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Cellular Toxicity Associated with Exposure to Perfluorinated Carboxylates (PFCAs) and Their Metabolic Precursors

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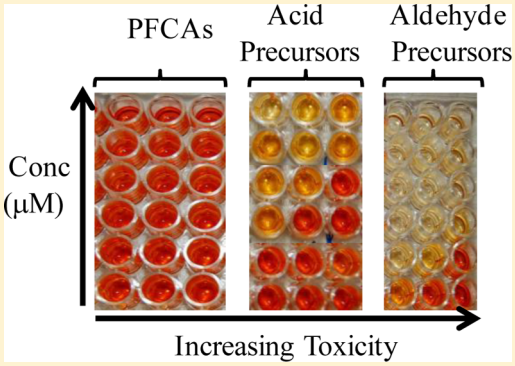
Supporting Information

ABSTRACT: The biotransformation of fluorotelomer based compounds yields saturated and unsaturated fluorotelomer aldehydes (FTALs and FTUALs, respectively) and carboxylic acids (FTCAs and FTUCAs, respectively) as intermediate metabolites that subsequently transform to perfluorinated carboxylic acids (PFCAs). Previous studies have demonstrated that the FTCAs and FTUCAs are 1 to 5 orders of magnitude more toxic than PFCAs after exposure to aquatic organisms. Additionally, FTUALs have demonstrated reactivity with proteins, which may be associated with toxicity through the inhibition of protein function. The purpose of this study was to carry out a comprehensive assessment of the relative toxicity between PFCAs and their intermediate precursor metabolites: the FTALs, FTUALs, FTCAs, and FTUCAs. Analytes were separately incubated with human liver epithelial (THLE-2) cells to assess how varying the functional group and the fluorinated chain length affects cell viability. For each analyte, dose–response EC_{50} values were calculated. The EC_{50} values for FTUCAs and FTCAs were similar, with values ranging from 22 ± 9 and 24 ± 9 μM for the 10:2 congeners to 1004 ± 20 and 1004 ± 24 μM for the 4:2 congeners, respectively. The EC_{50} values for the PFCAs ranged from 65 ± 41 (PFDA) to 1361 ± 146 (PFBA) μM. The range of toxicity between PFCAs and their acid precursors were similar. However, the comparative toxicity between the 6:2 and 8:2 congeners and their corresponding PFCA had toxicity thresholds that varied depending on the functional headgroup, where FTUALs ≥ FTALs > FTUCAs ≥ FTCAs > PFCAs. For all PFCAs and acid precursors, toxicity depended on the length of the fluorinated chain, where the longer chain lengths yielded greater bioaccumulation and enhanced toxicity, results which agreed with those previously reported. By contrast, FTALs and FTUALs were the most toxic of all the analytes examined, where toxicity was enhanced at shorter chain lengths, with EC_{50} values of 7 ± 1 μM (6:2 FTUAL) and 8.6 ± 0.8 μM (6:2 FTAL). DNA adducts were not detectable for the aldehyde precursors, using a quantitative long-range PCR method. Our data provide the first evidence that aldehyde intermediates have demonstrated toxicity in cellular systems that is more significant than PFCAs and their corresponding acid intermediates.



INTRODUCTION

Human use of per- and polyfluorinated substances (PFAS) has resulted in global human exposure, where classes of PFAS are found at low μg/L concentrations in human sera.[1−4] One class of PFAS is the polyfluoroalkyl phosphate esters (PAPs), which are used as commercial grease-repellent surfactants to coat food-contact papers. PAPs, specifically the polyfluoroalkyl phosphate diesters (diPAPs), have been observed to migrate from food contact material to food, serving as a potential source of contamination for humans.[5−7] Evidence of diPAP congeners in human sera have been recently observed; research from our group found concentrations ranging from 0.035 to 0.136 μg/L for the most dominant congeners (6:2, 6:2/8:2, 8:2).[8] Humans may also be exposed to volatile PFAS through inhalation, including the fluorotelomer alcohol (FTOH) and fluorotelomer acrylate (FTAC), used as starting materials in the production of commercial fluorotelomer-based polymers. In the commercial product, these fluorotelomer-based compounds may be present as unreacted starting materials and available for human exposure, although they have never been directly measured in humans.[9] Residual off-gasing from fluorochemical products has been observed from high concentrations of 8:2 FTOH (ng/m^3)[10−12] and 8:2 FTAC (ng/m^3)[12] contributing to indoor air contamination, compared to typical outdoor environmental concentrations of pg/m^3.[13]

Human contamination as a result of diPAP, FTOH, and FTAC exposure can result in biotransformation to produce perfluorinated carboxylates (PFCAs) with varying fluorocarbon chain lengths.[14,15] The general pathway for this is shown in Figure 1. The production of PFCAs is of concern because PFCAs with carbon chain lengths ≥ C7 are bioaccumulative and biologically persistent. For example, the human sera half-life of perfluorooctanoate (PFOA) is 3.5 years.[16] PFCAs are considered to be major contributors to the burden of human PFAS contamination, given their tendency toward bioaccumu-

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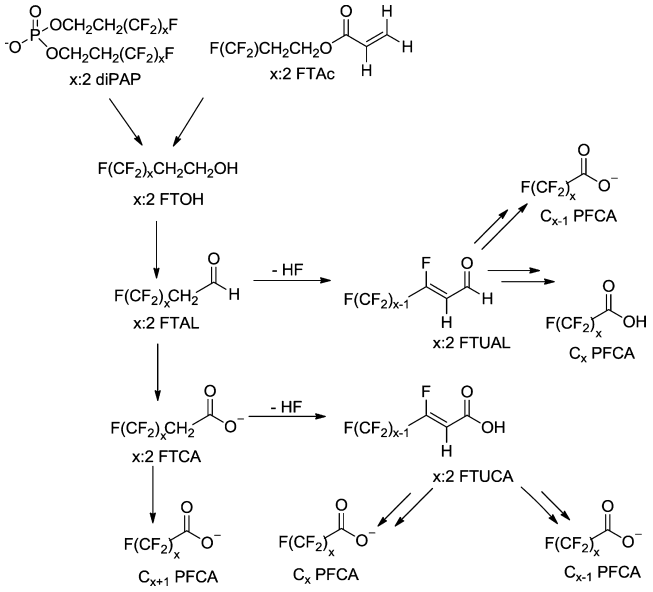

Figure 1. General biotransformation pathway of fluorotelomer-based compounds (i.e., *x*:2 diPAP). In this work, the toxicity of FTALs (x = 6, 8), FTUALs (x = 6, 8, 10), FTCAs (x = 4, 6, 8, 10), FTUCAs (x = 4, 6, 8, 10), and PFCAs (x = 3–9) was examined.

lation and toxicity. As such, industrial producers are phasing out the production of 8:2 FTOH to eradicate emissions by 2015[17,18] and are shifting to lower hexyl- and butyl-chain length congeners.[19,20]

Many studies have demonstrated that PFCAs, especially those with longer chain lengths (i.e., ≥C8), have toxicity in laboratory animals. PFCAs act as peroxisome proliferators, which have been associated with the onset of hepatotoxicity, in addition to causing developmental toxicity, immunotoxicity, and hormonal effects.[21,22] Compared to the interest in PFCA toxicity, limited studies have reported the toxicity of PFCA precursors. However, the few studies describing PFCA precursor toxicity have yielded interesting results. A study by Phillips et al.[23,24] demonstrated that PFCA precursor metabolites, the fluorotelomer carboxylic acids (FTCAs) and fluorotelomer unsaturated carboxylic acids (FTUCAs), had toxicity thresholds that ranged up to 10 000 times more than PFCAs in aquatic organisms. Toxicity depended on chain length, where longer carbon chains resulted in greater toxicity, proposed to be due to enhanced bioaccumulation.[23,25,26] FTCAs were the most toxic, and the relative toxicity of FTCAs compared to PFCAs was suggested to be associated with the loss of the inherently toxic hydrogen fluoride (HF).[27,28] Additional metabolic intermediates that might contribute to cellular toxicity are the fluorotelomer unsaturated aldehydes (FTUALs);[29,30] however, this remains to be elucidated. Currently, the relative toxicity among FTUALs, FTCAs, FTUCAs, and PFCAs is unknown. Nevertheless, these studies have provided evidence that metabolic intermediates have demonstrated toxicity in cellular systems and can be more toxic than PFCAs. This is important since PFCAs by themselves exhibit toxicity, but cellular exposure to PFCAs in conjunction with the metabolic intermediates may lead to enhanced toxicity with uncertain consequences.[30,31]

One mechanism for cellular toxicity is through the bioactivation of drugs or environmental pollutants to produce reactive intermediates that can covalently bind to cellular nucleophiles such as proteins or DNA.[32−35] Covalent binding to proteins may yield inactivation and loss of function, potentially leading to toxicity. Binding to DNA is also associated with toxicity,[33] for example, the toxicity that results from benzene oxide-DNA adducts following the bioactivation of benzene.[36] Fluorotelomer-based compounds are bioactive through the production of FTUALs and FTUCAs and subsequent conjugation to glutathione (GSH).[14,29−31,37] The FTUCAs are significantly less reactive than FTUALs, where reactivity is limited to GSH and other small thiol-containing nucleophiles.[38] Previous research in our lab has shown that FTUALs react with various functional groups, such as thiols, primary amines, imidazoles on amino acids, and proteins.[39] Additionally, protein binding has been observed in rat liver microsomes upon 8:2 FTOH exposure as a result of 8:2 FTUAL production.[40] Because of the degree of protein binding associated with the bioactivation of these fluorotelomer compounds and given the variety of functional groups targeted by FTUALs, exploring the potential for forming covalent DNA adducts is worthwhile.

The aim of this study was to provide a comprehensive comparison between the metabolites produced from the transformation of fluorotelomer-based compounds, including the FTCAs, FTUCAs, FTUALs, fluorotelomer saturated aldehydes (FTALs), and PFCAs. Since the production of intermediate metabolites mainly occurs in the liver, human liver epithelial (THLE-2) cells were used to generate dose−response EC_{50} values to interrogate how chain length and functional group correlate with observed toxicity. By using one cellular platform to compare the toxicity of PFCAs and intermediates (acids and aldehydes) of different chain lengths, the need for cross-platform extrapolation is eliminated. Additionally, because 8:2 FTOH is bioactivated to form FTUALs that bind to proteins, the potential for FTUALs to form DNA adducts was also assessed.

MATERIALS AND METHODS

Materials. All chemicals were used as received. Perfluorobutanoate (PFBA), perfluoropentanoate (PFPeA, >98%), perfluorohexanoate (PFHxA, >98%), and perfluoroheptanoate (PFHpA, >98%) were obtained from SynQuest Laboratories, Inc. (Alachua, FL, USA). Perfluorooctanoate (PFOA, 96%), perfluorononanoate (PFNA, 97%), and perfluorodecanoate (PFDA, 98%) were purchased from Sigma Aldrich (Oakville, ON, Canada). The 4:2, 6:2, 8:2, and 10:2 FTUCAs and FTCAs were synthesized following a method reported by Achilefu et al. and by methods previously described.[41,42] The 6:2, 8:2, and 10:2 FTUALs and 6:2 and 8:2 FTAL were synthesized based on a method by Leveque et al.,[43] the specific methods of which are provided in previous studies.[38,39] Samples were prepared using Hybri-Max dimethyl sulfoxide (DMSO, ≥99.7%) obtained from Sigma Aldrich (Oakville, ON, Canada), Omni-Solv acetonitrile (ACN, ≥99.9%), or acetone (≥99.9%) from BDH Chemicals (West Chester, PA, USA). The 6:2 FTUAL, due to its high reactivity and low stability, was synthesized *in situ* from reaction of triethylamine with 6:2 FTAL, as previously described.[38]

Cell Culture and Treatments. THLE-2 cells, a cell line derived from normal human liver cells, were purchased from American Type Culture Collection (Manassas, VA, USA). Previous *in vitro* research has focused on the PFCAs, their activation of multiple nuclear receptors, and the modulation of metabolic pathways associated with their exposure.[25,44] Since the purpose of this study was to understand the comparative sensitivity among PFCAs, the lesser studied FTUCAs and FTCAs, and the unstudied FTALs and FTUALs, THLE-2 cells were chosen as a platform to study general toxicity using cell viability as an end point. THLE-2 cells express characteristics of adult liver epithelial cells and have less enzymatic activity than primary human

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hepatocytes.[45,46] By using this cellular platform, we were able to test the inherent toxicity of the intermediate metabolites without additional metabolism. The use of these cells for generating toxicity data also captures what is likely to occur in most cell types (where phase I−III enzyme activities are generally low). Future studies using primary hepatocytes would allow insight into the role of metabolism in altering toxicity, comparison of the results with previous studies regarding the biological activation of PFCAs, and determination of effects related to any nuclear receptor targets of the PFCA precursors.

The cells were cultured in a Lonza (Walkersville, MD, USA) bronchial epithelial cell basal medium (BEBM) supplemented with the following proprietary mixture of components in their BEGM SingleQuot Kit: 0.5 mL of transferrin, 5 ng/mL human recombinant epidermal growth factor (rhEGF), 0.5 mL of triiodothyronine (T3), 0.5 mL of retinoic acid, 0.5 mL of hydrocortisone, 2 mL of bovine pituitary extract (BPE), and 10% fetal bovine serum. The cell line was incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were plated in 96 well plates at a density of 10 000 cells/100 μL and cultured for 24 h prior to dosing.

Most solutions were prepared using DMSO, but due to solubility issues, solutions of 10:2 FTCA, 10:2 FTUCA, PFDA, 6:2 FTAL, 8:2 FTAL, and 8:2 FTUAL were prepared using ACN, and the 10:2 FTUAL solution was prepared using acetone. All solvents were used as procedural blanks and did not significantly affect cell viability. Final test concentrations were made from serial dilution of the stock solutions, followed by cell dosing, where the amount of solvent was 0.6% (v/v) of the total volume. For PFCAs, final test concentrations ranged from 50 μM−4000 μM. Final concentrations of the FTALs, FTCAs, FTUALs, and FTUCAs ranged from 10−150 μM, 1−4000 μM, 0.010−150 μM, and 5−4000 μM, respectively. Concentration ranges varied depending on each analyte, but at least 6 concentrations were used to generate each dose−response curve, with a sample size of $n = 3$ at each concentration. Most sample replicates were within wells on the same plate, although the few replicates on different plates did not provide a varying response. This suggests that the application of replicates to the same plate did not significantly affect the experimental outcome. The specific concentration ranges for each analyte are given in Tables S1−S3 (Supporting Information). Since overall toxicity is a function of both uptake and inherent toxicity, cell uptake for each analyte was not quantified; after incubating for 24 h, analytes were presumed to reach uptake equilibrium, although this would be useful to verify in future studies. In addition, to ensure that the final dose concentrations for PFCAs, FTCAs, and FTUCAs were accurate, a series of cell incubations were extracted with acetonitrile and analyzed using LC-MS/MS to determine analyte concentration. The concentration of analytes incubated with cells was compared to the concentration of analytes incubated in culture media without cells. Initial dose concentrations were not measured, but analytes were presumed to be in their free form after the addition of acetonitrile to remove any noncovalently bound analytes from serum proteins present in the media. Methods similar to this have been used to analyze PFCAs and their acid precursors in biological matrices.[37,47] The concentrations quantified using LC-MS/MS are found in Table S4 of the Supporting Information.

The stability of the FTUALs in aqueous solution was examined using ^{1}H NMR. As presented in the Supporting Information, 8:2 FTUAL was exposed to water, and its stability was monitored over 24 h (Figure S1, Supporting Information). Results showed that the FTUAL formed a hydrate from reaction with water, a reversible reaction in the presence of additional nucleophilic groups such as proteins or DNA. No additional degradation products of the FTUAL were observed. Additionally, evidence of metabolism to FTUCAs, FTCAs, and PFCAs from the aldehyde cellular incubations was not observed. Trace amounts of the acid metabolites were detected in both the cellular incubations and culture medium controls (Table S4, Supporting Information). Solvent controls containing an equal volume of solvent as the dosed cells were run ($n = 2$) with each set of test incubations. To generate the response curves, responses from the analyte incubations were normalized to the average response of the controls. All of the cell viability measurements described below were conducted independently for each of the experiments.

Cell Viability Assays. The number of viable cells was measured by the Cell Counting Kit-8 (CCK-8) assay from Sigma Aldrich (St. Louis, MO, USA). Briefly, 10 μL of the CCK-8 solution, containing a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8) was added at 37 °C to each well containing the cell suspension. The plate was incubated for 1−3 h in a CO_2 incubator. Subsequently, absorbance was measured at 450 nm using a BMG Labtech FluoStar Optima (Cary, NC, USA). Viability response values were obtained by using a 6-point cell calibration curve ranging from 1000−25 000 cells.

Statistical Analysis. Statistical tests for all viability results were performed using SigmaPlot 11.0 (San Jose, CA, USA). Comparison among EC_{50} values (the concentration of 8:2 FTUAL needed to decrease cell viability by 50%) within each compound class (i.e., PFCAs, FTUCAs, FTCAs, FTALs, and FTUALs) was accomplished by a one-way analysis of variance (ANOVA). If the one-way ANOVA produced a result that was statistically significant, a pairwise comparison was done using the Holm−Sidak test. The overall significance level for all tests was $p = 0.05$. A summary of the p-values calculated for all EC_{50} concentrations is provided in Tables S5−S12 (Supporting Information).

qPCR Determination of Adduct Formation with DNA. Cells (75 000 cells/well) were cultured, by the methods described above, for 3, 6, and 24 h with 0.05, 0.5, or 2.5 μM 8:2 FTUAL. The 8:2 FTUAL was chosen for DNA adduct detection because it produced a small EC_{50} value. Concentrations of 8:2 FTUAL were chosen based on the dose−response curve, where dosing with 0.5 μM 8:2 FTUAL resulted in a cell viability of 95%. The 0.05 and 2.5 μM concentrations were chosen so that 100% and 75% of the cells remained viable, respectively, in order to maximize the amount of DNA extracted and exclude detection of DNA damage occurring as a secondary effect of cell death. Since this was a preliminary range-finding study to assess DNA damage, $n = 2$ samples were used per time point. A procedural solvent blank containing no dose chemical was used to ensure the solvent was not damaging the cellular DNA. After treatment, the BEBM medium (1 mL) was removed, cells were washed once in Gibco 1× Dulbecco's phosphate buffered saline (DPBS) obtained from LifeTechnologies (Burlington, ON, Canada), and trypsinized. BEBM medium (1 mL) was added to the trypsinized cell solution, which was then centrifuged at 5000*g* for 5 min. The supernatant was discarded, and the resulting cell pellet was stored at −80 °C until DNA extraction. A detailed description of the DNA extraction procedure and the qPCR method to determine nuclear and mitochondrial DNA damage can be found in the Supporting Information.

TOF-CIC Determination of Adduct Formation with DNA. Samples extracted for the qPCR reactions and additional 8:2 FTUAL, 8:2 FTUCA, and PFOA incubations were sent to the University of Toronto for analyses using total organic fluoride-combustion ion chromatography (TOF-CIC). Details of the cell incubations, extraction method, and the general method for the TOF-CIC method are provided in the Supporting Information. The specific parameters of the TOF-CIC can be found in studies by Yeung et al. and Rand and Mabury.[39,48]

RESULTS AND DISCUSSION

Effect of PFCAs and Their Metabolic Precursors on Cell Viability. As this was the first study to compare the toxicity of PFCAs and their metabolic precursors, viability was measured as a general end point. The specific mode of action for the aldehydes is unknown, and the acid intermediates have only been proposed to act similarly to PFCAs.[24] To determine the effect of PFCAs, FTUCAs, FTCAs, FTUALs, and FTALs on cell viability, dose−response curves were generated where the viability of the dosed cells was normalized with respect to the viability of the controls (cells containing only the transfer solvent) over a range of chemical concentrations, as presented

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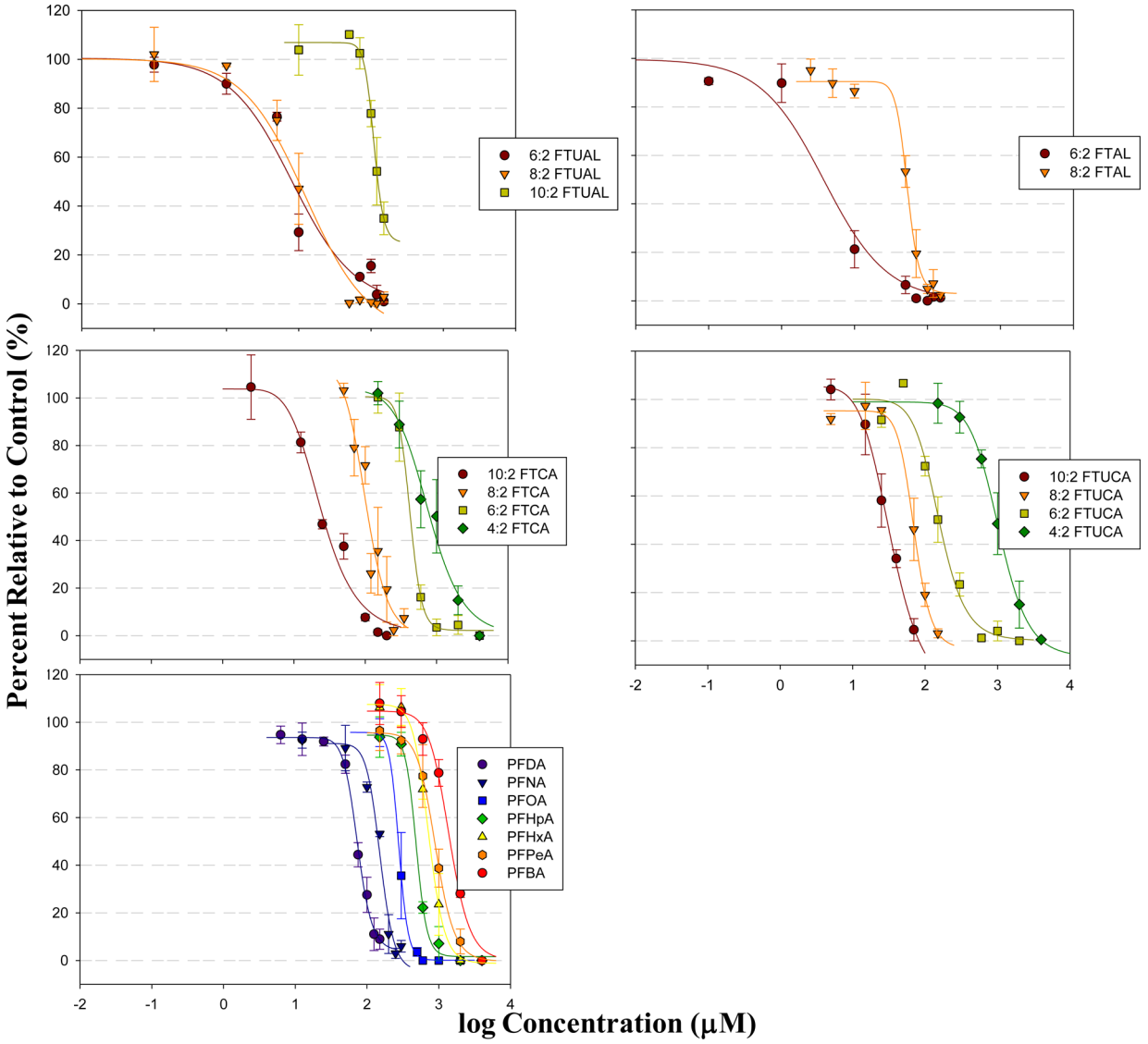

Figure 2. Cellular dose–response curves for FTUALs, FTALs, FTUCAs, FTCAs, and PFCAs. Error bars represent the standard error for $n = 3$ sample replicates.

in Figure 2. Additionally, to ensure that the final dose concentrations of each analyte were accurate, a series of cell incubations were extracted and analyzed using LC-MS/MS to determine analyte concentration (Table S4, Supporting Information). In general, because the quantified concentrations were similar to the original measured values, concentrations remained unaltered. For all of the acid congeners, cell viability was influenced by the length of the fluorinated chain, where in general, toxicity increased with increasing chain length. For example, increased concentrations of shorter chain length acids were required to generate a dose–response curve, compared to longer chain length acids that produced dose–response curves at lower concentrations (Figure 2). The PFCA congeners tested ranged from the C4 acid (PFBA) to the C10 acid (PFDA), where PFBA was the most soluble and PFDA the least, potentially explaining the large standard error of the measured EC_{50} value for PFDA (Figure 2). EC_{50} values for PFCAs varied over 1 order of magnitude, from 65 ± 41 to 1361 ± 146 μM (Figure 3). Toxicity of PFCAs has previously been proposed to be related to the length of the fluorinated tail, where longer chain lengths have been associated with greater hydrophobicity, increased bioaccumulation, and increased toxicity.[23,26,49,50] Additionally, effects of PFCAs have been associated largely with chain lengths that are ≥C7 (i.e., ≥ PFOA). For example, one route of toxicity associated with PFCA exposure is their involvement in peroxisome proliferation activated receptor-alpha (PPAR-α) activation.[21] Toxicity of PFCAs through activation of PPAR-α leads to cell proliferation, oxidation, and formation of hepatic tumors in rats.[21] However, the chain length of the PFCAs in these studies greatly influenced the relative toxicity, where PFCAs having chain lengths ≥ C7 carbons were associated with more effects from PPAR-α activation and other modes of action, compared to those PFCAs with shorter chain lengths.[25,51,52] The results here corroborate what other studies have shown, that toxicity is associated with the length of the fluorinated tail.

A similar trend was noted for the PFCA acid metabolic precursors: the FTUCAs and FTCAs. For both FTUCAs and FTCAs, the 10:2 congener was more toxic than the 4:2 congener. For the FTUCAs, EC_{50} ranged from 22 ± 9 (10:2 FTUCA) to 1044 ± 20 μM (4:2 FTUCA), where cells were about 47-times more sensitive to the 10:2 FTUCA than the 4:2

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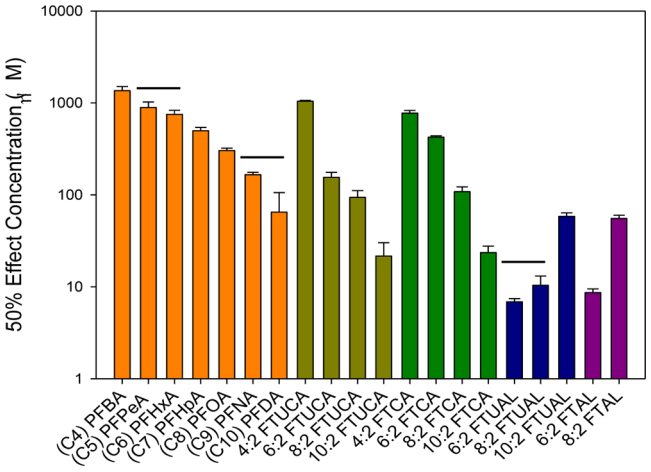

Figure 3. 50% Effect concentrations (EC_{50}) for cell viability calculated from the dose−response curves shown in Figure 2 for PFCAs, FTUCAs, FTCAs, FTUALs, and FTALs. Error bars represent the standard error for $n = 3$ sample replicates. The line over bars indicates groups which were not significantly different (Holm−Sidak statistical test, $p > 0.05$).

FTUCA. Similarly, the cells were most sensitive to the 10:2 FTCA (EC_{50} = 23.6 ± 4.0) and approximately 42-times less sensitive to the 4:2 FTCA (EC_{50} = 1004 ± 20 μM). Although the mechanisms for FTUCA and FTCA toxicity have not been elucidated, it has been suggested due to their similar structure that their toxic effects may be similar to those of PFCAs, for which toxicity may be associated with altered metabolism, mitochondrial respiration, and gap junctional intercellular communication.[24,50,53] The results shown here are in agreement with what was previously shown by Phillips et al.[23] in the first study to demonstrate toxicity using PFCA precursors. In that study, growth inhibition and viability of FTUCA and FTCA exposure in aquatic organisms was generally associated with the length of the fluorinated tail.[23] Here, we demonstrate that fluorinated chain length may be a main contributor to the observed toxicity, although the specific mechanism for toxicity of FTUCAs and FTCAs has yet to be elucidated.

Toxicity for the PFCAs, FTCAs, and FTUCAs in this study as well as previous studies was observed to be directly associated with the length of the fluorinated tail. By contrast, cells were most sensitive to FTUALs and FTALs with shorter chain lengths. For the FTUALs (6:2, 8:2, and 10:2), toxicity was greatest for the 6:2 FTUAL and smallest for the 10:2 FTUAL, with EC_{50} values of 7 ± 1 μM and 58 ± 5 μM, respectively, a difference of a factor of 8. For the FTALs, the 6:2 FTAL was approximately 6-times more toxic than the 8:2 FTAL, with EC_{50} values of 9 ± 1 μM and 55 ± 5 μM, respectively. These results indicate that for the aldehyde metabolites, toxicity may not be associated with bioaccumulation and chain length but rather with reactivity at the α- and β-carbon for the FTALs and FTUALs, respectively. For these aldehydes, a longer chain length may sterically hinder nucleophilic attack at the electrophilic centers, while the shorter chain length may allow greater reactivity and thus greater toxicity. A previous study of ours also demonstrated that reactivity of FTUALs with GSH depended on the length of the fluorinated tail: the 6:2 and the 8:2 FTUAL both depleted the GSH concentration, resulting in similar EC_{50} concentrations. However, the 10:2 FTUAL had less effect, with an EC_{50} concentration greater by a factor of 9 and 6 compared to the 8:2 FTUAL and 6:2 FTUAL, respectively.[38] Here, a similar result was observed, where the cellular exposure of 6:2 and 8:2 FTUAL gave EC_{50} values within an order of magnitude, but the 10:2 FTUAL was approximately 1 order of magnitude less toxic. Although no studies have shown the potential for FTALs to react with biological nucleophiles, their α-carbon may be attacked by endogenous amines (i.e., lysine residues on proteins) to form an imine (alternatively known as a Schiff-base). For example, 4-hydroxynonenal, an unsaturated aldehyde derived from the metabolism of polyunsaturated fatty acids, has been observed to form protein adducts primarily resulting from the reactivity of its β-carbon (1,4-Michael addition adducts) but to a lesser extent from reactivity with its α-carbon (Schiff-base adducts).[54]

Comparison of EC_{50} Values: PFCAs vs Metabolic Acid Precursors vs Metabolic Aldehyde Precursors. Previously, Phillips et al.[23] reported that FTUCAs and FTCAs were 10−10 000 times more toxic than their corresponding PFCAs, and generally FTCAs were more toxic than FTUCAs. To compare this trend with our results, we assessed the EC_{50} concentrations of PFCAs relative to their corresponding acid and aldehyde precursors, as shown in Figure 4. Generally, FTUCAs and FTCAs were significantly more toxic than their corresponding PFCA. For example, the effect of PFOA on cells produced an EC_{50} value of 303 ± 18 μM, compared to 94 ± 17 μM and 108 ± 14 μM for the 8:2 FTUCA and 8:2 FTCA, respectively. Although this relative trend was similar to what Phillips et al. had reported, there were some significant differences. First, the FTUCAs and FTCAs were observed to produce statistically similar EC_{50} values, except for the 6:2 congeners, where the 6:2 FTUCA was more toxic than the 6:2 FTCA ($p = 0.45$ (Tables S10−S12, Supporting Information)). Phillips et al.[23,24] suggested that the FTCAs were more toxic than the FTUCAs due to the production of HF. One explanation for this discrepancy is that the THLE-2 cellular system may not be as sensitive to HF production as compared to the organisms used by Phillips et al. Alternatively, the deficiency of metabolizing enzymes in the THLE-2 cell line may hinder the production of HF during our 24 h exposure period, as indicated by the lack of metabolites present within all cell incubations after analysis using LC-MS/MS (Table S4, Supporting Information). Second, in the study by Phillips et al.,[23] FTCAs had toxicity thresholds that were 5 orders of magnitude more than their corresponding PFCAs. It was proposed that the production of HF, as well as the combined exposure to FTCAs, FTUCAs, and PFCAs as a result of FTCA metabolism may lead to increased toxicity. Although we also observed the FTCAs to be more toxic than the PFCAs, threshold values were only 1.7−3 times greater than those of PFCAs. Phillips et al.[23] noted that the toxicity thresholds generated depended on the organism, where some organisms (i.e., *D. magna*) were more sensitive to FTCAs and FTUCAs than others. The results presented here, compared to those in other studies, are consistent with species differences.

The 6:2 and 8:2 FTUALs were the most toxic compared to their corresponding acid intermediates and PFCAs. Generally, relative toxicity was as follows: FTUALs ≥ FTALs > FTUCAs ≥ FTCAs > PFCAs. For the 6:2 and 8:2 FTUALs, EC_{50} values were approximately 98% less than their corresponding FTUCAs, 90% less than their corresponding FTCAs, and approximately 99% less than PFHxA and 97% less than PFOA. The lifetime of FTUALs has never accurately been measured, although one study observed the complete loss of 8:2 FTUAL after 2 h in rat hepatocytes.[29] The half-lives of the acid

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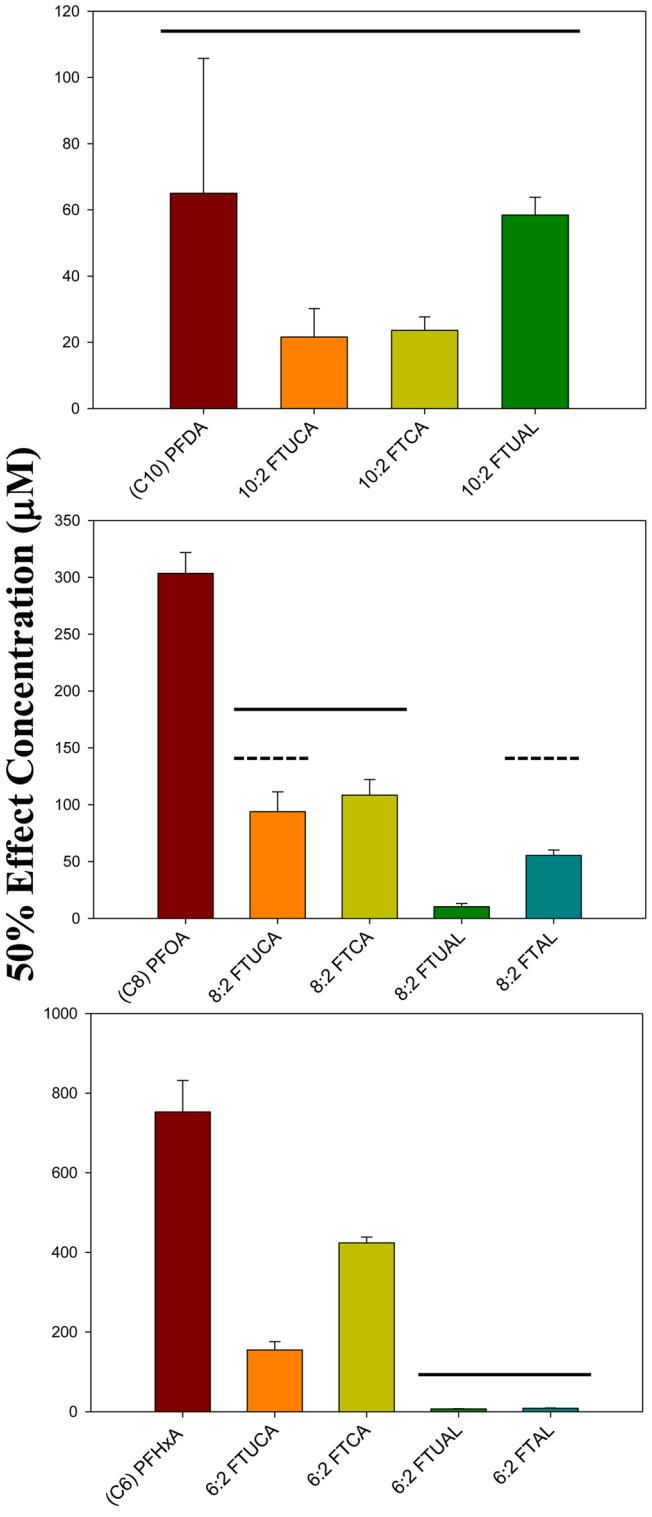

Figure 4. Relative EC_{50} concentrations of PFCAs with their corresponding acid (FTUCA and FTCA) and aldehyde (FTUAL and FTAL) precursors. Error bars represent the standard error for n = 3 sample replicates. The line over the bars indicates groups which were not significantly different (one-way ANOVA and Holm−Sidak statistical tests, $p > 0.05$).

intermediates and PFCAs are at least several hours;[15,30,55] they are proposed to be much longer lived than their corresponding aldehydes. Thus, despite their proposed short lifetime *in vivo*, these aldehydes have demonstrated toxicity greater than those of PFCAs. This toxicity is proposed to be driven primarily by their inherent reactivity with endogenous nucleophiles, although additional mechanisms may be involved. Although FTUALs have been shown to react with GSH, which may act as a detoxifying mechanism, in our recent studies FTUALs have also been shown to undergo reaction with proteins, which may lead to toxicity through protein deactivation.[39,40]

The relative toxicity among 10:2 FTUCA, FTCA, and FTUAL, and PFDA yielded different results compared to the toxicity of the 6:2 and 8:2 congeners and their corresponding PFCA. Here, the toxicity profile was less pronounced for the 10:2 FTUAL than its corresponding metabolites. This may result from toxicity that is increased due to the longer chain length for PFDA, 10:2 FTUCA, and 10:2 FTCA, yet diminished due to the steric hindrance of reactivity for the 10:2 FTUAL. Comparing the EC_{50} values for all of the C10 congeners showed little statistical significance (Table S12, Supporting Information). This finding suggests that the toxicity of PFCAs, FTUCAs, and FTCAs becomes equal to or greater than FTUALs as chain length increases.

For this study, the use of THLE-2 cells provided a general platform to understand the relative toxicity of PFCAs and their metabolic precursors. However, the results we obtained may vary by cell type. For example, several studies have indicated that after cellular uptake, some PFCAs (i.e., PFOA and PFHxA) activate nuclear receptors and modify enzyme expression[25,44] and that they are likely to be subject to cell-type-specific metabolism and transport. THLE-2 cells do not contain high levels of some of the enzymes that may be sensitive to PFCA exposure and involved in PFCA toxicokinetics. This may increase THLE-2 viability after exposure to PFCAs, relative to other cell lines that express these enzymes at a higher level; the degree of increase may also follow a different pattern of chain length specificity. It will be important to also investigate the effect of PFCA precursors on nuclear receptors and enzyme expression; future studies using primary hepatocytes should help to determine the nuclear receptor targets of PFCA precursors and the influence of higher levels of metabolic and transport proteins.

Reactivity of FTUALs with DNA. Since THLE-2 cells were most sensitive to the 8:2 FTUAL, the 8:2 FTUAL was incubated over 24 h to determine whether DNA adduction was a targeted mechanism for toxicity. In order to assess the extent of cellular mitochondrial or nuclear DNA damage after exposure to 8:2 FTUAL, a qPCR assay was used. Both the mitochondrial and nuclear qPCR assays yielded results that were not significantly different from those of the ACN control (Tables S15, Supporting Information), indicating that detectable DNA lesions did not result from 8:2 FTUAL exposure (the limit of detection of the assay is ∼1 lesions/100 000 nucleic acid bases). To evaluate whether any covalently bound fluoride from 8:2 FTUAL exposures was associated with DNA, these samples were also analyzed using TOF-CIC. Additional THLE-2 cell incubations were also compared, where 8:2 FTUAL, 8:2 FTUCA, and PFOA were exposed to cells at concentrations that resulted in 95% and 50% cell viability over 24 h yet showed no difference with respect to the controls. This indicated that exposure to FTUALs does not result in detectable DNA damage, as evidenced in previous studies examining DNA damage using the comet assay and *umuC* gene expression assay from exposure to FTOHs.[56,57]

Implications. The results presented in this study provide additional evidence that PFCA precursors are more toxic than

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PFCAs. This was the first time where FTUALs have been scrutinized with any toxicity assay, and this study thus provides initial evidence that FTUALs are up to 200-times more toxic than FTUCAs, FTCAs, and PFCAs. Although FTUALs were not shown to form adducts with DNA, FTUALs have been observed to readily bind with proteins *in vitro* (i.e., liver microsomes and blood plasma).[40] Additionally, our previous paper demonstrated that a significant part of the mass balance of 8:2 FTOH transformation is associated with the protein fraction, proposed to be due to 8:2 FTUAL covalent protein adduct formation.[40] This work offers an extension of the previous study, where the inherent reactivity of FTUALs may be intrinsically linked with toxicity. This is important, as exposure to fluorotelomer-based compounds such as diPAPs, FTOHs, or FTAC monomers have been proposed to be sources of PFCAs. Currently, most scientific and regulatory interest has focused on the toxicological risk of exposure to PFCAs. By contrast, limited exposure and risk assessment studies have considered the toxicology PFCA precursors.[58−60] To our knowledge, no studies have highlighted exposure to FTALs or FTUALs in the context of risk assessment. However, because we may be constantly exposed to fluorotelomer-based commercial products,[15] it is important to understand not only the risk of exposure to PFCAs but also their metabolic precursors.

With the industrial movement to produce shorter chain length PFAS, such as C4- and C6-based products, we need to elucidate the consequence of exposure to these shorter chain length compounds. The industry justified this movement because shorter chain length compounds are less bioaccumulative. However, the toxicology of the intermediates may not have been considered. The research herein indicates the shift to shorter chain length compounds has brought unexpected challenges through the increased toxicity of the FTUALs. We currently do not know the relative importance of persistent PFCAs compared to the short-lived aldehyde intermediates over a long period of time, nor whether PFCAs would be more toxic from a longer exposure period than the aldehydes. Here, we have shown that in 24 h, FTUALs are more toxic than PFCAs. As diPAPs have been found in human sera, this indicates that humans may be constantly exposed to fluorotelomer-based commercial products. Constant exposure to these products and consequent production of aldehydes may yield significant toxicity responses compared to those of the longer-lived but less reactive PFCAs. It is necessary to consider how short-lived intermediates and long-lived PFCAs compare in their effects over a long exposure period and to elucidate specific toxicity mechanisms in order to recognize the consequences of exposure to fluorotelomer-based compounds.

■ ASSOCIATED CONTENT

Supporting Information

Description of the qPCR method, dose−response concentrations for analytes, LC-MS/MS analyte concentrations, statistical measurements, gene targets and primer pairs for qPCR, thermocycler conditions, and fluorescence values for samples from the qPCR assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

FTAL, fluorotelomer aldehyde; FTUAL, fluorotelomer unsaturated aldehyde; FTCA, fluorotelomer carboxylic acid; FTUCA, fluorotelomer unsaturated carboxylic acid; PFCA, perfluorinated carboxylic acid; THLE-2, human liver epithelial cell line; EC_{50}, 50% effect concentration; PFDA, perfluorodecanoate; PFBA, perfluorobutanoate; PCR, polymerase chain reaction; PFAS, per- or poly fluorinated substances; PAP, polyfluoroalkyl phosphate esters; diPAP, polyfluoroalkyl phosphate diesters; FTOH, fluorotelomer alcohol; FTAC, fluorotelomer acrylate; PFOA, perfluorooctanoate; HF, hydrogen fluoride; PFPeA, perfluoropentanoate; PFHxA, perfluorohexanoate; PFHpA, perfluoroheptanoate; PFNA, perfluorononanoate; CCK, cell counting kit; qPCR, quantitative polymerase chain reaction; BEBM, bronchial epithelial cell basal medium; rhEGF, human recombinant epidermal growth factor; BPE, bovine pituitary extract; TOF-CIC, total organic fluoride-combustion ion chromatography

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