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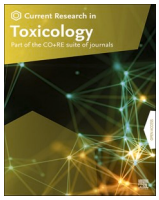

Effects of short-chain per- and polyfluoroalkyl substances (PFAS) on human cytochrome P450 (CYP450) enzymes and human hepatocytes: An *in vitro* study

Megan E. Solan, Ramon Lavado *

Department of Environmental Science, Baylor University, Waco, TX 76798, United States

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ABSTRACT

Short-chain per- and polyfluoroalkyl substances (PFAS) have been developed as alternatives to legacy long-chain PFAS, but they may still pose risks due to their potential to interact with biomolecules. Cytochrome P450 (CYP450) enzymes are essential for xenobiotic metabolism, and disruptions of these enzymes by PFAS can have significant human health implications. The inhibitory potential of two legacy long-chain (PFOA and PFOS) and five short-chain alternative PFAS (PFBS, PFHxA, HFPO-DA, PFHxS, and 6:2 FTOH) were assessed in recombinant CYP1A2, − 2B6, −2C19, −2E1, and −3A4 enzymes. Most of the short-chain PFAS, except for PFHxS, tested did not result in significant inhibition up to 100 μM. PFOS inhibited recombinant CYP1A2, −2B6, −2C19, and −3A4 enzymes. However, concentrations where inhibition occurred, were all higher than the averages reported in population biomonitoring studies, with IC_{50} values higher than 10 μM. We also evaluated the activities of CYP1A2 and CYP3A4 in HepaRG monolayers following 48 h exposures of the short-chain PFAS at two concentrations (1 nM or 1 μM) and with or without an inducer (benzo[a]pyrene, BaP, for CYP1A2 and rifampicin for CYP3A4). Our findings suggest that both 1 nM and 1 μM exposures to short-chain PFAS can modulate the CYP1A2 activity induced by BaP. Except for PFHxS, the short-chain PFAS appear to have little effect on CYP3A4 activity. Understanding the effects of PFAS exposure on biotransformation can shed light on the mechanisms of PFAS toxicity and aid in developing effective strategies for managing chemical risks, enabling regulators to make more informed decisions.

1. Introduction

Several decades ago, synthetic chemistry innovations led to the development of a versatile class of compounds known as per- and polyfluoroalkyl substances (PFAS) (Teaf et al. 2019; USEPA, 2016). This class of chemicals is characterized by chains of carbon–fluorine bonds that lend to the unique structural properties that make them suitable for use in a broad range of chemical products. However, widespread use and frequent exposure to PFAS have warranted much concern by the public and regulators.

PFAS exposure in humans primarily occurs through contaminated drinking water sources near fluorochemical manufacturing locations, military bases, and airport facilities that utilize aqueous film-forming foam (AFFF) and ingesting contaminated food (DeLuca et al. 2022; Garrett et al. 2022). Dermal contact with household cleaning products and inhalation of dust particles have also been reported as routes of exposure (East et al. 2023). PFAS are known to accumulate in serum and tissues. Exposure has been linked to hepatotoxic, immunotoxic, and neurotoxic effects, including hepatic peroxisome proliferation, altered antibody synthesis, and hepatic hypertrophy (De Silva et al. 2021).

While phasing out of the long-chain PFAS has been mostly successful, alternative compounds with similar useful properties have been developed. These compounds are considered short-chain PFAS because they have fewer than seven carbon–fluorine bonds (Brendel et al. 2018). While the short-chain structure appears to be less prone to tissue accumulation, they are still resistant to degradation and persist in the environment, contributing to the large-scale global contamination of PFAS in the environment. The contamination is considered problematic in risk mitigation for areas with high concentrations of PFAS, especially since shorter-chain variants may have a higher potential to interact with biomolecules due to less steric hindrance than the longer-chain PFAS (Sunderland et al. 2019).

* Corresponding author at: Baylor University, Department of Environmental Science, One Bear Place #97266, Waco, TX 76798-7266, United States.
E-mail address: Ramon_Lavado@baylor.edu (R. Lavado).

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Cytochrome P450 (CYP450) monooxygenases, sometimes called oxidoreductases, are a large family of cytoplasm-bound catalytic enzymes that generally operate through similar modes of operations. CYP450 enzymes play a crucial role in clearing endogenous molecules and lipophilic hydrocarbons (Coleman 2010). Thousands of CYP enzymes discovered across all biological organisms; of these, humans encode 57 individual enzymes in 18 families (Guengerich 2008). The pharmacodynamic potency of a drug heavily relies on biotransformation processes and nearly 60% of pharmaceuticals are cleared by only six CYP450 isozymes (CYP1A2, CYP2C9, CYP2B6, CYP2C19, CYP2D6, and CYP3A4) (Coleman 2010). Thus, elucidating the consequences of chemical mixtures often focuses predominantly on those key CYPs.

Biotransformation enzymes are not known to be able to modify PFAS to facilitate their elimination from the body (Kemper and Nabb 2005). The inability to biotransform these compounds may contribute to the long half-life of serum elimination times of PFAS in humans; for example, the estimated average for PFOA is 3.5 years (Olsen et al. 2007). Evidence for the need to study metabolism enzymes for this class of compounds was made apparent by an *in vitro* study using perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) that investigated three cytochrome P450 enzymes (CYP1A2, CYP2C19, and CYP3A4) and two conjugation enzymes (glutathione-*S*-transferase (GST-M1) and UDP-glucuronosyltransferase (UGT-1A1)) (Franco et al. 2020). Expression of all CYP enzymes was significantly reduced from exposure to both PFOA and PFOS after 48 h and concentrations as low as 40–50 ng/L, with CYP3A4 also presenting the lowest activity.

In general, short-chain PFAS toxicity data is limited. Mechanism-driven information can facilitate the creation of comprehensive toxicological profile and accurate *in vitro* to *in vivo* extrapolations that can be applied to elements of the risk assessment process.

Considering the critical role of CYP450 enzymes, the main objective of this study was to assess the influence of PFASs on CYP450 enzymes. We hypothesized that exposure to short-chain PFAS would inhibit clinically relevant metabolism enzymes based on previous studies that have indicated the inhibitory potential of PFOA and PFOS (Franco et al. 2020). Therefore, we assessed the activities of CYP1A2 and CYP3A4 in HepaRG monolayers using five short-chain PFAS compounds: perfluorobutanesulfonic acid (PFBS), perfluorohexanoic acid (PFHxA), [undecafluoro-2-methyl-3-oxahexanoic acid (HFPO-DA)], 6:2 fluorotelomer alcohol (6:2 FTOH) and perfluorohexanesulfonic acid (PFHxS) following 48 h exposures of each PFAS at 1 nM and 1 μM, with and without an inducer: benzo[a]pyrene (BaP) for CYP1A2 and rifampicin for CYP3A4. Additionally, the inhibitory potential of the short-chain PFAS was assessed in recombinant CYP1A2, − 2B6, −2C19, −2E1, and −3A4 enzymes.

Although many *in vitro* studies of CYP450 activities use liver cell lines, many of these have been conducted in the HepG2 cell line (Behr et al. 2020; Dale et al. 2022; Ojo et al. 2021; Wen et al. 2020; Wielsoe et al. 2015). While HepG2 cells have demonstrated many liver-specific functions, they lack the functional expression of several relevant human liver cytochrome P450s (Skolik et al. 2021). In contrast, the HepaRG human liver cell line maintains essential hepatic functions after undergoing differentiation, including high expression of biotransformation enzymes and drug transporters, providing metabolic competence comparable to primary human hepatocytes (Franzosa et al. 2021).

2. Materials and methods

2.1. Chemicals

Perfluorobutanesulfonic acid (PFBS), perfluorohexanoic acid (PFHxA), [undecafluoro-2-methyl-3-oxahexanoic acid (HFPO-DA)], 6:2 fluorotelomer alcohol (6:2 FTOH), perfluorohexanesulfonic acid (PFHxS), Rifampicin (CAS: 13292–46-1), ethanol (EtOH), and dimethyl sulfoxide (DMSO), methanol (MeOH), acetonitrile (ACN) were obtained from Sigma-Aldrich (St. Louis, MO). More detailed information on the PFAS chemicals is in Table 1. Benzo[a]pyrene (CAS: 50–32-8) was obtained from Thermo Scientific Chemicals (Waltham, MA). The fluorogenic substrates and standards for inhibition experiments were 7-benzyloxy-4-(trifluoromethyl) coumarin (7-BFC); 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC); 7-methoxy-4-(trifluoromethyl) coumarin (7-MFC); 7-hydroxy-4-(trifluoromethyl)coumarin (HFC); 7-methoxyresorufin; and resorufin sodium salt (Sigma-Aldrich, St. Louis, MO). All chemicals and reagents were of analytical grade. William's E Medium, Glutamax™, hydrocortisone hemisuccinate, and insulin were obtained from Gibco Life Technologies (ThermoFisher Scientific, Waltham, MA). Hydrocortisone hemisuccinate and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture and exposures

Undifferentiated HepaRG cells were obtained from BioPredic International (Paris, France) (distributors in the USA: Lonza Walkersville Inc., Walkersville, MD) and maintained at 37 °C and 5% CO_2. The growth medium for HepaRG consisted of Gibco-formulated William's E Medium supplemented with 2 mM Glutamax, 10 % fetal bovine serum (FBS), 5 μg/mL insulin, and 50 μM hydrocortisone hemisuccinate. Undifferentiated HepaRG cells were plated at 1×10^5 cells/mL in 96-well flat clear bottom white 96-well microplates and maintained in growth media for 14 days with media renewals every 3 days before undergoing differentiation. After 14 days, the cells were shifted to growth medium supplemented with 1.7% DMSO (differentiation medium). Culturing cells with the differentiation medium for 14 days led to confluent, differentiated cultures containing equal proportions of hepatocyte-like and progenitors/primitive biliary-like cells.

Exposures were conducted in growth medium without phenol-red and contained a reduced serum content (2% FBS) as it is known that PFAS have a high affinity for albumin, which can influence affect the uptake and intracellular availability during treatment (Bangma et al. 2020; Yang et al. 2023; Zhang et al. 2020). PFAS exposures were conducted for 48 h at 1 μM and 1 nM either alone or in a binary mixture with an inducer (10 μM rifampicin for CYP3A4 experiments and 10 μM BaP for CYP1A2 experiments). BaP is a potent CYP1A2 inducer in humans with well-established properties (Guengerich 2022), while rifampicin is a potent CYP3A4 with well-characterized effects (Chattopadhyay et al. 2018). The potency of the inducer treatments was selected following previous literature estimates for concentrations where induction has been observed without significantly compromising cell viability (Buick et al. 2021; Jennen et al. 2010).

The PFAS concentrations were selected below the median effective concentrations (EC_{50}) for cytotoxicity values determined by Solan et al. (2022). DMSO (0.1% v/v) was the solvent vehicle for 6:2 FTOH, PFBS, PFHxA, PFHxS, Rifampicin, and BaP. EtOH was used as the solvent vehicle (0.1% v/v) for HFPO-DA due to the rapid degradation of this compound in aprotic, polar solvents (Liberatore et al. 2020). The experiments were performed with biological (plates of different passages) and technical (wells) replicates. Four biological with four technical replicates were used.

2.3. CYP1A2 and 3A4 enzyme activities in HepaRG cells

CYP1A2 and CYP3A4 enzyme activities in differentiated HepaRG monolayers were determined using luminescence-based P450-Glo™ assay kits supplied by Promega Corporation (Madison, WI, USA) were used following the 48 h exposures. Reagents were prepared according to the manufacturer's instructions. The protocol for the lytic P450-Glo™ assays using cultured cells in monolayers was followed. Cell monolayers were washed twice with phosphate-buffered saline (PBS), then 50 μL of medium containing 3 μM luciferin-IPA (CYP3A4) or PBS (supplemented with 3 mM salicylamide) containing 6 μM luciferin-1A2 was added to all wells and incubated 37 °C, 5% CO_2 for 60 min. Next, an equal volume of luciferin detection reagent was added to the wells and briefly mixed on a

Table 1
PFAS chemicals used in this study. All chemicals were of analytical grade (≥97%) (CAS: Chemical Abstracts Service. IUPAC: International Union of Pure and Applied Chemistry. MW: Molecular Weight (g/mol)).

Preferred name	Abbreviation	CAS	IUPAC	Formula	MW	Provider
Undecafluoro-2-methyl-3-oxahexanoic acid	HFPO-DA	13252–13-6	2,3,3,3-Tetrafluoro-2-(heptafluoropropoxy) propanoic acid	$C_6HF_{11}O_3$	330.05	Synquest Laboratories (2121–3-13)
Perfluorobutanesulfonic acid	PFBS	375–73-5	Nonafluorobutane-1-sulfonic acid	$C_4HF_9O_3S$	300.09	Sigma-Aldrich (562629)
Perfluorohexanoic acid	PFHxA	307–24-4	Undecafluorohexanoic acid	$C_6HF_{11}O_2$	314.05	Sigma-Aldrich (43809)
Perfluorohexanesulfonic acid potassium salt	PFHxS	3871–99-6	1,1,2,2,3,3,4,4,5,5,6,6,6-tridecafluorohexane-1-sulfonate	$C_6HF_{13}KO_3S$	438.20	Sigma-Aldrich (50929)
6:2 Fluorotelomer alcohol	6:2 FTOH	647–42-7	Potassium 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctan-1-ol	$C_8H_5F_{13}O$	364.10	Sigma-Aldrich (370533)
Perfluorooctane sulfonic acid potassium salt	PFOS	2795–39-3	Potassium heptadecafluorooctane-1-sulfonate	$CF_3(CF_2)_7SO_3K$	538.22	Cayman Chemical Company (DRE-C15987122)
Perfluorooctanoic acid	PFOA	335–67-1	Pentadecafluorooctanoic acid	$C_8HF_{15}O_2$	414.07	Alfa Aesar (L08862)

plate shaker to form a lysate. The plate was allowed to equilibrate to room temperature for 15 min, then the luminescence signal (RLU) was read directly from the plates using a Varioskan Lux multimode microplate reader (Thermo Fisher, Waltham, MA). The net signals were calculated by subtracting background luminescence values from background wells (no-cells) from the treatment and solvent control values. The percent change was determined by dividing net treated values by net untreated values and multiplying by 100.

2.4. Human recombinant CYP450 enzyme inhibition assays

The Corning Supersomes™ were prepared by thawing and making a 4X solution in 100 mM potassium phosphate buffer (pH 7.4), with optimized concentrations of CYP protein as specified in Table 2. To prepare test inhibitor solutions (4X), PFAS were dissolved in ACN (MeOH for HFPO-DA) and diluted in 100 mM potassium phosphate buffer (pH 7.4) to obtain a final solvent concentration of 1% v/v in the reaction mixtures. The concentrations used were 1 pM, 100 pM, 10 nM, 1 μM, and 100 μM. The assay was performed by adding 25 μL of the 4X Supersome™ and 25 μL of the 4X test inhibitor solutions to each well of a black 96-well plate. After a 10-minute incubation at room temperature, 50 μL of 100 mM potassium phosphate buffer (pH 7.4) containing the appropriate substrate (Table 2) and NADPH Regenerating System (Promega, Madison, WI, USA) was added to each well to start the reaction. The plate was read immediately in kinetic mode at appropriate wavelengths every minute for 60 min at 37 °C. Each plate included background wells, negative controls (solvent controls), and positive controls (without the NADPH regenerating system). Background fluorescence was corrected with a reading from control blank samples. The corrected fluorescence signals were used to calculate the percent inhibition of the respective CYP isoform. The inhibition experiments were performed independently in triplicate with three technical replicates per plate.

2.5. Statistical analysis

Data were analyzed before statistical analysis to meet the homoscedasticity and normality assumptions. Statistical analyses and graphing were carried out using GraphPad Prism version 9 (GraphPad Software, San Diego, CA). The statistical significance of CYP1A2 and 3A4 enzyme activities in HepaRG cells was assessed using two-way ANOVA tests. Post-hoc analyses were performed using Tukey's multiple comparisons tests. Inhibition data were fitted to a log(inhibitor) vs. response–variable slope model, and the IC_{50} values were obtained from the best-fit values where able to be determined. All values were calculated using the average of the assessed endpoint of the independent experimental results and their associated errors. A p-value of < 0.05 was considered statistically significant unless otherwise indicated.

3. Results

3.1. CYP1A2 activity in HepaRG

The mean CYP1A2 activities (presented as % of the solvent control) following 48 h exposures to the short-chain PFAS are presented in Fig. 1. The two independent variables tested in this experiment were the concentration of PFAS (with 1 nM and 1 μM) and the presence of an inducer (with or without 10 μM BaP). Exposure levels chosen were within environmentally relevant ranges (including for human exposure).

The two-way ANOVA of the CYP1A2 activity of treatments with PFBS (Fig. 1A) demonstrated an overall statistically significant interaction effect between PFBS and the inducer, 10 μM BaP ($p < 0.0001$). However, the main effects of PFBS alone and the presence or absence of 10 μM BaP were not statistically significant ($p = 0.1726$ and $p = 0.1005$, respectively). In addition, Tukey's multiple comparisons tests demonstrated that single exposures to 1 nM PFBS and 1 μM PFBS increased the CYP1A2 activities significantly compared to the solvent control with 205.4% and 204.4%, respectively ($p < 0.0001$). In contrast, treatments with 1 nM PFBS and 1 μM PFBS in binary mixtures with 10 μM BaP were lower than the treatments of 10 μM BaP alone, with activities of 130.9% and

Table 2
Summary of the parameters for the CYP Supersome™ inhibition assays.

Supersome™	Substrate	Vehicle	Substrate concentration (μM)	Metabolite	Ex/Em (nm)	P450 conc. (pmol/mL)	Reference
CYP1A2	7-MR	DMSO	1	Resorufin	530/580	5	(Ghosal et al. 2003) (Turpeinen et al. 2006)
CYP2B6	7-EFC	ACN	2.5	7-HFC	409/530	7.5	(Turpeinen et al. 2006)
CYP2C19	7-BFC	ACN	25	7-HFC	409/530	25	(Turpeinen et al. 2006)
CYP2D6	7-MFC	ACN	100	7-HFC	409/530	15	(Lee et al. 2012) (Turpeinen et al. 2006)
CYP2E1	7-MFC	ACN	100	7-HFC	409/530	15	(Ghosal et al. 2003) (Turpeinen et al. 2006)
CYP3A4	7-BFC	ACN	50	7-HFC	409/530	75	(Turpeinen et al. 2006)

ACN: acetonitrile; DMSO: dimethylsulfoxide; BFC: 7-benzyloxy-4-(trifluoromethyl) coumarin; EFC: 7-ethoxy-4-(trifluoromethyl)coumarin; MFC: 7-methoxy-4-(trifluoromethyl) coumarin; MR: 7-methoxyresorufin; HFC: 7-hydroxy-4-(trifluoromethyl)coumarin.

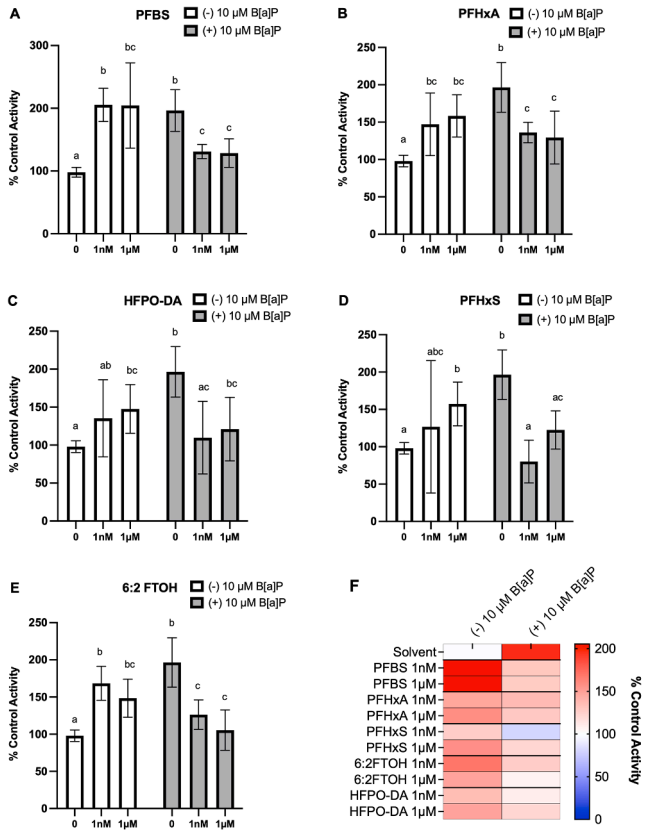

Fig. 1. Bar plots of the CYP1A2 enzyme activities determined using luminescence-based P450-Glo™ assays in differentiated HepaRG monolayers following 48 h exposures to (A) PFBS, (B) PFHxA, (C) HFPO-DA, (D) PFHxS, and (E) 6:2 FTOH at 1 nM and 1 μM and with or without 10 μM BaP). A heatmap summary of the data (presented as % of the solvent control ± standard deviation) is depicted in Fig. 1F. The statistical significance was assessed using two-way ANOVA with Tukey's post-hoc comparisons. Different letter denotes significant differences between exposure groups. A *p*-value of < 0.05 was considered statistically significant.

128.5%, respectively ($p < 0.01$), indicating a significant inhibitory effect of PFBS on the CYP1A2 induction by BaP despite an apparent induction in the single exposures.

The results of treatments with PFHxA (Fig. 1B) also showed a significant interaction effect ($p < 0.0001$), indicating that the effect of PFHxA on CYP1A2 activity depends on the presence of the inducer and vice-versa. In addition, significant differences were observed between the solvent control and single exposures of 1 nM PFHxA ($p = 0.0195$) and 1 μM PFHxA ($p = 0.0023$), resulting in increased CYP1A2 activity. On the other hand, there were no significant differences in mean CYP1A2 activity between solvent control and binary mixture of either 1 nM or 1 μM PFHxA with 10 μM BaP, indicating an inhibitory effect of PFHxA on the CYP1A2 induction by BaP.

While the interaction between HFPO-DA (Fig. 1C) and BaP presence was considered significant ($p < 0.0001$), neither concentration of HFPO-DA nor inducer presence alone had a significant main effect on CYP1A2 activity. However, similar to PFBS and PFHxA, the decreased activity observed in the binary mixtures with BaP indicated an inhibitory effect.

Similar to observations in the exposures with PFBS and PFHxA, the two-way ANOVA analysis revealed that the interaction between PFHxS (Fig. 1D) and inducer presence had a significant effect on CYP1A2 activity ($p < 0.0001$). The main effect of concentration by PFHxS was also significant on CYP1A2 activity ($p = 0.0188$). There were no significant differences between the solvent control and any treatments, including PFHxS ($p > 0.05$). The binary mixtures of 1 nM PFHxS and 1 μM PFHxS with 10 μM BaP were significantly lower than 10 μM BaP alone ($p < 0.05$), with mean activities that were 80.17% and 122.5%, respectively, of the solvent control activity. The decreased activity values in treatments that included the inducer and lack of significance relative to the solvent control indicated an inhibitory effect on CYP1A2 activity.

The statistical analyses for treatments containing 6:2 FTOH (Fig. 1E) revealed a significant interaction effect between 6:2 FTOH and inducer presence on CYP1A2 activity ($p < 0.0001$). The main effect of 6:2 FTOH concentration was also significant ($p = 0.0391$), indicating that 6:2 FTOH significantly affected CYP1A2 activity. The CYP1A2 activities of 1 nM and 1 μM 6:2 FTOH were significantly increased, with means of 168.4% and 148.4% of the solvent control activity ($p < 0.05$). In binary mixtures, the mean CYP1A2 activities were lower, with the main effect of 10 μM BaP not being considered significant, which indicated an inhibition effect by 6:2 FTOH on the CYP1A2 induction by BaP despite an apparent induction in the single exposures.

3.2. CYP3A4 activity in HepaRG

Fig. 2 presents the mean CYP3A4 activities expressed as a percentage of the solvent control after 48 h of exposure to short-chain PFAS. Similar to the CYP1A2 experiments, there were two independent variables: PFAS concentration (1 nM and 1 μM) and inducer presence (with or without 10 μM rifampicin).

For all five of the short-chain PFAS tested, neither 1 nM nor 1 μM treatments of the compounds alone had mean CYP3A4 activity levels that were statistically different from the solvent controls, signifying the short-chain PFAS did not affect the activities in single exposures. However, the mean difference between the (negative) solvent control and 10 μM rifampicin (positive) control was significant in all of the experiments ($p < 0.0001$).

The results of the two-way ANOVA showed that the interaction between PFBS (Fig. 2A) concentration and inducer were not significant, nor was the main effect of concentration. However, the inducer presence had a significant main effect on the response variable ($p < 0.0001$).

The two-way ANOVA of experiments with PFHxA (Fig. 2B) demonstrated that there was a significant effect of the concentration of PFHxA ($p = 0.0013$) and inducer presence ($p < 0.0001$) on CYP3A4 activity. However, there was no significant interaction between the two factors ($p > 0.05$), suggesting PFHxA did not affect CYP3A4 activity in the binary mixtures.

No significant interaction effects were observed in exposures that included HFPO-DA (Fig. 2C), indicating that HFPO-DA did not influence the CYP3A4 activity.

Experiments with PFHxS (Fig. 2D) had significant interaction effects between both concentration and inducer presence ($p < 0.0001$). The main effects of PFAS concentration and inducer presence were also considered significant ($p < 0.0001$). Both 1 nM and 1 μM mixtures of PFHxS with rifampicin had CYP3A4 activity significantly lower relative to rifampicin alone ($p < 0.05$), indicative of an inhibitory effect by PFHxS on the expected induction by rifampicin.

There were no statistically significant differences between the solvent control and the treatments with 6:2 FTOH alone (Fig. 2E), additionally, the binary mixtures of 6:2 FTOH and rifampicin were not statistically different from the inducer nor the solvent control.

3.3. Inhibition of human recombinant CYP450 enzymes by PFAS

Inhibition experiments with the selected Supersomes™ were conducted over a range of five concentrations for each PFAS (1 pM, 100 pM, 10 nM, 1 μM, and 100 μM) to determine the concentration of PFAS that resulted in 50% inhibition of the solvent control fluorescence signal of the probe metabolite (expressed as IC_{50}). The mean IC_{50} values and associated confidence intervals of three independent experiments evaluating the effects of seven PFAS on CYP1A2, −2B6, −2C19, −2E1, and −3A4 are presented in Table 3.

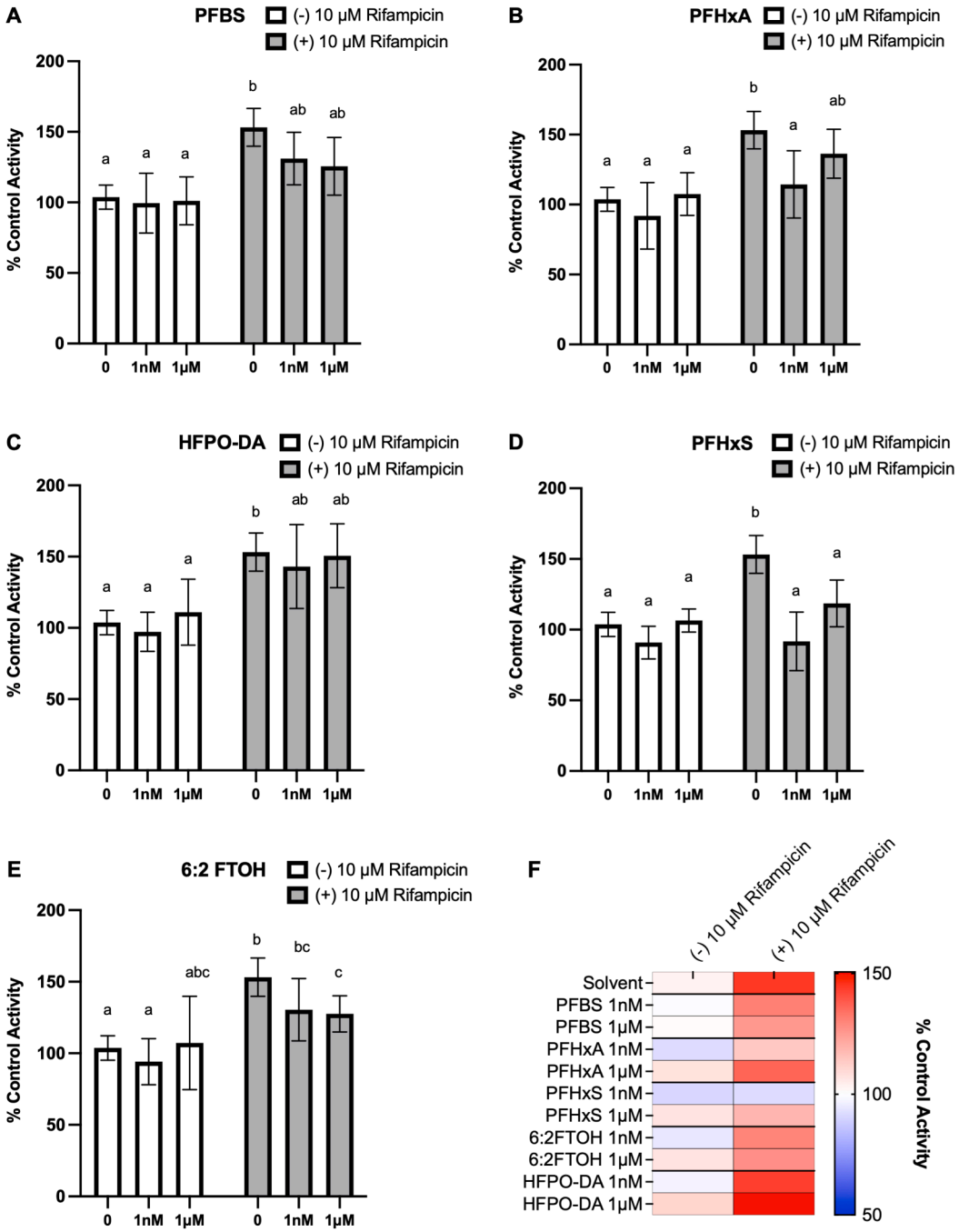

Fig. 2. Bar plots of the CYP3A4 enzyme activities determined using luminescence-based P450-Glo™ assays in differentiated HepaRG monolayers following 48 h exposures to (A) PFBS, (B) PFHxA, (C) HFPO-DA, (D) PFHxS, and (E) 6:2 FTOH at 1 nM and 1 μM and with or without 10 μM rifampicin). A heatmap summary of the data (presented as % of the solvent control ± standard deviation) is depicted in Fig. 2F. The statistical significance was assessed using two-way ANOVA with Tukey's post-hoc comparisons. Different letter denotes significant differences between exposure groups. A *p*-value of < 0.05 was considered statistically significant.

None of the PFAS tested (PFBS, PFHxA, HFPO-DA, PFHxS, 6:2 FTOH, PFOA, and PFOS) significantly inhibited CYP2E1 within the concentration range tested. Of the short-chain PFAS tested, only PFHxS resulted in inhibition that could be confidently determined for the CYP enzymes evaluated. The IC_{50} values (95% CI) determined for PFHxS were 13.8 (5.77–33.1) μM and 51.6 (27.5–96.9) μM for CYP1A2 and CYP2C19, respectively.

Two legacy long-chain PFAS were also assessed, PFOA and PFOS. Only CYP2B6 was inhibited by PFOA, with a mean IC_{50} of 96.9 (68.7–137) μM. Experiments with PFOS, however, demonstrated quantifiable inhibition of CYP1A2, −2B6, −2C19, and −3A4 activities. The lowest IC_{50} for experiments with PFOS was for CYP3A4, with a mean IC_{50} of 23.3 (14.6–37.2) μM, and the highest was CYP2C19 with a mean IC_{50} of 46.6 (14.4–150) μM.

4. Discussion

This study investigated the effects of short-chain PFAS on the activities of CYP1A2 and CYP3A4 in HepaRG monolayers following 48 h exposures of PFBS, PFHxA, HFPO-DA, PFHxS, 6:2 FTOH at 1 nM and 1 μM, with and without an inducer (BaP for CYP1A2 and rifampicin for CYP3A4). Additionally, recombinant CYP1A2, −2B6, −2C19, −2E1, and

Table 3
IC_{50} values for experiments determining inhibition of human recombinant CYPs by PFAS; data presented as IC_{50} values and the associated 95% CI. (n = 3). Daggers (†) denote treatments that did not result in >50% inhibition within the concentration tested.

	IC50 (μM)				
PFAS	CYP1A2	CYP2B6	CYP2C19	CYP2E1	CYP3A4
PFBS	†	†	†	†	†
PFHxA	†	†	†	†	†
HFPO-DA	†	†	†	†	†
PFHxS	13.8 (5.77–33.1)	†	51.6 (27.5–96.9)	†	†
6:2 FTOH	†	†	†	†	†
PFOA	†	96.9 (68.7–137)	†	†	†
PFOS	25.7 (4.69–141)	30.6 (22.7–41.2)	46.6 (14.4–150)	†	23.3 (14.6–37.2)

−3A4 were utilized to assess the potential of PFAS to directly inhibit the catalytic activity of CYP450 enzymes in an isolated system. Combining cell-based measurements and recombinant enzyme assays facilitates a more complete picture of PFAS effects on CYP450 enzymes by providing insight into enzyme-targeted inhibition and off-target effects (e.g., possible nuclear receptor interactions) indicated by activity in the HepaRG cells.

Inhibition experiments with the human recombinant CYP450 enzymes indicate that most of the PFAS tested did not significantly inhibit CYP1A2, −2B6, −2C19, −2E1, and −3A4 up to 100 μM. However, PFHxS showed significant inhibition of both CYP1A2 and CYP2C19. The long-chain PFAS, PFOA, and PFOS demonstrated inhibition of CYP2B6 and CYP1A2, −2B6, −2C19, and −3A4 for PFOS. These findings suggest that short-chain PFAS may not significantly impact CYP enzyme activity, while long-chain PFAS may have more potent inhibitory effects. The inhibition of the human recombinant CYP450 enzymes supports previous findings by Franco et al. (2020) that found PFOS and, to some extent, PFOA are inhibitors of CYP1A2.

Similar to the approach we used with the Supersomes™, Amstutz et al. (2022) used Vivid® CYP2E1, CYP2D6, CYP2C19, and CYP3A4 screening kits to assess the inhibitory potential of several PFAS on CYP450 BACULOSOMES®. Our results were mostly consistent with their findings for PFBS, PFHxA, PFHxS, and 6:2 FTOH. However, the PFOS IC_{50} we derived for CYP3A4 was roughly 30 times lower than the value given by Amstutz et al. (2022). This discrepancy could be due to differences in experimental approach and possible differences in sensitivity of Supersomes™ and BACULOSOMES®. Regardless, the concentrations reported to result in significant CYP3A4 inhibition here, and in previous studies are both higher than the average quantified PFOS serum concentration (4.7 μg/L or 8.7 nM) for the U.S. population from the National Health and Nutrition Examination Survey (2011–2018) (NHANES 2018).

Recombinant CYP450 enzymes are suitable for studying inhibition involving binding site interactions but not for evaluating other endpoints associated with CYP450 metabolism, such as induction or inhibition that may result from nuclear receptor interactions that regulate the enzyme expression. Cell-based CYP450 activity assays, on the other hand, may also use a probe substrate metabolism. However, the changes in activity can indicate potential interactions of xenobiotics with other cellular components, including the receptor responsible for enzyme synthesis. This is highlighted by the findings of the PFBS, PFHxA, and 6:2 FTOH exposures that resulted in increased CYP1A2 activity in the HepaRG cells that is suggestive of induction – a mechanism which is typically modulated through the aryl hydrocarbon receptor (AhR) (Guo et al. 2021).

Additionally, the significant relevance of PFBS, PFHxA and 6:2 FTOH inducing CYP1A2 expression more prominently than BaP lies in the potential implications for human health and environmental risk assessment. CYP1A2-mediated metabolism is a vital determinant of drug efficacy, toxicity, and the activation or detoxification of environmental chemicals. If PFAS compounds induce CYP1A2 more robustly than BaP, it implies that PFAS may modulate the metabolism of co-administered drugs and co-exposed environmental pollutants differently. This discrepancy in enzyme induction could potentially influence the pharmacokinetics, efficacy, and toxicity of various therapeutic agents and enhance the risk of adverse health outcomes in exposed individuals.

Despite the prototypical activator of AhR being polycyclic aromatic hydrocarbons (PAHs), like BaP, bioactivity profiling of PFAS has suggested that some PFAS may also be capable of indirect AhR activation (Houck et al. 2021). Over the last decade, several studies have presented evidence suggesting that planar geometry and aromatic/heteroaromatic rings are not necessary for AhR agonism (Dolciami et al. 2020; Endirlik et al. 2023; Guo et al. 2021). Furthermore, AhR is also known to participate in cellular redox balance and act as a modulator of redox signaling (Grishanova and Perepechaeva 2022). We have previously demonstrated that PFBS, PFHxA, and 6:2 FTOH are associated with biomarkers of oxidative stress at the same concentrations tested here (Solan et al. 2023).

Few studies on the effect of PFAS on the AhR function have been reported (Hu et al. 2003; Liu et al. 2008; Watanabe et al. 2009). Specifically, the lack of direct transactivation of AhR by long-chain PFAS has been observed in the mouse hepatoma cell line Hepa1.12cR cells, as noted by Long et al. (2013). Two potential explanations for the observed activation of AhR by short-chain PFAS are proposed: a) short-chain PFAS may exhibit different modes of action compared to long-chain PFAS, or b) short-chain PFAS may activate AhR through an indirect mechanism. Although the chemical behavior of PFAS, including solubility, is influenced by the length of their chains, no distinct effects or mechanisms of action have been described (Mokra 2021). The second explanation appears more plausible, as certain PFAS have been shown to indirectly activate AhR as an alternative to direct ligand-binding mechanisms, as described by Houck et al. (2021).

It is noteworthy that the induction of CYP1A2 may not solely be attributed to AhR (aryl hydrocarbon receptor) activation. Research has shown that other nuclear receptors, such as liver X receptor alpha (LXRα), can also stimulate the expression of CYP1A2 in hepatocytes (Araki et al. 2012). Moreover, while peroxisome proliferator-activated receptors (PPARs) have primarily been associated with sensing lipid metabolism, it has been demonstrated that PPARα also regulates several genes involved in biotransformation. Specifically, PPARα plays a role in the transcriptional control of CYP4, which is crucial for the metabolism of biologically significant compounds like fatty acids. In human hepatocytes, PPARα activation leads to the expression of various members of the CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, CYP2J, and CYP3A subfamilies, as well as certain conjugating enzymes (e.g., EPHX2, GSTA, and UGT1A9) (Cizkova et al. 2012; Rakhshandehroo et al. 2010).

It should be emphasized that assessing the impact of 6:2 FTOH (perfluorohexanoic acid) is highly intricate due to its demonstrated rapid metabolism into PFHxA (perfluorohexanoic acid), 5:3 fluorotelomer carboxylic acid, and PFHpA (perfluoroheptanoic acid) (Kabadi et al. 2018; Rice et al. 2020).

The results of the exposures of HepaRG cells to PFAS mixtures with an inducer yielded surprising results. The results showed that at 1 nM and 1 μM, PFBS, PFHxA, and 6:2 FTOH exposures increased CYP1A2

activity when tested alone but decreased CYP1A2 activity when tested in combination with the inducer BaP. In contrast, neither concentration of HFPO-DA nor PFHxS had a significant effect on CYP1A2 activity alone, but in the presence of the CYP1A2 inducer, BaP, significant decreases in CYP1A2 activity were also observed.

Exposure of cells to BaP results in complex transcription responses that involve multiple cellular signaling cascades, many of which are cell-specific (Hockley et al. 2007). The decrease in CYP1A2 activity observed in the binary mixtures of the PFAS with BaP may have resulted from the rapid biotransformation of this AhR ligand; however, AhR-mediated transcriptional repression is another possibility. When this occurs, AhR can act as a transcriptional repressor by modifying chromatin structures and preventing the binding of transcriptional activators, inhibiting gene expression (Safe 1995). However, further studies employing transcriptional data would need to be used to elucidate possible mechanisms of the activity further decreases here.

The results of the CYP3A4 activity measurements in the HepaRG were also unexpected. None of the five short-chain PFAS tested significantly affected CYP3A4 activity when tested alone at 1 nM and 1 μM alone. However, the PFBS exposures resulted in a concentration-dependent decrease of CYP3A4 activity when exposed to the inducer, with effects only being significant at 1 μM. In addition, significant decreases in CYP3A4 activity were observed only in cells exposed to lower (1 nM) concentrations of PFHxA with rifampicin but not in those exposed to the higher (1 μM) concentrations of PFHxA with rifampicin. The findings also showed no significant interaction effects in exposures that included HFPO-DA, indicating that this compound did not influence CYP3A4 activity.

Regulation and expression of the CYP3A4 enzyme occur through the pregnane X receptor (PXR) (Rashidian et al. 2022). Behr et al. (2020) used luciferase-based reporter gene assays to determine the capacity of several PFAS (including PFOA, PFOS, PFBS, PFHxA, and PFHxS) to activate human nuclear receptors and determined that PXR, among several others, were not affected by PFAS. These findings are supported by the lack of CYP3A4 activity in the exposures conducted with PFAS alone. Furthermore, because PFAS are not known to be PXR agonists, the decreased activity of CYP3A4 seen in mixtures of PFHxS and PFHxA with the CYP3A4 inducer rifampicin is likely not the rapid clearance of the agonist that has been observed when co-administered with CYP3A4 inducers (Kapetas et al. 2019).

While the activation of PXR by ligands such as rifampicin is well established, the mechanism behind ligand-mediated inhibitory or antagonistic effects on PXR is not as well understood. The bioactivity profiling conducted by Houck et al. (2021) and evaluations of bioactivity profiling of nuclear receptors by Behr et al. (2020) both employed the HepG2 cell line, which has low expression of PXR and constitutive androstane receptor (CAR) (Gerets et al. 2012). While the *in vitro* studies with HepG2 indicated that CAR was not affected by PFAS, studies in mice indicated that PFHxS activated CAR despite having little effect on PXR (Kublbeck et al. 2020; Oshida et al. 2015). CAR activation by PFAS in HepaRG may have resulted in marked inhibition of CYP3A4 activity in the presence of an inducer via recruitment of corepressors to the CYP3A4 promoter or induction of drug-metabolizing enzymes, for example, CYP2B6 (Olack et al. 2022). Further studies exploring the effects of PFAS on other clinically relevant biotransformation enzyme activities and their expression profiles may give insight into the possibility of crosstalk and modulation in these complex exposure scenarios.

Understanding the effects of PFAS on CYP450 enzymes may help elucidate the mechanisms of hepatoxicity by PFAS. We have previously demonstrated that exposures to the short-chain PFAS studied here are associated with biomarkers of oxidative stress at the same concentrations tested here (Solan et al. 2023). The properties of the heme group in CYP450 enzymes that are adequately expressed in HepaRG cells can facilitate ROS generation through reaction uncoupling or via reactive intermediates which modify endogenous substrates, including lipids, proteins, and nucleic acids, leading to oxidative stress (Veith and Moorthy 2018).

Alterations to the expression and activity of CYP have also been implicated in the etiology of inflammatory diseases, such as cancer (Stipp and Acco 2021). The Adverse Outcome Pathways (AOPs) framework can be used to understand the relationship between CYP450 enzymes, oxidative stress, and cytotoxicity implicated in the carcinogenic of potential PFAS. AOPs are a conceptual framework that links information from molecular initiating events and intervening key events to an adverse outcome at higher levels of biological organization (Ankley et al. 2010). For example, modulation of CYP450 enzymes and the production of reactive oxygen species resulting in cytotoxicity in hepatocytes have been established as an AOP leading to liver tumor formation (https://aopwiki.org/aops/220).

Additionally, information on alterations to biotransformation enzymes can provide insight into the possibility of PFAS exposure causing disruptions to endogenous functions and drug metabolism by CYP450 enzymes. CYP enzymes play a crucial role in the metabolism of xenobiotics, and changes in their activity or expression can affect the toxicity and efficacy of pharmaceuticals (Rey-Bedon et al. 2022). Disruptions to biotransformation pathways can also lead to dysfunctions in intracellular processes created by an inability to regulate fatty acid metabolism and the accumulation of unmetabolized harmful substrates (Deodhar et al. 2020). While CYP450 enzymes play a critical role in biotransformation, many other enzymes are involved. To fully understand the impact of PFAS on drug-metabolizing enzymes, it will be necessary to comprehensively profile other biotransformation enzymes, such as UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), and glutathione-*S*-transferases (GSTs). Future studies should consider including these and the other major CYP450 enzymes.

5. Conclusions

This study highlights the importance of considering the interactions between PFAS and other chemicals in assessing their effects on drug metabolism and toxicity. The findings here suggest that short-chain PFAS can modulate CYP1A2 activity in a complex manner, with some compounds acting as inducers and others as inhibitors. Except for PFHxS, the short-chain PFAS appear to have little effect on CYP3A4 activity. The study also highlights the limitations of recombinant CYP450 enzymes for evaluating other endpoints associated with CYP450 metabolism, such as induction or inhibition that may result from binding to the receptor that regulates the enzyme's activity. PFAS-mediated alterations to biotransformation enzymes can affect drug metabolism and disrupt cellular processes. CYP450 enzymes are necessary for both endogenous and xenobiotic metabolism. While CYP450 enzymes are crucial, other enzymes like UGTs, SULTs, and GSTs should also be studied to fully understand the effects of PFAS on drug-metabolizing enzymes. For many pollutants, including PFAS, understanding how exposure affects CYP450 enzymes can help develop more effective strategies for managing the risks associated with chemical exposure, allowing for more informed regulatory decisions.

6. Availability of data and materials

The data that support the findings of this study are available from the corresponding author, RL, upon request.

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CRediT authorship contribution statement

Megan E. Solan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft,

Writing – review & editing, Visualization. **Ramon Lavado:** Conceptualization, Methodology, Validation, Investigation, Supervision, Funding acquisition, Data curation, Writing – original draft, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

Amstutz, V.H., Cengo, A., Sijm, D., Vrolijk, M.F., 2022. The impact of legacy and novel perfluoroalkyl substances on human cytochrome P450: an *in vitro* study on the inhibitory potential and underlying mechanisms. Toxicology 468, 153116. https://doi.org/10.1016/j.tox.2022.153116.

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J. E., Villeneuve, D.L., 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. 29, 730–741. https://doi.org/10.1002/etc.34.

Araki, K., Watanabe, K., Yamazoe, Y., Yoshinari, K., 2012. Liver X receptor α bidirectionally transactivates human CYP1A1 and CYP1A2 through two cis-elements common to both genes. Toxicol. Lett. 215, 16–24. https://doi.org/10.1016/j.toxlet.2012.09.021.

Bangma, J., Szilagyi, J., Blake, B.E., Plazas, C., Kepper, S., Fenton, S.E., 2020. An assessment of serum-dependent impacts on intracellular accumulation and genomic response of per- and polyfluoroalkyl substances in a placental trophoblast model. Environ. Toxicol. 35, 1395–1405. https://doi.org/10.1002/tox.23004.

Behr, A.C., Plinsch, C., Braeuning, A., Buhrke, T., 2020. Activation of human nuclear receptors by perfluoroalkylated substances (PFAS). Toxicol. In Vitro 62, 104700. https://doi.org/10.1016/j.tiv.2019.104700.

Brendel, S., Fetter, E., Staude, C., Vierke, L., Biegel-Engler, A., 2018. Short-chain perfluoroalkyl acids: environmental concerns and a regulatory strategy under REACH. Environ. Sci. Eur. 30, 9. https://doi.org/10.1186/s12302-018-0134-4.

Buick, J.K., Williams, A., Meier, M.J., Swartz, C.D., Recio, L., Gagné, R., Ferguson, S.S., Engelward, B.P., Yauk, C.L., 2021. A modern genotoxicity testing paradigm: integration of the high-throughput CometChip® and the TGx-DDI transcriptomic biomarker in human HepaRGTM cell cultures. Front. Public Health 9, 694834. https://doi.org/10.3389/fpubh.2021.694834.

Chattopadhyay, N., Kanacher, T., Casjens, M., Frechen, S., Ligges, S., Zimmermann, T., Rottmann, A., Ploeger, B., Höchel, J., Schultze-Mosgau, M.H., 2018. CYP3A4-mediated effects of rifampicin on the pharmacokinetics of vilaprisan and its UGT1A1-mediated effects on bilirubin glucuronidation in humans. Br. J. Clin. Pharmacol. 84, 2857–2866. https://doi.org/10.1111/bcp.13750.

Cizkova, K., Konieczna, A., Erdosova, B., Lichnovska, R. and Ehrmann, J., 2012. Peroxisome proliferator-activated receptors in regulation of cytochromes P450: new way to overcome multidrug resistance? J. Biomed. Biotechnol. 2012, 656428. https://doi.org/10.1155/2012/656428.

Coleman, M., 2010. How oxidative systems metabolize substrates. Wiley, Human Drug Metabolism, pp. 23–64.

Dale, K., Yadetie, F., Horvli, T., Zhang, X., Froysa, H.G., Karlsen, O.A., Goksoyr, A., 2022. Single PFAS and PFAS mixtures affect nuclear receptor- and oxidative stress-related pathways in precision-cut liver slices of Atlantic cod (*Gadus morhua*). Sci. Total Environ. 814, 152732 https://doi.org/10.1016/j.scitotenv.2021.152732.

De Silva, A.O., Armitage, J.M., Bruton, T.A., Dassuncao, C., Heiger-Bernays, W., Hu, X.C., Karrman, A., Kelly, B., Ng, C., Robuck, A., Sun, M., Webster, T.F., Sunderland, E.M., 2021. PFAS exposure pathways for humans and wildlife: a synthesis of current knowledge and key gaps in understanding. Environ. Toxicol. Chem. 40, 631–657. https://doi.org/10.1002/etc.4935.

DeLuca, N.M., Minucci, J.M., Mullikin, A., Slover, R., Cohen Hubal, E.A., 2022. Human exposure pathways to poly- and perfluoroalkyl substances (PFAS) from indoor media: a systematic review. Environ. Int. 162, 107149 https://doi.org/10.1016/j.envint.2022.107149.

Deodhar, M., Al Rihani, S.B., Arwood, M.J., Darakjian, L., Dow, P., Turgeon, J., Michaud, V., 2020. Mechanisms of CYP450 inhibition: understanding drug-drug interactions due to mechanism-based inhibition in clinical practice. Pharmaceutics 12, 846. https://doi.org/10.3390/pharmaceutics12090846.

Dolciami, D., Ballarotto, M., Gargaro, M., Lopez-Cara, L.C., Fallarino, F., Macchiarulo, A., 2020. Targeting Aryl hydrocarbon receptor for next-generation immunotherapies: selective modulators (SAhRMs) versus rapidly metabolized ligands (RMAhRLs). Eur. J. Med. Chem. 185, 111842 https://doi.org/10.1016/j.ejmech.2019.111842.

East, A., Egeghy, P.P., Hubal, E.A.C., Slover, R., Vallero, D.A., 2023. Computational estimates of daily aggregate exposure to PFOA/PFOS from 2011 to 2017 using a basic intake model. J. Expo. Sci. Environ. Epidemiol. 33, 56–68. https://doi.org/10.1038/s41370-021-00374-w.

Endirlik, B.U., Wincent, E., Dreij, K., 2023. Non-additive mixture effects of benzo[a]pyrene and pesticides *in vitro* and *in vivo*: role of AhR signaling. Environ. Pollut. 316, 120510 https://doi.org/10.1016/j.envpol.2022.120510.

Franco, M.E., Sutherland, G.E., Fernandez-Luna, M.T., Lavado, R., 2020. Altered expression and activity of phase I and II biotransformation enzymes in human liver cells by perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS). Toxicology 430, 152339. https://doi.org/10.1016/j.tox.2019.152339.

Franzosa, J.A., Bonzo, J.A., Jack, J., Baker, N.C., Kothiya, P., Witek, R.P., Hurban, P., Siferd, S., Hester, S., Shah, I., Ferguson, S.S., Houck, K.A., Wambaugh, J.F., 2021. High-throughput toxicogenomic screening of chemicals in the environment using metabolically competent hepatic cell cultures. npj Syst. Biol. Appl. 7 https://doi.org/10.1038/s41540-020-00166-2.

Garrett, K.K., Brown, P., Varshavsky, J., Cordner, A., 2022. Improving governance of "forever chemicals" in the US and beyond. One Earth 5, 1075–1079. https://doi.org/10.1016/j.oneear.2022.10.003.

Gerets, H.H., Tilmant, K., Gerin, B., Chanteux, H., Depelchin, B.O., Dhalluin, S., Atienzar, F.A., 2012. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. Cell Biol. Toxicol. 28, 69–87. https://doi.org/10.1007/s10565-011-9208-4.

Ghosal, A., Hapangama, N., Yuan, Y., Lu, X., Horne, D., Patrick, J.E., Zbaida, S., 2003. Rapid determination of enzyme activities of recombinant human cytochromes P450, human liver microsomes and hepatocytes. Biopharm. Drug Dispos. 24, 375–384. https://doi.org/10.1002/bdd.374.

Grishanova, A.Y., Perepechaeva, M.L., 2022. Aryl hydrocarbon receptor in oxidative stress as a double agent and its biological and therapeutic significance. Int. J. Mol. Sci. 23, 6719. https://doi.org/10.3390/ijms23126719.

Guengerich, F.P., 2008. Cytochrome P450 and chemical toxicology. Chem. Res. Toxicol. 21, 70–83. https://doi.org/10.1021/tx700079z.

Guengerich, F.P. 2022. Chapter One - Roles of cytochrome P450 enzymes in pharmacology and toxicology: Past, present, and future. In: H. Yamazaki (Ed), Advances in Pharmacology, Academic Press pp. 1-47.

Guo, J., Zhu, X., Badawy, S., Ihsan, A., Liu, Z., Xie, C., Wang, X., 2021. Metabolism and mechanism of human cytochrome P450 enzyme 1A2. Curr. Drug Metab. 22, 40–49. https://doi.org/10.2174/1389200221999210101233135.

Hockley, S.L., Arlt, V.M., Brewer, D., te Poele, R., Workman, P., Giddings, I., Phillips, D. H., 2007. AHR-and DNA-damage-mediated gene expression responses induced by benzo(a)pyrene in human cell lines. Chem. Res. Toxicol. 20 (12), 1797–1810.

Houck, K.A., Patlewicz, G., Richard, A.M., Williams, A.J., Shobair, M.A., Smeltz, M., Clifton, M.S., Wetmore, B., Medvedev, A., Makarov, S., 2021. Bioactivity profiling of per- and polyfluoroalkyl substances (PFAS) identifies potential toxicity pathways related to molecular structure. Toxicology 457, 152789. https://doi.org/10.1016/j.tox.2021.152789.

Hu, W., Jones, P.D., DeCoen, W., King, L., Fraker, P., Newsted, J., Giesy, J.P., 2003. Alterations in cell membrane properties caused by perfluorinated compounds. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 135, 77–88. https://doi.org/10.1016/s1532-0456(03)00043-7.

Jennen, D.G., Magkoufopoulou, C., Ketelslegers, H.B., van Herwijnen, M.H., Kleinjans, J. C., van Delft, J.H., 2010. Comparison of HepG2 and HepaRG by whole-genome gene expression analysis for the purpose of chemical hazard identification. Toxicol. Sci. 115, 66–79. https://doi.org/10.1093/toxsci/kfq026.

Kabadi, S.V., Fisher, J., Aungst, J., Rice, P., 2018. Internal exposure-based pharmacokinetic evaluation of potential for biopersistence of 6:2 fluorotelomer alcohol (FTOH) and its metabolites. Food Chem. Toxicol. 112, 375–382. https://doi.org/10.1016/j.fct.2018.01.012.

Kapetas, A.J., Sorich, M.J., Rodrigues, A.D., Rowland, A., 2019. Guidance for rifampin and midazolam dosing protocols to study intestinal and hepatic cytochrome P450 (CYP) 3A4 induction and de-induction. AAPS J. 21, 1–11. https://doi.org/10.1208/s12248-019-0341-y.

Kemper, R.A., Nabb, D.L., 2005. *In vitro* studies in microsomes from rat and human liver, kidney, and intestine suggest that perfluorooctanoic acid is not a substrate for microsomal UDP-glucuronosyltransferases. Drug Chem. Toxicol. 28, 281–287. https://doi.org/10.1081/dct-200064468.

Kublbeck, J., Niskanen, J., Honkakoski, P., 2020. Metabolism-disrupting chemicals and the constitutive androstane receptor CAR. Cells 9, 2306. https://doi.org/10.3390/cells9102306.

Lee, W., Woo, E.R., Choi, J.S., 2012. Effects of myricetin on the bioavailability of carvedilol in rats. Pharm. Biol. 50, 516–522. https://doi.org/10.3109/13880209.2011.611141.

Liberatore, H.K., Jackson, S.R., Strynar, M.J., Mccord, J.P., 2020. Solvent suitability for HFPO-DA ("GenX" parent acid) in toxicological studies. Environ. Sci. Technol. Lett. 7, 477–481. https://doi.org/10.1021/acs.estlett.0c00323.

Liu, Y., Wang, J., Wei, Y., Zhang, H., Liu, Y., Dai, J., 2008. Molecular characterization of cytochrome P450 1A and 3A and the effects of perfluorooctanoic acid on their mRNA levels in rare minnow (*Gobiocypris rarus*) gills. Aquat. Toxicol. 88, 183–190. https://doi.org/10.1016/j.aquatox.2008.04.008.

Long, M., Ghisari, M., Bonefeld-Jørgensen, E.C., 2013. Effects of perfluoroalkyl acids on the function of the thyroid hormone and the aryl hydrocarbon receptor. Environ. Sci. Pollut. Res. Int. 20, 8045–8056. https://doi.org/10.1007/s11356-013-1628-7.

Mokra, K., 2021. Endocrine disruptor potential of short- and long-chain perfluoroalkyl substances (PFASs)-a synthesis of current knowledge with proposal of molecular mechanism. Int. J. Mol. Sci. 22 (4), 2148.

NHANES, 2018. Serum Perfluorooctane sulfonic acid (PFOS) (2011 - 2018). National Health and Nutrition Examination Survey https://www.cdc.gov/exposurereport/data_tables.html.

Ojo, A.F., Xia, Q., Peng, C., Ng, J.C., 2021. Evaluation of the individual and combined toxicity of perfluoroalkyl substances to human liver cells using biomarkers of oxidative stress. Chemosphere 281, 130808. https://doi.org/10.1016/j.chemosphere.2021.130808.

Olack, E.M., Heintz, M.M., Baldwin, W.S., 2022. Dataset of endo-and xenobiotic inhibition of CYP2B6: comparison to CYP3A4. Data Brief 41, 108013. https://doi.org/10.1016/j.dib.2022.108013.

Olsen, G.W., Burris, J.M., Ehresman, D.J., Froehlich, J.W., Seacat, A.M., Butenhoff, J.L., Zobel, L.R., 2007. Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ. Health Perspect. 115, 1298–1305. https://doi.org/10.1289/ehp.10009.

Oshida, K., Vasani, N., Jones, C., Moore, T., Hester, S., Nesnow, S., Auerbach, S., Geter, D.R., Aleksunes, L.M., Thomas, R.S., Applegate, D., Klaassen, C.D. and Corton, J.C., 2015. Identification of chemical modulators of the constitutive activated receptor (CAR) in a gene expression compendium. Nucl. Recept. Signal. 13, e002. https://doi.org/10.1621/nrs.13002.

Rakhshandehroo, M., Knoch, B., Müller, M., Kersten, S., 2010. Peroxisome proliferator-activated receptor alpha target genes. PPAR Res 2010, 1–20.

Rashidian, A., Mustonen, E.K., Kronenberger, T., Schwab, M., Burk, O., Laufer, S.A., Pantsar, T., 2022. Discrepancy in interactions and conformational dynamics of pregnane X receptor (PXR) bound to an agonist and a novel competitive antagonist. Comput. Struct. Biotechnol. J. 20, 3004–3018. https://doi.org/10.1016/j.csbj.2022.06.020.

Rey-Bedon, C., Banik, P., Gokaltun, A., Hofheinz, O., Yarmush, M.L., Uygun, M.K., Usta, O.B., 2022. CYP450 drug inducibility in NAFLD via an *in vitro* hepatic model: understanding drug-drug interactions in the fatty liver. Biomed. Pharmacother. 146, 112377 https://doi.org/10.1016/j.biopha.2021.112377.

Rice, P.A., Aungst, J., Cooper, J., Bandele, O., Kabadi, S.V., 2020. Comparative analysis of the toxicological databases for 6:2 fluorotelomer alcohol (6:2 FTOH) and perfluorohexanoic acid (PFHxA). Food Chem. Toxicol. 138, 111210 https://doi.org/10.1016/j.fct.2020.111210.

Safe, S.H., 1995. Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds. Pharmacol. Ther. 67, 247–281. https://doi.org/10.1016/0163-7258(95)00017-B.

Skolik, R.A., Solocinski, J., Konkle, M.E., Chakraborty, N. and Menze, M.A., 2021. Global changes to HepG2 cell metabolism in response to galactose treatment. Am. J. Physiol. Cell Physiol. 320, C778-C793. https://doi.org/10.1152/ajpcell.00460.2020.

Solan, M.E., Senthilkumar, S., Aquino, G.V., Bruce, E.D., Lavado, R., 2022. Comparative cytotoxicity of seven per- and polyfluoroalkyl substances (PFAS) in six human cell lines. Toxicology 477, 153281. https://doi.org/10.1016/j.tox.2022.153281.

Solan, M.E., Koperski, C.P., Senthilkumar, S., Lavado, R., 2023. Short-chain per- and polyfluoralkyl substances (PFAS) effects on oxidative stress biomarkers in human liver, kidney, muscle, and microglia cell lines. Environ. Res. 223, 115424 https://doi.org/10.1016/j.envres.2023.115424.

Stipp, M.C., Acco, A., 2021. Involvement of cytochrome P450 enzymes in inflammation and cancer: a review. Cancer Chemother. Pharmacol. 87, 295–309. https://doi.org/10.1007/s00280-020-04181-2.

Sunderland, E.M., Hu, X.C., Dassuncao, C., Tokranov, A.K., Wagner, C.C., Allen, J.G., 2019. A review of the pathways of human exposure to poly- and perfluoroalkyl substances (PFASs) and present understanding of health effects. J. Expo. Sci. Environ. Epidemiol. 29, 131–147. https://doi.org/10.1038/s41370-018-0094-1.

Teaf, C.M., Garber, M.M., Covert, D.J., Tuovila, B.J., 2019. Perfluorooctanoic acid (PFOA): environmental sources, chemistry, toxicology, and potential risks. Soil Sediment Contam. 28, 258–273. https://doi.org/10.1080/15320383.2018.1562420.

Turpeinen, M., Korhonen, L.E., Tolonen, A., Uusitalo, J., Juvonen, R., Raunio, H., Pelkonen, O., 2006. Cytochrome P450 (CYP) inhibition screening: comparison of three tests. Eur. J. Pharm. Sci. 29, 130–138. https://doi.org/10.1016/j.ejps.2006.06.005.

USEPA, 2016. Drinking water health advisory for perfluorooctanoic acid. EPA 822-R-16-005.

Veith, A., Moorthy, B., 2018. Role of cytochrome P450s in the generation and metabolism of reactive oxygen species. Curr. Opin. Toxicol. 7, 44–51. https://doi.org/10.1016/j.cotox.2017.10.003.

Watanabe, M.X., Jones, S.P., Iwata, H., Kim, E.Y., Kennedy, S.W., 2009. Effects of co-exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin and perfluorooctane sulfonate or perfluorooctanoic acid on expression of cytochrome P450 isoforms in chicken (*Gallus gallus*) embryo hepatocyte cultures. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 149, 605–612. https://doi.org/10.1016/j.cbpc.2009.01.001.

Wen, Y., Mirji, N., Irudayaraj, J., 2020. Epigenetic toxicity of PFOA and GenX in HepG2 cells and their role in lipid metabolism. Toxicol. In Vitro 65, 104797. https://doi.org/10.1016/j.tiv.2020.104797.

Wielsoe, M., Long, M., Ghisari, M., Bonefeld-Jorgensen, E.C., 2015. Perfluoroalkylated substances (PFAS) affect oxidative stress biomarkers *in vitro*. Chemosphere 129, 239–245. https://doi.org/10.1016/j.chemosphere.2014.10.014.

Yang, Y.D., Tian, R., Lu, N., 2023. Binding of serum albumin to perfluorooctanoic acid reduced cytotoxicity. Sci. Total Environ. 876, 162738 https://doi.org/10.1016/j.scitotenv.2023.162738.

Zhang, R., Zhang, H., Chen, B., Luan, T., 2020. Fetal bovine serum attenuating perfluorooctanoic acid-inducing toxicity to multiple human cell lines via albumin binding. J. Hazard. Mater. 389, 122109 https://doi.org/10.1016/j.jhazmat.2020.122109.