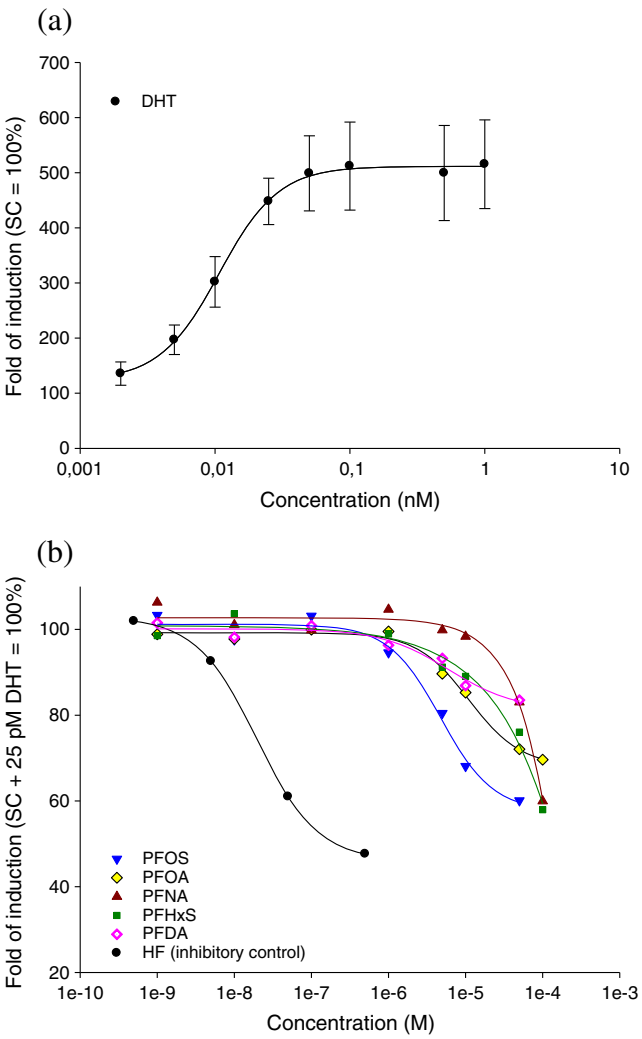


Fig. 2 AR transactivity in CHO-K1 cells. Concentration-response curves for **a** the agonist control DHT and **b** the inhibitory control HF and PFOS, PFOA, PFNA, PFHxS, and PFDA antagonizing the DHT-induced AR transactivity. Data represent mean of at least three independent experiments each performed in triplicate. *SC* solvent control (maximum 0.1 %)

relatively close to 1 (Table 4), supporting the finding of an additive mixture effect. At the 90 and 85 % inhibition levels (IC_{90} and IC_{85}, respectively), the predicted effect concentrations were found outside the 95 % confidence band for the observed effects, and were higher than observed effect concentrations, indicating an effect more than additive (synergistic) of the mixture (Fig. 3). This observation was substantiated by isobole coefficients below 1 (Table 4).

Aromatase activity

Cytotoxicity

Effects of the PFAAs on aromatase activity were analyzed in JEG-3 cells. PFOS, PFOA, PFNA, PFDA, PFUnA, and PFDoA were found to be toxic to the cells at concentrations $\geq 1\times10^{-4}$ M, whereas an incipient toxicity of PFHxS towards the JEG-3 cells was observed at 1×10^{-4} M.

Table 4 Evaluation of mixture effects on DHT-induced AR transactivity

	IC_{95}	IC_{90}	IC_{85}
Single PFAAs			
EC_{PFHxS} (M)	3.4×10^{-6}	9.8×10^{-6}	1.9×10^{-5}
EC_{PFOS} (M)	1.2×10^{-6}	2.1×10^{-6}	3.2×10^{-6}
EC_{PFOA} (M)	2.6×10^{-6}	5.6×10^{-6}	9.6×10^{-6}
EC_{PFNA} (M)	2.0×10^{-5}	3.2×10^{-5}	4.4×10^{-5}
EC_{PFDA} (M)	2.2×10^{-6}	6.9×10^{-6}	2.4×10^{-5}
Mixture of PFAAs			
Observed EC_{mix} (M)	3.0×10^{-6}	4.1×10^{-6}	5.1×10^{-6}
Predicted EC_{mix} (M)	3.5×10^{-6}	7.5×10^{-6}	1.3×10^{-5}
Isobole coefficients[a]	0.9	0.5	0.4

Concentrations of the individual PFAAs (EC_{PFHxS}, EC_{PFOS}, EC_{PFOA}, EC_{PFNA}, and EC_{PFDA}) and the mixture (observed EC_{mix}) causing down to 95, 90, and 85 % inhibition of DHT-induced AR transactivity (IC_{95}, IC_{90}, and IC_{85}, respectively). All observed concentrations were estimated from the respective concentration-response curves (Figs. 2b and 3). Since no competitive effects on DHT-induced AR activity were observed for PFUnA and PFDoA, no data are shown for these PFAAs. The predicted effect concentrations (predicted EC_{mix}) and isobole coefficients were calculated as described in the "Materials and methods" section

[a] An isobole coefficient equal to 1 indicates an additive mixture effect, a value below 1 indicates a synergistic mixture effect, and a value above 1 indicates an antagonizing mixture effect

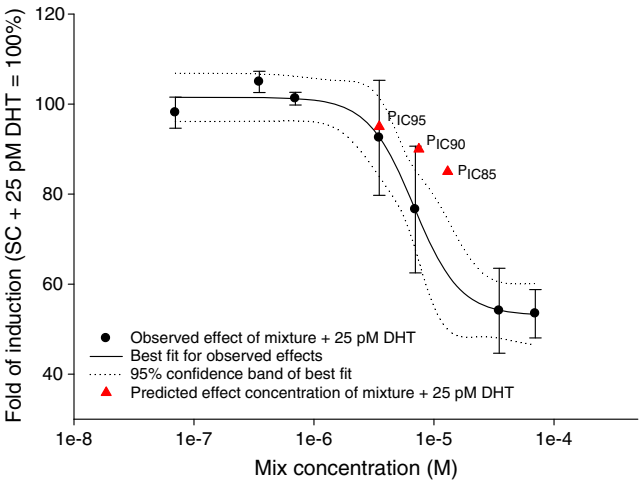


Fig. 3 Observed antiandrogenic effects and predicted effect concentrations of the PFAA mixture. Calculations of predicted effect concentrations, causing inhibition down to 95, 90, and 85 % effect levels (P_{IC95}, P_{IC90}, and P_{IC85}, respectively), were described in the "Materials and methods" section. Data for observed effects represent mean ± SD of three independent experiments each performed in triplicate. *SC* solvent control (maximum 0.1 %)

PFAAs tested as single compounds

An effect was observed only for PFDA; a significant (p= 0.002) decrease of the aromatase activity down to 85 % compared to the solvent control (set to 100 %) at 1×10^{-5} M (data not shown). Since the down-regulating effect was only seen at this relatively high concentration of PFDA, it cannot be ruled out that it may be due to an incipient cytotoxicity of the compound.

Discussion

Within the past decade, considerable attention has been paid to PFCs because of their worldwide presence in humans, wildlife, and in the environment. Concern regarding the public health implications of exposure to these ubiquitous global contaminants has emerged. The underlying biochemical mechanisms of action responsible for observed toxicological effects of PFCs in humans have not yet been thoroughly described. Thus, there is an urgent need to clarify whether these environmental contaminants have the potential to disrupt the homeostatic balance between sex hormones via endpoints such as steroid hormone receptors and steroidogenic enzymes.

In the present in vitro study, we demonstrated the potential of three PFAAs (PFHxS, PFOS, and PFOA) to act as ER agonists, and five PFAAs (PFHxS, PFOS, PFOA, PFNA, and PFDA) to act as AR antagonists. Moreover, we observed a down-regulating effect of PFDA on the aromatase enzyme activity. The equimolar mixture of the seven PFAAs weakly induced the ER transactivity at only two concentrations; both alone and upon co-exposure with the natural estrogen E2, whereas the equimolar mixture antagonized the DHT-induced AR transactivity in a dose-dependent manner. The combined action of the PFAAs was found to have a more than additive (synergistic) impact on AR function compared with the AR-antagonizing effect concentrations of the single compounds.

In the present study, PFHxS, PFOS, and PFOA induced the ER transactivity in human MVLN breast carcinoma cells, whereas no significant effect on ER function was observed for PFNA, PFDA, PFUnA, and PFDoA. Reports on disrupting effects of PFAAs on ER function in vitro are relatively scarce. To our knowledge, we report here, for the very first time, the ability of PFHxS to act as an ER agonist, whereas contradictory in vitro data concerning the estrogenic potential of PFOS and PFOA exist (Benninghoff et al. 2011; Henry and Fair 2011; Du et al. 2012; Maras et al. 2006; Ishibashi et al. 2007).

Recent studies reported estrogenic activity of PFOS and PFOA in human cell lines; both PFAAs were found to significantly induce transactivation of human ERα in HEK-293T embryonic kidney cells (Benninghoff et al. 2011), and to cause a significant increase in proliferation of human MCF-7 BOS breast cancer cells (Henry and Fair 2011), suggesting that PFOS and PFOA may be potent ER agonists. Moreover, PFOS has been found to induce transactivation of the ER in monkey CV-1 kidney cells (Du et al. 2012). These findings are in accordance with our results and are further substantiated by studies indicating that PFOS and PFOA are inducers of the estrogen-responsive biomarker protein vitellogenin (Benninghoff et al. 2011; Liu et al. 2007; Wei et al. 2007). In contrast, other in vitro studies did not observe any estrogenic activity of PFOS and PFOA since these PFAAs were neither found to induce proliferation of human MCF-7 breast cancer cells nor to cause any estrogenic effects in yeast cells modified by incorporation of human ERs (Maras et al. 2006; Ishibashi et al. 2007). Overall, the discrepancy between these reported in vitro activities of PFOS and PFOA on ER function might be due to varying designs and/or sensitivities of the assays as well as differences in experimental conditions such as choice of cell line and incubation period.

Previous studies have sought to clarify possible biological endpoints involved in the observed estrogenicity of PFOS and PFOA. Based on competitive ER-binding assays, both PFAAs have shown to be weak ligands for the trout liver ER and additionally, they were found to bind efficiently to human-, mouse- and trout ERα proteins in silico, forming a hydrogen bond in a manner similar to what is seen for the environmental estrogens bisphenol A and nonylphenol (Benninghoff et al. 2011). In view of these findings and the results from our ER transactivation analyses which are based on an ER-mediated transactivity, it seems likely that PFOS and PFOA can interact directly with the ER to exert their estrogenic effects. In contrast, we did not observe any effects of PFOS and PFOA on aromatase activity in JEG-3 cells. Contradicting data concerning the effect of PFOS on aromatase activity in human H295R adrenocarcinoma cells exist (Du et al. 2012; Kraugerud et al. 2011). A significant induction of the *CYP19* (aromatase) gene expression was observed for PFOS (Du et al. 2012) but not for PFOA (Rosenmai et al. 2012).

Upon co-exposure with E2, we observed that PFHxS, PFOS, and PFOA significantly (p <0.05) enhanced the E2-induced ER response in human MVLN breast cancer cells. This further inducing capacity of PFOS has previously been seen in monkey CV-1 kidney cells, using a similar ER-mediated reporter gene assay (Du et al. 2012). In contrast, PFOS and PFOA were found to significantly inhibit E2-induced proliferation of MCF-7 BOS cells, suggesting anti-estrogenicity of these PFAAs (Henry and Fair 2011). Moreover, PFOS showed antiestrogenic capacity in a noncompetitive enzyme-linked immunosorbent assay using vitellogenin induction in primary cultured hepatocytes of freshwater male tilapia (Liu et al. 2007).

Recently, it was reported that PFNA, PFDA, and PFUnA are weak xenoestrogens in vitro and in vivo (Benninghoff et al. 2011). PFNA and PFDA were found to significantly induce ERα-dependent transcriptional activation in human HEK-293T embryonic kidney cells, and furthermore, all three PFAAs were shown to completely displace E2 from the ER in a trout hepatic binding study (Benninghoff et al. 2011). Moreover, PFNA, PDFA, and PFUnA were found to be potent inducers of the estrogen-responsive biomarker protein vitellogenin in vivo using juvenile rainbow trout (Benninghoff et al. 2011). In contrast, we did not observe any effects of these compounds on ER transactivity in human MVLN breast cancer cells. Different cell lines and analysis setups might explain the data differences.

In conclusion, our data suggest that PFHxS, PFOS, and PFOA possess an estrogenic potential in vitro mediated via the ER. PFCs are known to cross the human placenta causing an exposure of the developing fetus (Needham et al. 2011; Apelberg et al. 2007a; Fei et al. 2007), being of great concern since estrogen hormone signaling is known to play an important role during fetal development. Moreover, disruption of ER signaling pathways may contribute to adverse health effects such as reproductive failure including infertility and endocrine-related cancers (Mueller 2004; Sikka and Wang 2008).

To our knowledge, we report here for the very first time the in vitro potency of PFHxS, PFOS, PFOA, PFNA, and PFDA to antagonize the AR transactivity in a concentration-dependent manner. A previous study did not observe any effects of PFOS on 1 nM DHT-induced AR transcriptional activity in human MDA-kb2 breast carcinoma cells (Du et al. 2012). The discrepancy, between these data and our finding of an antiandrogenic potential of PFOS in CHO-K1 cells upon co-treatment with 25 pM DHT, might be due to varying designs and/or sensitivities of the assays as well as differences in experimental conditions such as choice of cell line, reporter plasmids and co-treatment conditions. A recent study did not report any effects of PFOA ($\leq 5\times10^{-5}$ M) on 100 pM methyltrienolone (R1881)-induced AR transactivity in Chinese hamster ovary (CHO) cells (Rosenmai et al. 2012) which is in contrast to our results. Despite the fact that our study and the Rosenmai et al. (2012) study utilized similar assay conditions, such as origin of cell line, choice of plasmids, and incubation period, there were notable differences in co-treatment conditions, 25 pM DHT vs. 100 pM R1881, respectively. We have previously used the synthetic AR antagonist R1881 in our laboratory and suspect that PFAAs such as PFOA do simply not have the potency to compete with 100 pM R1881, since the AR might be fully saturated at this agonist concentration. This might explain the diverging results presented in the two studies.

Our findings of an antiandrogenic potential of five of seven PFAAs investigated are of concern, since growing evidence suggests a link between AR disruptors and disorders of male health (Luccio-Camelo and Prins 2011). The AR is the key regulatory element of androgen cell signaling. AR-regulated gene expression is responsible for male sexual differentiation in utero and male reproductive function and development, including spermatogenesis (Dehm and Tindall 2007; Gao et al. 2005). Moreover, epidemiological reports have suggested that PFAAs negatively affect sperm quality (Toft et al. 2012; Joensen et al. 2009).

Effects of the PFAA mixture on ER function were observed at only two test concentrations (3.5×10^{-5} M and 7×10^{-5} M), whereas the mixture was toxic to the MVLN cells at concentrations $>7\times10^{-5}$ M. Consequently, we were not able to construct concentration-response curves and predict any possible combination effects of the mixture on the ER. In contrast, the mixture showed concentration-dependent AR-antagonizing effects. Since the PFAAs were assumed to act on the same molecular target (receptor), we applied the model of CA to predict possible mixture effects. This model has shown to be a valid tool for in vitro assessment of mixture effects of xenobiotics (Birkhoj et al. 2004; Ghisari and Bonefeld-Jorgensen 2009; Kruger et al. 2008; Payne et al. 2000; Rajapakse et al. 2002). For the AR system, we predicted mixture concentrations at effect levels corresponding to 95, 90, and 85 % of inhibition. We did not predict effect concentrations for effect levels below 85 % of inhibition since the largest effect level predictable with the CA model (i.e., the lowest inhibition effect level percentage-wise) is determined by the mixture component with the lowest maximal effect (i.e., 84 % for PFDA, Table 3). We conclude that a mixture of PFAAs may have a more than additive (synergistic) impact on the individual AR-antagonizing effect concentrations of the single antiandrogenic components of the mixture. Moreover, we have shown that antiandrogenic PFAAs are able to act together to produce significant effects, when combined at concentrations below their individual LOECs. Based on these results, the biological effects of single weak endocrine-disrupting PFAAs cannot be considered as negligible.

The in vitro assays applied in this study are tools for initial screening of endocrine-disrupting compounds. The present findings have some limitations in terms of assay conditions which show short-term interactions, but do not reflect the in vivo situation where bioaccumulation and metabolism of the PFAAs may greatly influence their intracellular concentrations. Thus, in vivo studies are needed to further elucidate the endocrine-disrupting effects of the PFAAs.

We conclude that our data indicate that the analyzed PFAAs have the potential in vitro to interfere with sex steroid hormone receptor transactivity and thus, have the potency to disrupt endocrine homeostasis. Our observation, that PFHxS, PFOA, and PFOA elicited an effect on both the ER and AR, might suggest an enhanced biological effect in the intact organism, since the final response is likely to be determined by the interaction of implicated pathways.

The data presented in this study were obtained at relatively high concentrations compared to levels found in humans. Moreover, the PFAAs generally seem to be far less potent than the natural hormone with respect to the ER. However, due to the persistent and highly bioaccumulative nature of PFCs, long human elimination half-lives, as well as the life-long human exposure scenario to complex mixtures of chemicals, it is conceivable that many environmental contaminants may act together and affect the effect of the single compounds. Therefore, the biological effects of the PFAAs on the ER and AR presented in this study might play a role in endocrine disruption, with especial concern for the developing fetus, and must be taken into consideration, since they can contribute to the understanding of the toxicological mechanisms of PFAAs. It is increasingly recognized that knowledge of the toxicity of single chemicals is often inadequate for human risk assessment, and further studies of the concerted actions of PFCs at physiological levels are required to elucidate potential adverse human health effects.

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Conflict of interest The authors declare that there are no conflicts of interest.

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