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Cite this: *Environ. Sci.: Processes Impacts*, 2016, **18**, 538

Biotransformation of 8:2 fluorotelomer alcohol by recombinant human cytochrome P450s, human liver microsomes and human liver cytosol†

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Biotransformation of 8:2 fluorotelomer alcohol (8:2 FTOH) can form potentially more toxic metabolites. However, the responsible cytochrome P450 (CYP) isoform(s) and phase II metabolism have not been studied in humans. Here, we characterized the *in vitro* metabolism of 8:2 FTOH by recombinant human CYPs, human liver microsomes, and human liver cytosol. The results showed that among the 11 isoforms investigated, CYP2C19 was the only enzyme capable of catalyzing 8:2 FTOH with K_m and V_{max} values of 18.8 μM and 8.52 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450, respectively. The phase I metabolite was identified as 8:2 fluorotelomer aldehyde (8:2 FTAL). HLMs also catalyzed 8:2 FTOH transformation, with the V_{max} and intrinsic clearance (CL_{int}) values similar to those of CYP2C19 after the protein content is taken into account. Molecular docking showed that the hydroxyl group of 8:2 FTOH accesses the heme iron-oxo of CYP2C19 in an energetically favored orientation. 8:2 FTOH was also transformed by phase II enzymes to form *O*-glucuronide and *O*-sulfate conjugates. The CL_{int} values follow the order of sulfation > oxidation > glucuronidation, suggesting that conjugation is the major metabolic pathway, which explains the low yield of perfluoroalkyl acids (PFCAs). These results provide new insight into fluorotelomer alcohol biotransformation and indirect human exposure to PFCAs.

Received 6th February 2016
Accepted 26th April 2016

DOI: 10.1039/c6em00071a

rsc.li/process-impacts

Environmental impact

Fluorotelomer alcohols (FTOHs) are the precursors of perfluoroalkyl acids (PFAAs) which are now considered as global persistent organic pollutants and are therefore the potential source of PFAAs. CYP2C19 that we characterized plays a crucial role in the biotransformation of 8:2 FTOH in the human body to form a more toxic metabolite (8:2 fluorotelomer aldehyde). Phase II metabolism (glucuronidation and sulfation) showed higher efficiency than phase I metabolism, which indicates that forming conjugates is the major fate of 8:2 FTOH metabolism. These results will help future epidemiological studies in the assessment of indirect sources of human exposure to PFAAs and potential health risks of fluorotelomer alcohols.

1 Introduction

Poly- and perfluoroalkyl chemicals (PFCs) are a family of anthropogenic compounds consisting of a carbon bone of 4–14 in length, in which some or all of the carbon–hydrogen (C–H) bonds are replaced by carbon–fluorine (C–F) bonds. Due to their unique properties of stability, hydrophobicity and lipophilicity, these substances have been used extensively in consumer and industrial applications since the 1950s.¹ Traditionally, PFCs were produced *via* two major manufacturing processes: electrochemical fluorination (ECF)

and telomerization. Because of their persistence, toxicity and bioaccumulation, ECF-associated chemicals, perfluorooctane sulfonic acid (PFOS), its salts and perfluorooctane sulfonyl fluoride (POSF), were placed into the Annex B of the Stockholm Convention (SC) on Persistent Organic Pollutants (POPs) in 2009. ECF production has also been phased out gradually in the developed countries,² and was moved to China after 2003,^{3,4} which may have global implications. However, fluorotelomer production has continued to increase, reaching 1.2×10^7 kg annually in 2004.² Fluorotelomer alcohols (FTOHs, $\text{CF}_3(\text{CF}_2)_x\text{C}_2\text{H}_4\text{OH}$, $x = 3, 5, 7$, and 9) are the major fluorotelomer products used in the manufacture of fluoropolymers and fluorosurfactants. The annual production of FTOHs reached 5×10^6 kg in 2000–2002.⁵ 8:2 FTOH (where $x = 7$) has attracted the most attention for it has the largest production and has been implicated as a potential source of perfluorooctanoic acid (PFOA).⁶

Plenty of studies have demonstrated the metabolism of 8:2 FTOH in rats,^{6–11} mice,^{12,13} fish,¹⁴ humans,¹⁵ and micro-

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6em00071a

organisms.^{16,17} The biotransformation mechanisms and pathways have been reviewed by Butt *et al.*¹⁸ In general, the phase I metabolism of 8:2 FTOH in the human body would produce 8:2 FTAL, which is transient and then eliminates HF non-enzymatically to form 8:2 FTUAL. 8:2 FTAL and 8:2 FTUAL would further be oxidized to form 8:2 fluorotelomer carboxylic acid (8:2 FTCA) and 8:2 fluorotelomer α,β -unsaturated carboxylic acid (8:2 FTUCA).⁶ The final metabolites mainly contain PFOA and perfluorononanoic acid (PFNA).⁷

The toxicities of FTOHs and the related metabolites have drawn considerable attention in recent years. 8:2 FTOH and 6:2 FTOH ($x = 5$) were suggested to induce estrogenic effects in animal models.^{19–23} The intermediate metabolites, specifically 8:2 FTAL, 8:2 FTUAL, 8:2 FTCA and 8:2 FTUCA, showed higher toxicity than PFAAs in both aquatic organisms^{24,25} and human liver epithelial (THLE-2) cells following the order of FTUALs $\geq$ FTALs $>$ FTUCAs $\geq$ FTCAs $>$ PFAAs.²⁶ Martin *et al.* found that electrophilic aldehydes and acids were responsible for the depletion of glutathione (GSH) and increased protein carbonylation and lipid peroxidation in isolated rat hepatocytes after dosing 8:2 FTOH.²⁷ PFAAs, especially PFOA, have been reported to cause a wide range of toxic effects including hepatotoxicity, developmental toxicity, immunotoxicity and neonatal toxicity.^{28–30} Epidemiological studies have also found significant or suggestive associations between PFAA exposure and biochemical or physiological end points.^{28,31,32} Besides, although the overall yield of PFOA in humans and animals *via* the biotransformation of 8:2 FTOH is low,^{7,8,12} long-term exposure of 8:2 FTOH may accumulate PFOA and induce toxic effects since PFOA cannot be degraded and has a long elimination half-life time.^{12,15,33} Therefore, the biotransformation of 8:2 FTOH would produce remarkable effects on the toxicity of this class of compounds.

The initial biotransformation of 8:2 FTOH includes phase I metabolism (8:2 FTOH to 8:2 FTAL) and phase II metabolism (8:2 FTOH to 8:2 FTOH-glucuronide and 8:2 FTOH-sulfate),⁷ in which the former would produce more toxic metabolites, while phase II metabolites tend to be easily excreted. Some of the *in vitro* studies have suggested that P450 enzymes were responsible for the oxidative biotransformation,⁸ but the enzymology of both the phase I metabolism and phase II metabolism has not been elucidated.

The aim of the present investigation was to identify the specific human P450 enzyme(s) that contribute to the oxidative biotransformation of 8:2 FTOH in humans and to quantitatively assess the *in vitro* metabolic kinetics of 8:2 FTOH using recombinant human P450s, human liver microsomes (HLMs) and human liver cytosol (HLC). Metabolites were identified using HPLC-MS/MS. Attempts were also made to investigate the binding conformation of 8:2 FTOH in the active site of CYP2C19 using molecular docking.

2 Materials and methods

2.1 Sample preparation

We used crimp-sealed serum bottles (5 mL volume) with butyl rubber stoppers as the incubation vials. Acetonitrile pre-rinsed

C18 cartridges were used to capture the volatilized substrate. 8:2 FTOH was dissolved in acetonitrile, the amount of which introduced into the incubation solution did not exceed 1% (v/v) of the total system. Acetonitrile, up to 2% (v/v), has no apparent effects on enzyme activities according to the data from Eastbrook.³⁴ All the incubations were performed in triplicate.

The following control samples were included in the study: (1) enzymes incubated in the absence of 8:2 FTOH to determine the background level of 8:2 FTOH, (2) 8:2 FTOH incubated in the absence of enzyme proteins to determine any non-enzymatic transformation of the substrate and (3) CYPs incubated with 8:2 FTOH in the absence of a NADPH-regenerating system to confirm the CYP-dependent enzymatic biotransformation. Control samples were prepared in duplicate.

At multiple times, test vials were taken out of the incubator. The volatilized 8:2 FTOH in the headspace of the serum bottle was trapped onto a C18 cartridge using a similar technique to that described by Ruan *et al.*³⁵ A syringe that was fixed to a small cartridge containing C18 packing materials was inserted into the headspace of the vial and purged with about 20 mL of air. 100 μ L of ice-cold HCl (6 M) was added to inactivate and precipitate the protein. The mixtures were extracted twice with 600 μ L MTBE. After vortexing vigorously, the mixtures were centrifuged at $10\,000 \times g$ for 5 min and the organic layer was moved to another tube using a pipette. The C18 cartridge was eluted with 2 mL of MTBE and combined with the extract.

The MTBE extract was transferred into a glass GC vial, internal standard (8:1 FTOH) added, and analyzed by gas chromatography coupled with mass spectrometry (GC-MS).

2.2 Materials

HLMs and HLC pooled from 50 donors with mixed genders were obtained from Xenotech (Lenexa, KS, USA) and stored at $-70\text{ }^{\circ}\text{C}$ until use. Baculovirus-insect cell line microsomes (Corning Supersomes) containing individually expressed human P450 enzymes (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) with supplemental cDNA expressed reductase and cytochrome b5 were purchased from Corning Inc. Life Sciences (Tewksbury, MA, USA). β -Nicotinamide adenine dinucleotide phosphate (NADP), β -nicotinamide adenine dinucleotide hydrate (NAD^+), D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase, D-saccharic acid 1,4-lactone monohydrate, UDP-glucuronic acid (UDPGA) (trisodium salt), adenosine 3'-phosphate 5'-phosphosulfate (PAPs) and 2-mercaptoethanol were all purchased from Sigma Aldrich (St. Louis, MO, USA). Alamethicin and omeprazole were from J&K Scientific (Beijing, CN).

1H,1H,2H,2H-Perfluoro-1-decanol (8:2 FTOH) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Internal standards, 1H,1H-perfluorononan-1-ol (8:1 FTOH) and 1D,1D,2H,2H-¹³C₂-perfluoro-1-decanol (m8:2 FTOH), were purchased from Alfa Aesar (WardHill, MA, USA) and Wellington Laboratories (Guelph, ON, CA), respectively. 8:2 fluorotelomer aldehyde (8:2 FTAL) was synthesized as described by Napoli *et al.*³⁶ The gas chromatogram and mass spectrum of the synthesized 8:2 FTAL are shown in ESI Fig. S1.† The abbreviations and molecular

structures of 8:2 FTOH and the analogs are presented in ESI Table S1.†

2,4-Dinitrophenylhydrazine (DNPH) was from J&K Scientific (Beijing, CN). C18 cartridges (600 mg of sorbent) were obtained from Thermo Fisher Scientific (Ottawa, ON, CA). Acetonitrile was of LC-MS grade and methyl *tert*-butyl ether (MTBE) was of GC grade. Deionized water (18.2 MΩ) was generated on a Milli-pore Milli-Q water purification system.

2.3 In vitro studies of 8:2 FTOH metabolism

2.3.1 P450-specific metabolism of 8:2 FTOH. Recombinant human CYPs, including CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 (21.4 pmol mL⁻¹), were individually incubated with 4 μM 8:2 FTOH in a 200 μL NADPH-regenerating system (consisting of 100 mM potassium phosphate buffer, 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM MgCl₂, and 0.4 units per mL glucose-6-phosphatedehydrogenase at pH 7.4). The samples were pre-incubated at 37 °C for 5 min prior to the addition of ice-cold enzymes. The vials were immediately sealed and incubated at 37 °C for 0, 20, 40, 60, 120 and 240 min.

2.3.2 HLM-mediated phase I metabolism of 8:2 FTOH. Microsomes are vesicle-like artifacts re-formed from pieces of the endoplasmic reticulum when eukaryotic cells were subjected to differential centrifugation. HLMs contain various membrane-bound enzymes, including CYPs and UGTs. We used HLMs as the source of CYPs to investigate the phase I metabolism. 0.5 mg mL⁻¹ HLMs was incubated with 4 μM 8:2 FTOH using the same procedure as that described in 2.3.1 for up to 4 h.

Omeprazole is a selective inhibitor of CYP2C19 and CYP3A4. We incubated 8:2 FTOH with HLMs in the presence of omeprazole to further demonstrate the CYP2C19 contribution. Briefly, HLMs (0.5 mg mL⁻¹) were preincubated with omeprazole (50 μM) in the NADPH-regenerating system for 5 min, and then 8:2 FTOH (4 μM) was added and incubated at 37 °C as described above.

2.3.3 8:2 FTOH glucuronidation assay. HLMs were used to provide UGTs in this study. The glucuronidation reactions were carried out in a total volume of 200 μL containing phosphate buffer (0.1 M, pH 7.4), HLMs (0.5 mg mL⁻¹), MgCl₂ (10 mM), saccharolactone (5 mM) and 8:2 FTOH (4 μM). HLMs were previously activated by adding the pore-forming polypeptide alamethicin (50 μg mg⁻¹ protein) and incubated on ice for 30 min.³⁷ Saccharolactone was used to inhibit the glucuronide product hydrolysis caused by endogenous β-glucuronidases. After a 5 min preincubation, reactions were initiated by the addition of UDPGA (5 mM in incubation). The incubation vials were sealed and incubated at 37 °C for up to 2 h.

2.3.4 8:2 FTOH sulfation assay. The cytosolic fraction was produced by ultracentrifugation of eukaryotic cells and contains various soluble enzymes, including SULTs. We used cytosol as the source of SULTs in the present investigation. The sulfation reactions were carried out in a total volume of 200 μL potassium phosphate buffer (0.25 M, pH 7.0) containing 0.2 mM PAPs, 7.5 mM 2-mercaptoethanol, and 4 μM 8:2 FTOH. Cytosol (0.5 mg mL⁻¹ in incubation) was added to start the reaction, which was carried out at 37 °C for 0, 10 and 20 min.

2.4 Metabolic kinetics

The $T_{1/2}$ value was calculated according to the following equation:

$$T_{1/2} = \ln 2 / -k \quad (1)$$

where k represents the elimination rate constant and is calculated as the negative slope of the line defined by the linear regression of the natural logarithm of the percent remaining concentration of 8:2 FTOH over time.

For calculating the apparent kinetic parameters (K_m and V_{max}) of CYP2C19- and HLM-mediated phase I metabolism, as well as the HLM-mediated (glucuronidation) and cytosol-mediated (sulfation) phase II metabolism, recombinant human CYP2C19 (25 pmol mL⁻¹), HLMs (0.5 mg mL⁻¹) and HLC (0.5 mg mL⁻¹) were individually incubated with various 8:2 FTOH concentrations using the incubation conditions described above. The incubation time was previously optimized to ensure linear degradation of the substrate over time. The velocity of the reactions was obtained from the depletion of the parent compound, not the production of the metabolites, which are not commercially available. Using Sigma Plot version 12.5 (Systat Software, Inc., San Jose, CA, USA), we determined the kinetic values. The Michaelis-Menten equation is as follows:

$$v = \frac{V_{max} \times S}{K_m + S} \quad (2)$$

where v is the rate of reaction, K_m is the Michaelis-Menten constant, V_{max} is the maximum velocity, and S represents the substrate concentration.

The intrinsic clearance (CL_{int}) value was calculated according to the following equation:

$$CL_{int} = V_{max} / K_m \quad (3)$$

2.5 GC-MS analysis

The MTBE extracts were analyzed on an Agilent Technologies (Palo Alto, CA) 7890A GC system equipped with a 5975C mass detector (MSD) and a programmable temperature vaporizing (PTV) injection system. All samples were monitored in the electron impact (EI) ionization mode.³⁸ Compound separation and quantification were performed on an HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Company) using helium as the carrier gas. Samples (2 μL) were introduced into the capillary inlet (injector temperature 225 °C). The oven had an initial temperature of 60 °C which was maintained for 1 min, after which the temperature was increased to 130 °C at 5 °C min⁻¹, and then ramped to 300 °C at 50 °C min⁻¹, where it was held for 5 min. The ion source and quadrupole were at 230 and 150 °C, respectively.

The GC-MS was initially operated in the full-scan mode (scanning from m/z 50 to 600) to confirm the fragment ions (m/z) corresponding to 8:2 FTOH. Quantification was performed based on the single ion monitoring (SIM) mode using a standard calibration curve and internal standard 8:1 FTOH.

2.6 Structural conformation of the metabolites

2.6.1 Phase I metabolite. The phase I metabolite of 8:2 FTOH was identified as described by Nabb *et al.*⁶ with some modifications. Briefly, 8:2 FTOH (40 μM) and CYP2C19 (25 pmol mL^{-1}) were incubated in the presence of the NADPH-regenerating system as described in 2.3.2 for 40 min. The tubes were vortexed for 10 min after adding 400 μL acetonitrile and then centrifuged at $15\,000 \times g$ for 10 min at 10°C . The supernatant (300 μL) was transferred into another tube, mixed with 300 μL acetonitrile and 300 μL DNPH solution (consisting of 0.3% (w/v) DNPH, 51% (v/v) nanopure water, 29% (v/v) concentrated hydrochloric acid, and 20% (v/v) acetonitrile), and vortexed vigorously. The mixture was transferred into an HPLC vial and analyzed by HPLC/ESI-Q-TOF-MS.

2.6.2 Phase II metabolites. Glucuronide and sulfate conjugates were extracted as previously described⁷ with minor modifications. Briefly, the incubation solutions were extracted twice with 1 mL MTBE, blown to dryness under nitrogen, taken up in 1 mL of methanol– H_2O (1 : 1, v/v), and analyzed by HPLC/ESI-Q-TOF-MS.

2.6.3 HPLC/ESI-Q-TOF-MS assay. Both phase I and phase II metabolites were qualitatively measured in this study by HPLC coupled with ESI-Q-TOF-MS. Compound separation was performed on a Waters XTerra C18 (2.1×150 mm, 5 μm) column. The gradient HPLC method used HPLC grade water (A) and acetonitrile (B) each containing 0.15% acetic acid as mobile phases with a gradient of 10% for 1.0 min then to 50% B at 1.1 min held until 2 min then to 95% B at 9.5 min, held until 12.0 min, then to 10% B at 12.1 min, and held until 15.5 min with a flow rate of 0.2 mL min^{-1} . The MS system was operated in the negative ionization mode with capillary and sample cone voltages of 2500 V and 30 V, respectively. The collision energy was 20 eV. The source temperature was 80°C .

2.7 Quality assurance and quality control

For GC-MS analysis of 8:2 FTOH, calibration data were obtained in a linear range from 0.01 to $3\text{ }\mu\text{g mL}^{-1}$ of 8:2 FTOH. The standard calibration curve exhibited good and reproducible linearity with the R^2 value > 0.999 . We calculated the limits of detection (LODs) and limits of quantification (LOQs) of the GC-MS method for the detection of 8:2 FTOH based on the standard deviation of the signals arising from blanks. LODs and LOQs are defined as the minimum concentrations of 8:2 FTOH producing a signal-to-noise ratio (S/N) of 3 and 10, respectively. The LODs and LOQs are 0.5 and 2 ppb, respectively. The extraction recovery of 8:2 FTOH was determined by spiking m8:2 FTOH into the samples which were incubated with heat-inactivated enzymes (kept at 50°C for 30 min). The recovery was $(81.3 \pm 5.3)\%$ (mean $\pm$ SD, $n = 3$).

2.8 Molecular docking

The chemical structure of 8:2 FTOH was constructed in ChemDraw (CambridgeSoft Corporation, Cambridge, MA) and was MOPAC energy-minimized using Chem3D (CambridgeSoft).³⁹ The protein template of CYP2C19 was based on

the X-ray crystal structure 4GQS, an inhibitor (0XV) cocrystal structure of CYP2C19,⁴⁰ and was further modified by adding polar hydrogens, Kollman partial charges, and solvation parameters with AutoDock Tools. We applied AutoDock Vina 1.1.2 (The Scripps Research Institute, La Jolla, CA, USA)⁴¹ to define the substrate binding space in CYP2C19, a $25\text{ }\text{\AA} \times 25\text{ }\text{\AA} \times 25\text{ }\text{\AA}$ cubic region covering the active site cavity above the heme porphyrin, which precalculated the grids of van der Waals, hydrogen bonding, and electrostatic, torsional, and solvation interactions between the protein and 8:2 FTOH. The docking process was accomplished on Pfizer's high-performance computing Linux (Linux Torvalds, Santa Clara, CA) clusters using AutoDock 4.0 (Scripps Research Institute, La Jolla, CA, USA), which searched globally energetically optimized conformations and orientations of 8:2 FTOH using the Lamarckian genetic algorithm. A total of 100 million evaluations were performed for each output binding pose, followed by clustering 200 poses by a root-mean-square deviation (RMSD) method. The lowest energy pose in the proximity of heme iron was chosen for analysis using Pymol and Discovery Studio 4.5 Visualizer (Accelrys Inc., San Diego, CA).

The docking of 8:2 FTOH to the active sites of other P450 isoforms (PDB ID 4I8V, 2HI4, 3PMO, 3T3Q, 3QOA, 1PQ2, 1OG2, 4WNW, 3E4E, and 4K9W) was conducted by a similar method as described above.

3 Results and discussion

3.1 Identification of the P450 enzymes involved in the oxidative biotransformation of 8:2 FTOH

Previous studies have demonstrated that $\sim 75\%$ of enzymatic reactions with drugs are catalyzed by CYPs, while $\sim 90\%$ of the P450 reactions can be accounted for by a set of five CYPs, P450s, 1A2, 2C9, 2C19, 2D6, and 3A4. Besides, 1A1, 1A2, 1B1, 2A6, 2E1, and 3A4 accounted for 77% of the P450 activation reactions of carcinogens.⁴² Therefore, the eleven enzymes used in this study could cover most of the important CYPs responsible for the metabolism of drugs and contaminants in the human body.^{43,44} The enzymes were individually incubated with 8:2 FTOH to select only those enzymes with a relatively rapid biotransformation for the substrate. We considered 15% loss of the parent compound in 4 h as a minimum to reliably distinguish metabolism from experimental error. The results showed that only CYP2C19 metabolized 8:2 FTOH to a measurable extent (Fig. 1a and S2†), while other CYPs demonstrated low or no metabolism of 8:2 FTOH. Besides, the CYP2C19 selective inhibitor (omeprazole) could reduce the 8:2 FTOH depletion almost completely (Fig. 1c). These results demonstrated that 8:2 FTOH is eliminated primarily by CYP2C19. Even if there may be some other enzymes that could potentially catalyze 8:2 FTOH oxidation, they will not be significant because they are minimally expressed in humans. CYP2C19 is mainly expressed in human liver and is of clinical importance because it is involved in the metabolism of diverse drugs including omeprazole and other important proton pump inhibitors, certain tricyclic antidepressants such as imipramine and some barbiturates.⁴⁵ CYP2C19 is also of environmental significance for it is

Fig. 1 8:2 FTOH metabolism by CYP2C19 and HLMs. (a) 8:2 FTOH ($4 \mu\text{M}$) incubated with $21.4 \text{ pmol mL}^{-1}$ human CYP2C19 for up to 4 h. (b) Degradation velocity of 8:2 FTOH calculated when a range of 8:2 FTOH concentrations (0.5 to $50 \mu\text{g mL}^{-1}$) were incubated with 25 pmol mL^{-1} CYP2C19 for 40 min. (c) 8:2 FTOH ($4 \mu\text{M}$) incubated with 0.5 mg mL^{-1} HLMs for up to 4 h. (d) Degradation velocity of 8:2 FTOH calculated when a range of 8:2 FTOH concentrations (0.5 to $50 \mu\text{g mL}^{-1}$) were incubated with 0.5 mg mL^{-1} HLMs for 40 min. Samples were prepared in triplicate.

responsible for the bioactivation of some environmental toxins, including benzo[a]pyrene (B[a]P).⁴²

After 60 min of incubation, 53.2% of the parent compound was biotransformed, which indicates that CYP2C19 catalyzed 8:2 FTOH with a high efficiency. Fig. 1b shows the Michaelis-Menten kinetic parameters (K_m and V_{max}) obtained from the Michaelis-Menten plots based on the velocity of 8:2 FTOH degradation by CYP2C19. The values are $18.8 \mu\text{M}$ and $8.52 \text{ pmol min}^{-1} \text{ pmol}^{-1}$ P450, respectively. The CL_{int} value was calculated to be $0.453 \mu\text{L min}^{-1} \text{ pmol}^{-1}$ CYP2C19.

As shown in Fig. 1c and d, HLMs were also able to catalyze the biotransformation of 8:2 FTOH, although to a lesser extent. The K_m value is $18.5 \mu\text{M}$, which is close to that observed for CYP2C19. The V_{max} and CL_{int} values for HLM metabolism are $204 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein and $11.0 \mu\text{L min}^{-1} \text{ mg}^{-1}$ protein, respectively. The HLMs used in this study contained $\sim 470 \text{ pmol P450/mg protein}$,⁴⁴ in which CYP2C19 accounted for approximately 1–5% of the total CYPs.^{46,47} Therefore, the V_{max} and CL_{int} values for the HLM biotransformation are in the range of $(8.68\text{--}43.4) \text{ pmol min}^{-1} \text{ pmol}^{-1}$ CYP2C19 and $(0.469\text{--}2.35) \mu\text{L min}^{-1} \text{ pmol}^{-1}$ CYP2C19, respectively, which are in good agreement with the values observed for CYP2C19. The $T_{1/2}$ values, calculated according to eqn (1), are 77.9 min and 383 min for CYP2C19 and HLM incubation, respectively. Both show that 8:2 FTOH was rapidly metabolized under P450s catalysis.

In 2009, Martin and colleagues found that a selective CYP2E1 inhibitor, but not alcohol dehydrogenase (ADH) or CYP3A inhibitors, reduced the toxic effects caused by the intermediate metabolites in rats after dosing 8:2 FTOH and concluded that CYP2E1 controlled the metabolism of 8:2 FTOH in rats.²⁷ However, in our study, we did not find any degradation of 8:2

FTOH when incubated with human CYP2E1. This discrepancy can be attributed to the interspecies difference because the P450 family shows appreciable interspecies differences between humans and animal models; for example, rodents do not express CYP2C19 enzymes.⁴⁷ Our finding suggests that caution should be taken when extrapolating metabolism data of 8:2 FTOH from animal models to humans. Besides, CYP2C19 is a minor isoform in humans compared with CYP2C8 and CYP2C9 and is highly polymorphic. Poor metabolizers (PMs) of CYP2C19 represent approximately 3–5% of Caucasians, a similar percentage of African-Americans and 12–100% of Asian groups.^{45,47} People with a higher expression of CYP2C19 may produce higher levels of 8:2 FTAL and thereby, yield more downstream PFAAs and induce higher health risk. Additionally, D'Eon *et al.*² found that the elimination half-life of PFOA after the ECF phase out in 2000–2002 was longer than that in Olsen's results⁴⁸ and implicated that 8:2 FTOH metabolism may be of significance for PFOA exposure. However, some other studies claimed that 8:2 FTOH metabolism has a minimal contribution.^{6,49,50} This discrepancy may be related to the differential expression of CYP2C19 in the human body. Therefore, the genetic variability may also play an important role in the human metabolism of 8:2 FTOH.

3.2 Phase II metabolism of 8:2 FTOH

Phase II metabolizing enzymes, especially microsomal UDP-glucuronosyltransferases (UDPGTs) and cytosolic sulfotransferases (SULTs), contain a superfamily of enzymes. Phase II metabolism involves the attachment of additional molecules to the substrates to create inactive and more hydrophilic

compounds thereby enhancing their biliary and renal excretion. In general, glucuronidation and sulfation lead to the termination of pharmacological and toxicological potency, and phase II metabolism is primarily considered to be storage of the parent compound, although exceptions exist.⁵¹ Therefore, phase II metabolism plays an important role in the metabolism, elimination, and detoxification of both xenobiotics and endogenous substrates.

As shown in Fig. 2, 8:2 FTOH could also be biotransformed by the UDPGTs and SULTs. Using the standard incubation conditions described in 2.3.3 and 2.3.4, enzyme kinetic parameters for the glucuronidation and sulfation of 8:2 FTOH were determined in HLMs and HLC, respectively. The CL_{int} values of the HLM- and HLC-mediated metabolism are 9.11 and 17.3 $\mu\text{L min}^{-1} \text{mg}^{-1}$ protein, respectively.

Compared with the phase I metabolism of 8:2 FTOH, phase II metabolism showed higher efficiency following the order of sulfation > oxidation > glucuronidation, which is in good agreement with a previous study, that is, phase II metabolism often succeeds phase I metabolism.⁵² Therefore, a large portion of 8:2 FTOH would be excreted *via* feces upon dosing. This might be the reason that a significant portion of the mass balance of 8:2 FTOH transformation was associated with phase II conjugates and protein adducts, while PFOA, which is a downstream metabolite in phase I metabolism, is actually a minimal product.^{6,7}

3.3 Metabolite identification

3.3.1 Phase I metabolites. We added DNPH into the incubation, the aldehyde products could react with DNPH to form hydrazone derivatives. The metabolites were identified by HPLC/ESI-Q-TOF-MS. Fig. 3a and b show the retention time of the selected ion m/z 641 and the corresponding mass spectrum; both are identical to the DNPH derivatized 8:2 FTAL standard (Fig. S3†). Therefore, the metabolite of 8:2 FTOH catalyzed by CYP2C19 was identified as 8:2 FTAL. The later eluting peak at m/z 621 (12.56 min) in Fig. 3a and c was suggested to be DNPH derivatized 8:2 FTUAL according to Martin *et al.*⁷ Besides, based on relative instrument responses of the ions m/z 641 and m/z 621, we can conclude that 8:2 FTAL is quite unstable and easy to eliminate HF.

Fig. 3 HPLC-MS/MS chromatograms and product ion spectra of the DNPH derivatized metabolites of 8:2 FTOH after being incubated with CYP2C19 for 40 min. (a) Selective ion chromatograms at m/z 641 (12.29 min) and m/z 621 (12.56 min). (b) Product ion spectra of the m/z 641 peak. (c) Product ion spectra of the m/z 621 peak.

In vitro mechanistic investigations have demonstrated that the highly reactive FTUAL could form covalent adducts with GSH,⁵³ nucleophilic amino acids (including cysteine, histidine, lysine and arginine),⁵⁴ and proteins in liver microsomes and blood plasma.⁵⁴ An *in vivo* study also found protein adducts in the plasma, liver, and kidney of rats administered with 8:2 FTOH or 6:2 polyfluoroalkyl phosphate diester (6:2 diPAP).⁵⁵ Rand *et al.*⁵⁶ demonstrated that binding of 8:2 FTUAL to proteins might be the primary fate in the biotransformation of 8:2 FTOH. Covalent binding of FTUAL with proteins could affect protein functions and thereby induce serious toxic effects. Besides, FTUAL could be further oxidized and finally produce PFAAs; long term exposure of 8:2 FTOH would accumulate a high level of PFAAs and induce hepatotoxicity,¹² while the conjugate of FTUAL with GSH could be further transformed and finally produce a nephrotoxic thiol metabolite.^{9,57} Overall, the initial oxidative reaction is a bio-activation process for this kind of compound. Therefore, the CYP2C19 mediated oxidative biotransformation plays a vital role in the transformation and toxicity of 8:2 FTOH.

Fig. 2 (a) Metabolic kinetics of the 8:2 FTOH glucuronidation; HLMs (0.5 mg mL^{-1}) were incubated with 8:2 FTOH (0.5 to $80 \mu\text{g mL}^{-1}$) for 60 min (as described in 2.3.3). (b) Metabolic kinetics of the 8:2 FTOH sulfation; HLC (0.5 mg mL^{-1}) was incubated with 8:2 FTOH (0.5 to $80 \mu\text{g mL}^{-1}$) for 20 min (as described in 2.3.4). The degradation rates were determined based on the depletion of the 8:2 FTOH. Error bars represent standard error for $n = 3$ sample replicates.

3.3.2 Phase II metabolites. The structures of phase II metabolites were elucidated by LC-MS/MS using a combination of full scan and diagnostic interpretation of product ion spectra. For 8:2 FTOH-glucuronide, the expected molecular ion appeared at m/z 639 ($[M - H]^-$) in the negative ion mode. The mass spectrum of m/z 639 gave a fragment ion at m/z 193 (loss of the fluorinated carbon chain) (Fig. 4a). The product ion spectrum of 8:2 FTOH-sulfate told a similar story. The expected pseudomolecular ion appeared at m/z 543 ($[M - H]^-$) in the negative mode and yielded an abundant product ion at m/z 97, corresponding to sulfate (Fig. 4b). A neutral loss of 20 was detected in both instances, which was apparent in most poly-fluorinated metabolites.

3.4 Structure-based modeling of 8:2 FTOH with CYP2C19

In an attempt to rationalize the differences in the metabolism of 8:2 FTOH between CYP2C19 and other isoforms, 8:2 FTOH was docked to the active sites of the CYPs. Molecular docking is

a commonly used approach to elucidate the important interactions between substrates and active site residues of enzymes.^{39,58,59} After modeling, the poses with the lowest binding energy were chosen for analysis. For CYP-mediated oxidative metabolism to take place, the site of metabolism should be in proximity to the heme iron with a favored binding angle (90°) for electron transfer reactions to occur.^{39,58,59}

Fig. 5a shows the energetically favored binding pose of 8:2 FTOH at the active site of CYP2C19, which is lined with the helix I and B-C loop above the heme porphyrin. It appears that specific electrostatic interactions exist between 8:2 FTOH and the active residues including Val 208, Leu 233, Leu 237, Gly 296 and Ala 297 (Fig. 5b). With these key forces, 8:2 FTOH is juxtaposed above the heme porphyrin ring to expose its hydroxyl oxygen to the P450 compound (2.3 Å from the oxygen atom to heme iron atom of CYP2C19) with a favored binding angle (87.7° from the line of oxygen and iron atoms to the porphyrin plane). This conformation is very similar to that

Fig. 4 Product ion spectra of phase II metabolites of 8:2 FTOH after being incubated with HLMs and HLC, respectively. The products were identified as (a) 8:2 FTOH-glucuronide and (b) 8:2 FTOH-sulfate.

Fig. 5 (a) Molecular docking pose of 8:2 FTOH in the active site of CYP2C19. Conformation and orientation were predicted by AutoDock with the lowest energy-binding cluster close to the heme. CYP2C19 is shown in a cartoon format, with heme in blue and docked 8:2 FTOH in green. (b) 2D diagram shows the interactions between 8:2 FTOH and the active site residues of CYP2C19.

Table 1 Molecular docking results show the distances and angles of the energetically favored binding poses of 8:2 FTOH after docking to the active sites of eleven selected P450 enzymes

P450 isoforms	Distance ^a (Å)	Angle ^b
CYP2C19	2.3	87.7°
CYP1A2	3.9	54°
CYP2D6	4.9	49.7°
CYP1A1	5.5	64.7°
CYP1B1	6.9	62.9°
CYP2B6	7.0	104.6°
CYP2A6	7.5	105°
CYP2C8	7.6	91.7°
CYP2C9	8.0	95.4
CYP2E1	9.1	47.6°
CYP3A4	9.7	19.7°

^a Defined as the distance from the hydroxyl oxygen atom to the heme iron atom. ^b Defined as the angle from the line of oxygen and iron atoms to the porphyrin plane.

found for the inhibitor (0XV) molecule of the CYP2C19 cocrystal structure template.⁴⁰ However, the velocity of oxidation of 8:2 FTOH by other P450 isoforms would be expected to be low mainly because of the relatively long distance from the oxygen atom to the heme iron atom, as well as the unfavored angle (Table 1), for the formation of the tetrahedral intermediates, which is crucial for the oxidative transformation.

4 Conclusions

To conclude, the initial biotransformation of 8:2 FTOH includes phase I and phase II metabolism. Human CYP2C19 is the primary isoform engaged in the oxidative biotransformation of 8:2 FTOH. 8:2 FTAL was identified to be the product under CYP2C19 catalysis, which could eliminate HF non-enzymatically to form 8:2 FTUAL. 8:2 FTOH could also be catalyzed by microsomal UDPGTs and cytosolic SULTs, leading to the formation of glucuronide and sulfate conjugates. Overall, phase II metabolism showed higher efficiency than the phase I reaction, and the major fate of 8:2 FTOH could be excretion. These results provide new insight into fluorotelomer alcohol biotransformation by identifying the key human CYP isoform and quantifying metabolic kinetics. They will help future epidemiological studies in the assessment of indirect sources of human exposure to perfluoroalkyl acids and potential health risks of fluorotelomer alcohols.

Acknowledgements

This work was supported by the Chinese Academy of Sciences (XDB14040100) and National Natural Science Foundation of China (21321004, 21375143 and 91543203).

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