PERFLUOROALKYL ACIDS HAVE NO ENDOCRINE DISRUPTING ACTIVITIES VIA HUMAN STEROID HORMONE RECEPTORS AS DETERMINED BY A PANEL OF CALUX® BIOASSAYS

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Abstract
Perfluoroalkyl acids are a group of man-made compounds widely used as surfactants, lubricants, polymers, and fire-fighting foams. Recent studies have shown a wide-spread distribution of PFAAs in the environment, wildlife, and in humans. Toxicity of PFAAs in mammals is related to developmental toxicity, reproductive toxicity, immunotoxicity and hepatotoxicity. Furthermore these compounds are suspected endocrine disrupting compounds (EDCs). In this report we investigate the endocrine disrupting activities of several PFAAs (perfluorooctane sulfonate, perfluorohexanoic acid, perfluoroheptanoic acid, perfluorononanoic acid and perfluorooctanoic acid) on various human steroid hormone receptors by means of several *in vitro* reporter gene assays. A panel of U2-OS-based CALUX® bioassays was used measuring activities on the androgen receptor, estrogen receptors alpha and beta, progesterone receptor, and glucocorticoid receptor. The activities of the PFAAs were compared with earlier reported endocrine disrupting activities of other persisting compounds of concern, including several brominated flame retardants (BFRs) and pesticides. None of the PFAAs tested showed agonistic or antagonistic activities on any of the human hormone receptors tested, in contrast to the BFRs and pesticides. This report shows that the suspected endocrine disrupting activity, if any, of PFAAs does not act via direct interference with human steroid hormone receptors.

Introduction
Perfluoroalkyl acids (PFAAs) belong to a diverse family of man-made perfluorinated chemicals with surface-active properties, which have been manufactured for over half a century. Because of their unique properties of repelling both water and oil, PFAAs have been widely used as surfactants, fire-fighting foams, floor polishes, and shampoos[1]. Until recently, research investigating the potential toxicity and the environmental fate of PFAAs has been evaluated of minor importance as compared to that of chlorinated and brominated organic compounds (such as BFRs), because they are more biologically inert and therefore, less likely to have an impact on human health or the environment. The environmental persistence, the potential for long-term environmental effects, as well as the finding of PFAAs in blood of humans and wildlife tissues, raised a notable concern about PFAAs, especially the most widely known perfluorooctane sulfate (PFOS) and perfluorooctanoic acid (PFOA) [1, 2]. Toxicity of PFAAs in mammals has been mainly focussed on the latter two compounds and relate to developmental toxicity, reproductive toxicity, immunotoxicity and hepatotoxicity[1]. One mode of action of PFAAs might be their suspected endocrine disrupting activity: both PFOA and PFOS activate peroxisome proliferator-activated receptor (PPAR) subtypes[3], while also both compounds reduce total thyroid hormones (T3 and T4) in circulation[1].
We recently developed a panel of highly sensitive and selective CALUX® cell lines allowing efficient and convenient measurement of not only androgen-, but also estrogen (ERalpha and ERbeta)-, progesterone-, and glucocorticoid receptor interacting compounds [4,5]. This panel of bioassays uses the same cellular background (human U2-OS bone cell line) in which the activity of all major classes of steroid hormones can be determined specifically. Members of this panel were recently used to show agonistic and/or antagonistic activities of several environmental contaminants on various human steroid hormone receptors [4, 6, 7, 8].
In this report we investigate the endocrine disrupting activities of several PFAAs on several human steroid hormone receptors by means of the panel of U2-OS-based CALUX bioassays. The activities of the PFAAs were compared with the earlier reported endocrine disrupting activities of other persisting compounds of concern, such as several BFRs[8], and chloride containing pesticides[4].

Materials and Methods

Compounds
Perfluorooctane sulfonate (PFOS) potassium salt (> 98%), perfluorohexanoic acid (PFHxA > 98%), perfluoroheptanoic acid (PFHpA, 99%), perfluorononanoic acid (PFNA, 97%) and perfluorooctanoic acid (PFOA,

96%) were kind gifts from Prof. Dr. B. van Bavel (Orebro University, Sweden). All other compounds were described previously[4, 8].

CALUX® bioassays

The generation of androgen (AR), progestin (PR), estrogen (ERα and ERβ) and glucocorticoid (GR) responsive CALUX® bioassays was described previously[4]. Cells were plated in 96 well plates (12.000 cells/well) with phenol red free DF medium supplemented with 5% dextran coated charcoal stripped FCS (DCC-FCS) at a volume of 100 µl per well. After 24 h, medium was removed from the cells and 200 µl of DCC-FCS medium containing the compound(s) of interest (dissolved in DMSO), final dilution 1:1000) was added. After 24 hours the medium was removed, cells were lysed in 30 µl Triton-lysis buffer and measured for luciferase activity using a luminometer (Lucy2; Anthos Labtec Instruments, Wals, Austria) for 0.1 min/well.

Data analysis

Analysis of steroid receptor mediated luciferase activity in CALUX® cells was performed as followed. Luciferase activity per well was measured as relative light units (RLUs). Fold induction was calculated by dividing the mean value of light units from exposed and non-exposed (solvent control) wells. Luciferase induction as a percentage of maximal reference activity (DHT for AR, E2 for ERα and ERβ, Org 2058 for PR, and dexamethasone for GR CALUX®) was calculated by setting the highest fold induction of reference compound at 100%. Data are represented as mean values ± SEM from at least three independent experiments with each experimental point performed in triplicate. Dose-response curves were fitted using the sigmoidal fit $y = a_0 + a_1/(1 + \exp(-(x-a_2)/a_3))$ in GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA), which determines the fitting coefficients by an iterative process minimizing the c2 merit function (least squares criterion). At least 8 different concentrations covering the total S-curve were included for each compound. The EC_{50} and IC_{50} values were calculated by determining the concentration by which 50 percent of maximum (EC_{50}) or minimum (IC_{50}) activity was reached using the sigmoidal fit equation. The relative transactivation activity (RTA) of each compound tested was calculated as the ratio of maximal luciferase reporter gene induction values of each compound and the maximal luciferase reporter gene induction value of reference compound of each specific CALUX® bioassay. The transactivation activity of the reference compound was arbitrarily set at 100. Two dimensional hierarchical clustering on the base 10 logarithm of EC_{50} and IC_{50} data was performed using the correlation option within the clustergram function from the bioinformatics toolbox in Matlab (The Mathworks, the Netherlands).

Compound	AR agonistic logEC50 (M)	ERa agonistic logEC50 (M)	ERb agonistic logEC50 (M)	PR agonistic logEC50 (M)	GR agonistic logEC50 (M)	AR antagonistic logIC50 (M)	ERa antagonistic logIC50 (M)	ERb antagonistic logIC50 (M)	PR antagonistic logIC50 (M)	GR antagonistic logIC50 (M)
DHT (AR ref.)	-9.9	>-5	-6.2	-5.4	>-5	>-5	nd	nd	nd	nd
E2 (ER ref.)	-6.3	-10.8	-9.6	>-5	>-5	nd	>-5	>-5	nd	nd
org2058 (PR ref.)	>-5	>-5	>-5	-10.3	>-5	nd	nd	nd	>-5	nd
dex (GR ref.)	-7.8	>-5	>-5	>-5	-9.4	nd	nd	nd	nd	>-5
vinclozolin	>-5	>-5	>-5	>-5	>-5	-6.0	>-5	>-5	-5.6	>-5
HPTE	>-5	-7.1	>-5	>-5	>-5	-6.5	>-5	-6.0	-5.2	-5.5
o,p'DDT	>-5	-6.0	-6.2	>-5	>-5	-5.8	>-5	>-5	-6.1	-5.0
p,p'DDT	>-5	-5.0	>-5	>-5	>-5	-5.6	>-5	>-5	-5.5	>-5
Methoxychlor	>-5	-5.1	>-5	>-5	>-5	-5.1	>-5	-5.0	-5.4	-5.0
octa-BDE	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5
TCDD	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5
BDE19	>-5	-5.6	>-5	>-5	>-5	-7.2	>-5	-5.0	-6.1	>-5
BDE38	>-5	>-5	>-5	>-5	>-5	-5.7	>-5	-5.0	>-5	>-5
BDE47	>-5	>-5	>-5	>-5	>-5	-6.0	>-5	-5.0	>-5	>-5
6OH-BDE47	>-5	>-5	>-5	>-5	>-5	-5.5	-6.0	>-5	-5.3	>-5
BDE49	>-5	>-5	>-5	>-5	>-5	-6.2	>-5	>-5	-5.2	>-5
BDE100	>-5	-5.0	>-5	>-5	>-5	-7.0	>-5	-5.4	-5.5	>-5
BDE155	>-5	-5.0	>-5	>-5	>-5	-5.7	>-5	-5.0	-5.4	>-5
BDE181	>-5	>-5	>-5	>-5	>-5	-5.5	-5.0	-5.0	-5.4	>-5
BDE190	>-5	>-5	>-5	>-5	>-5	-5.1	-5.0	-5.0	-5.3	>-5
BDE209	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5
TBBPA	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5
2,4,6-TBP	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5
PFOS	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5
PFHxA	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5
PFOA	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5
PFNA	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5
PFHpA	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5	>-5

Table 1. LogEC_{50} and logIC_{50} values of agonistic and antagonistic compounds in AR, ERalpha, ERbeta, PR and GR CALUX® bioassays. Nd=not determined. Some of the data was adapted from references 4 and 8.

Results and Discussion

It has been found that a variety of environmental chemicals mimic steroid hormones or interfere in an antagonistic fashion with hormonal action, thereby possibly contributing to negative health effects in humans and wildlife. These include BFRs, acting on ER, AR and PR[8], and several pesticides (including DDT, methoxychlor and its metabolite HPTE)[4]. In this report, several PFAAs were studied for similar activities (agonistic and antagonistic. Stable CALUX® reporter cell lines were used expressing either the human androgen receptor (AR), the human estrogen receptor, type alpha (ERalpha), or beta (ERbeta), the human progesterone receptor (PR), or the human glucocorticoid receptor (GR) in combination with their cognate luciferase expressing reporter gene constructs. None of the tested PFAAs (PFOS, PFHxA, PFHpA, PFNA and PFOA) showed agonistic or antagonistic properties on AR, ERalpha, ERbeta, PR and GR (Table 1) or TRbeta (data not shown). No such activities were also found for TCDD, and the BFRs octa-BDE, 2,4,6-TBP, TBBPA and BDE209 (Table 1; references 4 and 8). On the other hand, the other compounds tested showed agonistic and/or antagonistic activities in one ore more CALUX® bioassays (Table 1). By means of hierarchical clustering, bioactivity relationships could be visualized (Figure 1). These include previously reported antagonistic activities of several BFRs on AR and PR[8] and several pesticides (vinclozolin, DDTs, methoxychlor and HPTE) on AR[4], and agonistic activities of these pesticides on ERalpha[4]. Furthermore, new agonistic activities (BDE19, BDE100 and BDE155) and antagonistic activities (6OH-BDE47, BDE181 and BDE190) were found on ERalpha as reported earlier for ER[8]. Weak antagonistic activities on ERbeta were found for BDE19, BDE38, BDE47, BDE100, BDE155, BDE181, BDE190, methoxychlor and HPTE (Table 1 and Figure 1). The latter two, together with vinclozolin and 'o,'p-DDT showed antagonism on PR, while methoxychlor, HPTE and 'o,'p-DDT possessed weak antagonistic activity on GR.

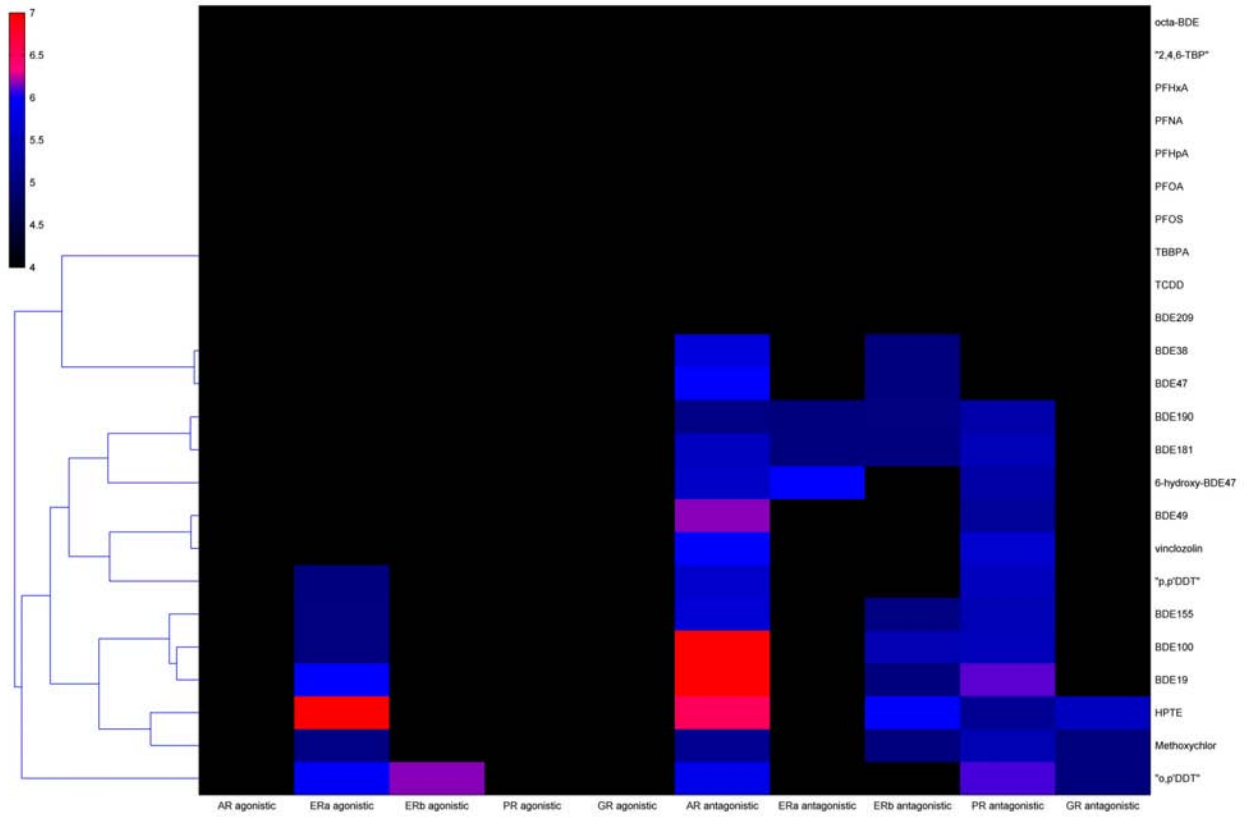

Figure 1. Profiling of PFAAs in relation to other contaminants by means of hierarchical clustering. EC_{50} and IC_{50} values of PFAAs, BFRs and several other contaminants (n=24; see Table I) determined in the AR, PR, ERalpha, ERbeta and GR CALUX ® bioassays were -10 log transformed and clustered according to the two-dimensional hierarchical clustering method. The intenser the red color, the higher the activity of the compound. Blue means weaker activity, black means no activity (see intensity bar).

In conclusion, none of the tested PFAAs showed *in vitro* agonistic and antagonistic activities in a panel of CALUX® steroid receptor-based bioassays, in contrast with other environmental contaminants such as BFRs and pesticides. This shows that the suspected endocrine disrupting activity of PFAAs does not act via direct interference with human steroid hormone receptors.

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