Evaluation of perfluorooctanoate for potential genotoxicity

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ABSTRACT

Perfluorooctanoate (PFOA) is a fully fluorinated eight-carbon fatty acid analog with exceptional stability toward degradation that has been used as an industrial surfactant and has been detected in environmental and biological matrices. Exposures to PFOA in the workplace and in the environment have continuously stimulated investigations into its potential human health hazards. In this article, the results of fifteen unpublished genotoxicity assays conducted with perfluorooctanoate (as either the linear or linear/branched ammonium salt (APFO) or the linear/branched sodium salt) are reported and include: seven mutation assays (three in vitro reverse mutation assays with histidine auxotrophic strains of Salmonella typhimurium, two in vitro reverse mutation assays with the tryptophan auxotrophic Escherichia coli WP2uvr strain, one in vitro mitotic recombination (gene conversion) assay with Saccharomyces cerevisiae D4, and an in vitro Chinese hamster ovary (CHO) HGPRT forward mutation assay); seven studies to assess potential for chromosomal damage (three in vitro CHO chromosomal aberration studies, an in vitro human whole blood lymphocyte chromosomal aberration study, and three in vitro mouse micronucleus assays); and an in vitro C3H 10T1/2 cell transformation assay. Although PFOA has not been demonstrated to be metabolized, all in vitro assays were conducted both in the presence and in the absence of a mammalian hepatic microsomal activation system. These assays were originally described in twelve contract laboratory reports which have been available via the United States Environmental Protection Agency public docket (Administrative Record 226) for over a decade; however, the details of these assays have not been published previously in the open scientific literature. With the exception of limited positive findings at high and cytotoxic concentrations in some assay trials which reflected the likely consequence of cytotoxic disruption of normal cellular processes and not a specific genotoxic effect, the results of the studies presented in this paper and other published results clearly demonstrate the absence of direct mutagenic or genotoxic risk associated with PFOA. This finding is consistent with the physical/chemical characteristics of PFOA and is supported by other published genotoxicity studies.

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1. Introduction

Perfluorooctanoate (PFOA, CF3(CF2)6CO2−, CASRN 335-67-1) is a perfluorinated carboxylate that has been used principally in the form of the ammonium salt (NH4+)PFOA or APFO, CF3(CF2)6CO2NH4, CASRN 3825-26-1) as an
emulsifier in the production of fluoropolymers such as polytetrafluoroethylene. The carbon-fluorine bonds impart extreme stability against degradation of the PFOA molecule. The stability of PFOA renders it persistent in environmental and biological matrices. PFOA has been found widely distributed in human blood at low part per billion levels \[1,2\] and has been identified in wildlife and environmental media \[3\].

The toxicology of PFOA has been studied extensively and detailed review have been published \[4–6\]. In laboratory toxicology study with mice, exposures to PFOA leads to decrease in body weight, triglyceride, and cholesterol. The serum elimination half-life is approximately 2–3 weeks in either male or female mice and the acute oral LD50 is approximately 450 mg/kg. PFOA is tightly bound to serum proteins and accumulates in the liver, which is the primary target organ for PFOA toxicity. In rodents such as mice, the hepatic changes are susceptible, largely due to nuclear xenosensor receptors PPARα activation which subsequently leads to hepatocellular hypertrophy and proliferation. PFOA can cause developmental delays and neonatal mortalities in mice when pregnant dams are exposed to PFOA during gestation; however, most developmental toxicities appeared to be PPARα-mediated \[7\] and were not observed when PPAR knockout mice were evaluated.

Even though the biological properties of PFOA, including genotoxicity, have been well characterized and reviewed \[4–6\], the details of many key studies conducted in the past that have examined PFOA for potential genotoxicity have not been published. Although the reports for these studies have been made public and available through the USEPA Administrative Record (Docket AR226), for greater accessibility, the experimental details and results of these key studies are summarized and presented herein. Information from other published studies is also included in the discussion to provide a more complete evaluation of perfluorooctanoate for potential genotoxicity.

### 2. Materials and methods

#### 2.1. Materials

Five perfluorooctanoate (PFOA) test materials were used in conducting the genotoxicity studies reported herein. The characteristics of these five samples and the specific studies in which they were used are summarized in Table 1. The purity of the solids used in all samples was ≥95%. Four of the PFOA samples were tested as the ammonium salt (APFO), and one was tested as the sodium salt (Na+ PFOA). Three of the APFO samples and the Na+ PFOA sample were produced by electrochemical fluorination (ECF) and contained approximately 22% branched isomers of PFOA; for example, as representative lot may contain 12.6% internal monomethyl (non-alpha); 9% isopropy; 0.2% tert-butyl; 0.1% gem-dimethyl; and 0.1% alpha monomethyl. Impurities in ECF-produced PFOA primarily consisted of lesser homologs (C4–C7) at approximately 2% with additional minor impurities (monohydro APFO, monounsaturated APFO, and undefined (possibly) substituted perfluorocyclo species). One of the APFO samples

| Sample ID | Salt form | Process Lot# | Purity(%) | Form | Study ID | Endpoint | Test systems | Test media | Identities | Final report date | Study ID | Genotoxicity | Test systems | Study ID | Genotoxicity | Test systems | Study ID | Genotoxicity | Test systems | Study ID | Genotoxicity |
|-----------|-----------|---------------|-----------|------|----------|----------|--------------|-------------|------------|-----------------|----------|--------------|--------------|----------|--------------|--------------|----------|--------------|--------------|----------|--------------|
| A         | NH₄⁺      | ECF           | 95        | Solid| 1        | Mutation | S. cerevisiae | CHO 101/2  | In vitro     | May 1993       | 2015    | 326-0429     |   |         |              |           |              |              |           |              |              |           |              |
| B         | NH₄⁺      | ECF           | 95        | Solid| 2        | Cell transformation | CHO 101/2 | In vitro     | May 1981       | 2015    | 326-0428     |   |         |              |           |              |              |           |              |              |           |              |
| C         | NH₄⁺      | ECF           | 95        | Solid| 3        | Mutation | S. cerevisiae | CHO 101/2  | In vitro     | May 1996       | 2015    | 326-0699     |   |         |              |           |              |              |           |              |              |           |              |
| D         | NH₄⁺      | ECF           | 95        | Solid| 4        | Mutation | S. cerevisiae | CHO 101/2  | In vitro     | May 2002       | 2015    | 6889.7       |   |         |              |           |              |              |           |              |              |           |              |
| E         | Na⁺       | ECF           | 95        | Solid| 5        | Mutation | S. cerevisiae | CHO 101/2  | In vitro     | May 1993       | 2015    | 326-0430     |   |         |              |           |              |              |           |              |              |           |              |
2.2. Assays to assess potential to cause point mutations

2.2.1. Prokaryotic assay systems

2.2.1.1. S. typhimurium (Sample A, Study 1; Sample D, Study 8; Sample E, Study 12). Three test materials were used in three separate assays to evaluate potential mutagenicity utilizing S. typhimurium test strains TA98, TA100, TA1535, TA1537, and TA1538 (Study 1 only), with and without the presence of a metabolic activation system. The genotypic composition of these test strains is detailed in Mortelmans and Zeiger [8]. The test methods employed were originally described by Ames et al. [9] and Maron and Ames [10].

Griffith and Long [11] previously provided a brief description of a study in which S. typhimurium histidine auxotrophic strains TA98, TA100, TA1535, TA1537, and TA1538 were used to test APFO for potential mutagenicity (Sample A, Study 1). A more detailed description of methods is provided here and study data not shown by Griffith and Long are presented in the results. Liver S9 extract was isolated from an adult male Sprague Dawley rat after Acorol™ 1254 treatment. S9 liver extract (0.10 mL) was mixed with triphosphopyridine nucleotide (TPN) sodium salt (4 μmol), glucose-6-phosphate (5 μmol), sodium phosphate dibasic (100 μmol), MgCl₂ (8 μmol), and KCl (33 μmol) prior to each assay. The study was performed with the test strains both in the presence and absence of S9 activation. Approximately 10⁸ cells from an overnight culture of each indicator strain were added to separate test tubes containing 2.0 mL molten agar supplemented with biotin and histidine. APFO concentrations tested ranged from 0.1 μg to 500 μg. When microsomal activation was required, 0.5 mL of S9 mix was added. The solution was gently mixed and overlaid onto minimal agar plates. After overlay had solidified, the plates were incubated for 48 h at 37 °C for S. typhimurium strains. Vehicle control (50 μL DMSO per plate) was used as the negative control for all the assays while the positive controls used for each strain, with or without S9 activation, are listed in Table 2. Plates were evaluated both macroscopically and microscopically, and the number of revertant colonies was counted and reported. Criteria for a positive response were, in general, based on data from at least three non-cytotoxic concentrations, a positive dose-response, and at least a 2–3 fold

| Table 2 | Positive controls used in mammalian microsomal reverse mutation assays (Studies 1, 8, and 12). |
|---------|---------------------------------------------------------------------------------|
| Strain  | Mutagen Tested                                      | Positive Control | Negative Control |
|---------|----------------------------------------------------|------------------|------------------|
| TA98    | 2-Aminoanthracene                                  | 10               | 10               |
|         | 2-Aminooxanthenone                                  | 10               | 10               |
|         | 4-Nitroquinoline-2-Oxide                            | 1               | 1                |
| TA100   | 2-Aminoanthracene                                  | 10               | 10               |
|         | 2-Aminooxanthenone                                  | 10               | 10               |
|         | 4-Nitroquinoline-2-Oxide                            | 1               | 1                |
| TA1535  | 2-Aminoanthracene                                  | 10               | 10               |
|         | 2-Aminooxanthenone                                  | 10               | 10               |
|         | 4-Nitroquinoline-2-Oxide                            | 1               | 1                |
| TA1537  | 2-Aminoanthracene                                  | 10               | 10               |
|         | 2-Aminooxanthenone                                  | 10               | 10               |
|         | 4-Nitroquinoline-2-Oxide                            | 1               | 1                |
| TA1538  | 2-Aminoanthracene                                  | 10               | 10               |
|         | 2-Aminooxanthenone                                  | 10               | 10               |
|         | 4-Nitroquinoline-2-Oxide                            | 1               | 1                |
| E. coli | 2-Aminoanthracene                                  | 10               | 10               |
|         | 2-Aminooxanthenone                                  | 10               | 10               |
|         | 4-Nitroquinoline-2-Oxide                            | 1               | 1                |
| S. cerevisiae | D4                             | 10               | 10               |
|         | 7-Azinozoanthenone                                  | 1               | 1                |

* Not evaluated. 
* Equivalent of 100 μmol.
increase in revertants per plate as compared to the vehicle control at the highest dose.

In two additional assays using APFO (Sample D, Study 8) or Na"PFOA (Sample E, Study 12), S. typhimurium strains TA98, TA100, TA1535, and TA1537 were used. The APFO sample assay was run in duplicate; whereas, the Na"PFOA sample was tested once. Following dose range-finding studies, doses of 30–150 μg per plate APFO (Sample D, Study 8) and 20–100 μg per plate of Na"PFOA (Sample E, Study 12) were tested using triplicate plates at each concentration. Deionized water was used as the negative control, and the positive controls used for each strain were as listed in Table 2. When the S9 metabolic activation system was not incorporated, aliquots of each testing strain (100 μL) and test material (50 μL) were added to 2.5 mL of molten selective top agar maintained at 45 ± 2 °C. When S9 activation was required, 0.1 mL Aroclor™ 1254-treated male Sprague Dawley rat liver S9 extract homogenates (Molecular Toxicology, Inc., Boone, NC) was mixed with water (0.70 mL), 1 M NaH₂PO₄/Na₂HPO₄, pH 7.4 (0.10 mL), 0.25 M glucose-6-phosphate (0.02 mL), 0.10 M NADP (0.04 mL), and 0.825 M KCl (0.2 M MgCl₂ (0.04 mL)). Aliquots of each testing strain (100 μL), test material (50 μL), and S9 mix (500 μL) were added to 2.0 mL of molten selective top agar maintained at 45 ± 2 °C. The mixtures prepared above were vortexed and overlaid onto 15 mm × 100 mm agar plates. After overlay had solidified, the plates were inverted and incubated for 52 ± 4 h at 37 ± 2 °C. The mean and standard deviation of the number of revertant colonies per plate were calculated from the individual revertant colony counts from triplicate cultures at each concentration by strain. Criteria for a positive response were as described above.

2.2.1.2. E. coli reverse mutation assay (Sample D, Study 8; Sample E, Study 12). The potential mutagenicity of APFO (Study 8) and Na"PFOA (Study 12) was studied in the E. coli test system using the tryptophan auxotrophic strain WP2uvrA. Methods employed were originally described by Green and Muriel [12]. Preparation of cultures, dose ranges, and enumeration of revertant colonies were as described above for the S. typhimurium assays conducted as part of Studies 8 and 12 (Section 2.2.2.1.1). The positive control for the metabolic activation system was 2-aminoanthracene (25 μg/plate). When the metabolic activation system was not used, 4-nitroquinoline-N-oxide (1 μg/plate) was used as the positive control. The mean number of revertant colonies per plate by concentration and strain were counted and recorded as described above, and the criteria for a positive response were as described above for the S. typhimurium test strains (Section 2.2.1.1).

2.2.2. Eukaryotic assay systems

2.2.2.1. S. cerevisiae mitotic recombination (gene conversion) assay (Sample A, Study 1). Also described briefly by Griffith and Long [11] was an assay in which APFO (Sample A) was tested for potential mutagenicity via non-specific DNA damage leading to mitotic recombination expressed as gene conversion to produce prototrophic revertants in the auxotrophic heteroallelic D4 strain of S. cerevisiae. The assays followed the S. typhimurium procedures described above for Study 1 (See 2.2.1.1). Plates were evaluated both macroscopically and microscopically and the number of revertant colonies per plate were counted and reported. Study data not shown by Griffith and Long are presented in the results.

2.2.2.2. Chinese hamster ovary/HGPRT forward mutation assay (Sample C, Study 5). APFO was also evaluated for its potential to induce mutation at HGPRT locus of Chinese hamster ovary (CHO) cells. CHO cells were grown at 37 °C in complete Ham's F-12 medium (10% fetal bovine serum (FBS), 1–2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) and were buffered with 10 mM HEPES. Liver S9 homogenates isolated from male Sprague Dawley rat liver were purchased from Molecular Toxicology, Inc. (Boone, NC).

Plates were seeded with cells and incubated at 37 °C for 24 h followed by exposure to positive controls (EMS for the non-activated and DMBA for the S9-activated), negative control (Ham's F-12 medium), or APFO (9.75–39 μg/mL) for 17 h (non-activated) or 5 h (S9 activated). After exposure, cells were washed twice with phosphate buffered saline (PBS) and incubated for additional 48 h (non-activated) or 64.5 h (S9 activated) in Ham's F-12 medium. The cells were subsequently harvested, re-plated, and passed every 48–72 h for 6 days for phenotypic expression. Following this, cells were grown in selective medium specific for mutant cells for 9 days allowing for colony formation. The cells were rinsed with PBS, fixed in methanol, and stained with Giemsa. The results of the assay were evaluated based on the number of thioguanine-resistant mutants per 10⁶ surviving cells. The significance of the test results were determined by using the statistical program, Tallarida, R.S. and R.B. Murray's Pharmacological Calculations Procedure, ANOVA, and Newman–Keuls test for confirmation. The statistical method determined if there was a significant (p < 0.05) increase in the mutation frequency of the test article compared to the negative control article.

2.3. Assays to assess potential for chromosomal damage

2.3.1. Chromosomal aberrations in CHO cells (Sample C, Study 4; Sample D, Study 7; and Sample E, Study 11)

CHO cells used in this assay were obtained from a permanent cell line from the University of California San Francisco. This cell line has an average cycle of 12–14 h with a modal chromosomal number of 21. The CHO cells were grown in McCoy's 5a culture medium supplemented with 10% FBS, 1% l-glutamine, 1% penicillin and streptomycin at approximately 37 °C in the atmosphere of 5% CO₂.

Three test materials were evaluated in three separate studies using CHO cells both in the presence and absence of a metabolic activation system and with varying test material exposure and harvest times, as summarized in Table 3. These were two APFO samples (Sample C, Study 4; Sample D, Study 7) and one Na"PFOA sample (Sample E, Study 11). Positive controls used were mitomycin C (MMC) for assays without metabolic activation and cyclophosphamide (CP) for assays with metabolic activation. Negative controls for cultures contained only cells and medium while the solvent controls employed the highest concentration of
Table 3
CHO chromosomal aberrations assay summary (Studies 4, 7, and 11). For aberration evaluation, 100 metaphase cells from each replicate at 4 dose levels were analyzed.

| Sample | S9 activation | Exposure length (h) | Harvest time (h) | Trials | Dose range (µg/mL) | Doses aberrations evaluated (µg/mL) | Non-cytotoxic doses | Cytotoxic doses | Description of cytotoxicity |
|--------|---------------|---------------------|------------------|--------|-------------------|--------------------------------------|---------------------|----------------|---------------------------|
| C      | No            | 17.6                | 20               | 1      | 12.5–200          | 75–200 ≤75                           | No                  | ≥100          | Reduced cell monolayer confluence and reduced mitotic index. |
| D      | No            | 17.8                | 20.1             | 2      | 18.75–600         | 30–300 ≤150                         | No                  | ≥180         | Mitotic index lowered. |
| D      | No            | 3                   | 44.2             | 1      | 15–360            | 30–180 ≤60                           | No                  | ≥120         | Unhealthy monolayers, reduced mitotic cells, ~15% reduction in monolayer confluence. |
| E      | No            | 17.6–17.8           | 20               | 2      | 25–400            | 150–400 <149                         | No                  | ≥149         | Toxicity ranged from a 95% reduction in mitotic index at 400 µg/mL to a 25–57% reduction at 150 µg/mL; typical at higher concentrations were unhealthy cell monolayers, loss of mitotic cells, floating dead cells and debris, reduction in cell layer confluence. |
| E      | No            | 41.8                | 44.1             | 1      | 25–398            | 50–199 ≤99.6                         | No                  | ≥149         | Toxicity ranged from a 79% reduction in mitotic index at 199 µg/mL to a 18% reduction at 149 µg/mL; higher concentrations were lethal. |
| E      | No            | 3                   | 20               | 1      | 50–996            | 50–498 ≤250                          | No                  | ≥498         | At 498 (µg/mL) a 30% reduction in confluency was observed. Severe toxicity at the next dose (748 µg/mL). At 748 µg/mL, only 38 metaphase cells were available from the combined duplicates hence data at this concentration were excluded; 18% of cells were observed to have aberrations. |
| E      | No            | 3                   | 44               | 1      | 50–996            | 100–748 ≤250                         | No                  | ≥498         | At 498 µg/mL, there was a 39% reduction in confluency. At 748 µg/mL, one of the two duplicate cultures was observed to have 11% of cells with aberrations, while the parallel duplicate had 4% (similar to the negative and solvent control values). Cytotoxicity at 748 µg/mL: 50% reduction in confluency, 57% reduction in mitotic index, and increased % polyploidal cells in one of the two duplicate cultures. |
| Table | Yes/No | Dose (μg/mL) | Cell Monolayer Confluence | Mitotic Index | Dead | Toxicity |
|-------|--------|---------------|--------------------------|--------------|------|---------|
| C     | Yes    | 2 20 1       | 125–1500 125–750 ≤500 No | ≥750 No       | Dead monolayers, no mitotic cells, reduced cell monolayer confluence. |
| D     | Yes    | 3 20–20.1 2 | 75–1200 75–750 ≤450 No  | ≥600 No/Yes No | A 12.5% increase over control observed in one of the two assays at 750 μg/mL. Dose-related reductions in cell monolayer confluence, with reductions in mitotic cells at higher concentrations. At 750, 675, and 600 μg/mL, monolayer confluence was reduced by 45%, 30%, and 15%, respectively, and mitotic index by 30%, 11%, and 15%, respectively. |
| D     | Yes    | 3 44.2 1    | 150–900 600–825 ≤600 No  | ≥675 Yes      | A 13% increase over control in aberrations was observed at 825 μg/mL. Dose-related reductions in cell monolayer confluence, with reductions in mitotic cells at higher concentrations. At 825, 750, and 675 μg/mL, monolayer confluence was reduced 55%, 45%, and 15%, respectively, with mitotic index reductions of 53%, 56%, and 17%, respectively. |
| E     | Yes    | 3 20 2     | 50–1000 250–1000 ≤500 No  | ≥750 Yes      | 14.5–42.7% increases over control in aberrations at 750 and 1000 μg/mL, respectively. 70–95% reduction in monolayer confluence at 1000 μg/mL and 14–47% reduction in mitotic index; slight toxicity noted at 750 μg/mL. |
| E     | Yes    | 3 44.1 1   | 100–994 100–748 ≤498 No  | ≥748 No       | At 994 μg/mL, % polyploidy cells increased and mitotic index reduced by 70%. |

Cells increased and mitotic index reduced by 70%.
solvent used in the test system. All assays were conducted using duplicate cultures, and data based on the average of duplicate cultures was reported. Multiple experimental trials in the absence and presence of a metabolic activation system were performed for Samples D and E (Studies 7 and 11, respectively), while only one trial for each metabolic activation condition was performed for Sample C (Study 4).

Assays conducted in the absence of a metabolic activation system were performed as follows. In two studies with APFO, test concentrations of APFO ranged up to 600 μg/mL and in the study with Na⁺PFOA, test concentrations ranged up to 996 μg/mL. The MMC positive control concentrations ranged from 0.08 to 1 μg/mL. In all cases, cells were cultured for 24 h prior to treatment by seeding approximately 0.8–1.2 × 10⁶ cells per 75 cm² flask into 10 mL of McCoy’s 5a culture media followed by incubation with the test articles at 37 °C for 3–41.8 h (Table 3). Following incubation with test articles, cultures were washed with PBS and incubated with fresh medium containing Colcemid™ (0.1 μg/mL) for 2 h, after which the cultures were harvested from 20 to 44.2 h, based on study (Table 3), and fixed with absolute methanol:glacial acetic acid (3:1) fixative, dried, and stained with 5% Giemsa solution for the analysis of the mitotic index and chromosomal aberrations.

Assays conducted in the presence of a metabolic activation system were performed as follows. In the two studies conducted with APFO, tested ranged up to 1500 μg/mL (Sample C, Study 4; and Sample D, Study 7), and, for Na⁺PFOA, concentrations tested ranged up to 1000 μg/mL (Sample E, Study 11). Concentrations used for the positive control agent, CP, ranged from 5 to 10 μg/mL. Cells were seeded at approximately 0.8–1.2 × 10⁶ cells per 75 cm² flask in 10 mL of McCoy’s 5a culture media and were cultured for 24 h prior to treatment, after which cells were incubated for 2–3 h at 37 °C in McCoy’s 5a medium without FBS with added test articles and 99 reaction mixture. Treated cells were then washed with PBS and fresh medium and incubated with Colcemid™ (0.1 μg/mL) during the last 2 h of incubation. The metaphase cells were harvested after 20–44.2 h, fixed with absolute methanol:glacial acetic acid (3:1), washed, dried, and stained with 5% Giemsa solution for the analysis of the mitotic index and chromosomal aberration.

Stained cells were selected for good morphology, and only cells with the number of centromeres equal to the modal number of 21 ± 1 were analyzed for mitotic index and chromosomal aberrations. A total of 100 cells per dose level from each treatment were analyzed for different types of chromosomal aberrations. The number of mitotic cells per 1000 was reported as the mitotic index, and, in evaluation of chromosomal aberration data, the percent of cells with aberration, the percent of cells with more than 1 aberration, and evidence for increasing amount of damage with increasing dose was considered, along with evidence of polyploidy and endoreduplication.

In these assays, statistical analysis employed the Fisher’s Exact Test [13] to compare the percentage of cells with aberrations in each treatment group with the results from the solvent controls. A linear trend test of increasing number of cells with aberrations with increasing dose [14] was also performed. Significance was established when \( p < 0.01 \).

2.3.2. Chromosomal aberrations in human whole blood lymphocytes (Sample E, Study 10)

Human venous whole blood from a healthy adult male donor was drawn into a heparinized Vacutainer® tube. Whole blood lymphocyte cultures were obtained after heparinized blood (0.6 mL) was incubated with RPMI 1640 culture medium (9.4 mL, supplemented with 15% FBS, 100U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 1% phytohemagglutinin) at 37 °C, and cultures were incubated for 2 days before treatment with test compound. Na⁺PFOA (prepared in water) was evaluated in the whole blood lymphocyte culture at concentrations ranging from 12.4 μg/mL to 600 μg/mL. Dose levels of Na⁺PFOA were selected for the study based on the preliminary range finder data which evaluated cytotoxicity at doses ranging from 0.033 to 1000 μg/mL. Fresh RPMI 1640 culture medium was used as the negative control for the assay. CP (20–50 μg/mL in water) and MMC (0.1–0.3 μg/mL in water) were used as the positive controls for the assay in the presence and absence of S9 metabolic activation, respectively.

In performing the assay in the absence of a metabolic activation system, whole blood lymphocyte culture was initiated for 2 days followed by exposure to Na⁺PFOA for up to 46 h. The cultures were then washed with PBS followed by incubation with RPMI 1640 medium containing 0.1 μg/mL Colcemid™. Two hours later, the cells were harvested, dried, and stained in 5% Giemsa solution for analysis of chromosomal aberrations.

When metabolic activation was used in the assay system, a preparation of rat S9 liver extract (15 μL, Molecular Toxicology, Inc., Boone, NC) was mixed with NADP (1.5 mg/mL) and isocitric acid (2.7 mg/mL). Following 3 h of incubation with Na⁺PFOA or control substances in the presence of the S9 activation system, cultures were washed with PBS, replenished with fresh RPMI 1640 culture medium, and incubated for up to 46 h. The cells were incubated for the rest of the culture period up to the time of harvest with 0.1 μg/mL Colcemid™ present during the last 2 h of incubation. The metaphase cells were harvested and the resulting cell pellets were re-suspended with 75 mM KCl hypotonic solution, fixed with absolute methanol:glacial acetic acid (3:1, v/v), and a portion spread onto a microscope slide. After air-drying, the slide was stained with 5% Giemsa solution and evaluated for mitotic index and chromosomal aberrations.

Assessments of chromosomal aberrations and mitotic indices were performed by first selecting cells for appropriate morphology. Only cells with 46 centromeres (normal number) were analyzed. A total of 100 cells per dose level from each treatment were analyzed for different chromosomal aberrations. Evaluation of cultures was performed as noted above the assays with CHO cells (Section 2.3.1).

Cochran-Armitage test for linear trend and Fisher’s Exact Test [15] were employed to compare the percentage of cells with aberrations, polyploidy, and
endoreduplication. Test article significance was established where \( p < 0.01 \).

2.3.3. In vivo mouse micronucleus assays (Sample C, Study 3; Sample D, Study 6; Sample E, Study 9)

Three separate in vivo studies were conducted to assess the potential for PFNA to increase micronuclei in polychromatic erythrocytes isolated from mouse bone marrow. The methods employed were first described by Heddle et al. [16]. For all three studies, adult male and female Crl:CD-1® (ICR) BR mice (6–8 weeks old) were purchased from Charles River Laboratories (Portage, MI). All mice were housed in standard cages; rodent chow (Purina® Certified Laboratory Pellets® #5002) and tap water were provided \textit{ad libitum}. Environmental controls for the animal room were set to maintain a temperature of 72 ± 6 °F, humidity of 55 ± 15%, and a 12-h light/dark cycle. The study facility was accredited by the Association for Assessment and for the Accreditation of Laboratory Animal Care International. All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee. Animal care and procedures followed guidelines as specified the U.S. Department of Health and Human Services Guide for the Care and the Use of Laboratory Animals [17].

The three test articles and dosages utilized were as follows:

1. Na⁺PFNA (Sample E, Study 9) dose levels of 250 mg/kg, 500 mg/kg, and 1000 mg/kg were selected based on the preliminary range finder data which evaluated the acute oral toxicity of Na⁺PFNA which ranged from 100 to 1000 mg/kg. Na⁺PFNA solutions (prepared in deionized water) were administered orally as a single dose to groups of mice \((n=5/sex/dose group/time point). \) CP (80 mg/kg) and deionized water were used as positive and negative controls, respectively.

2. APFO (Sample C, Study 3) dose levels of 200 mg/kg, 400 mg/kg, and 800 mg/kg were selected for the study based on the preliminary range finder data which evaluated the acute oral toxicity of APFO which ranged from 200 to 1000 mg/kg. APFO solutions (prepared in deionized water) were administered orally as a single dose to primary groups of mice \((n=5/sex/dose group/time point). \) A secondary group of mice \((n=5/sex)\) were also treated with 800 mg/kg APFO as possible replacements should mortality occur within the primary group. CP (80 mg/kg) and deionized water were used as positive and negative controls, respectively.

3. APFO (Sample D, Study 6) was tested in the same manner as for Sample C (Study 3) except that dose levels of 150 mg/kg, 300 mg/kg, and 600 mg/kg were used (based on the preliminary range finder data which evaluated the acute oral toxicity of APFO ranging from 300 to 900 mg/kg), and the secondary group was dosed with 600 mg/kg APFO.

At designated times (24, 48, and 72 h post dose), mice treated with APFO or Na⁺PFNA were euthanized by CO₂ asphyxiation, and bone marrow was flushed from the bone into a centrifuge tube containing 3–5 mL of bovine serum. Following centrifugation and the removal of supernatant, a portion of the resulting bone marrow pellet was spread onto a microscope slide, dried, and fixed in methanol and stained with May–Grunwald and Giemsa. The mice treated with both the positive and negative control solutions were euthanized at 24 h post dose only and bone marrow samples were also harvested and evaluated as described above. Bone marrow cells were analyzed for the presence of micronuclei in both the polychromatic erythrocyte (PCE), and the mature normochromatic erythrocyte (NCE). Approximately 1000 PCEs per mouse were scored and the frequency of micronucleated cells was expressed as % micronucleated cells based on the number of PCEs analyzed. The spontaneous (background) micronuclei frequency was determined based on the number of PCEs observed in the optic fields while scoring the first 1000 erythrocytes on the slide. The normal frequency of micronuclei in this Crl-CD-1 (ICR) BR strain is about 0–0.4%. The ratios of PCE to NCE were calculated.

The analysis of data was performed using ANOVA [18] on either untransformed (when variances are homogeneous) or rank transformed (when variances are heterogeneous) proportions of cells with micronuclei per animal. If ANOVA was significant \((p<0.05)\), a Dunnett’s t-test [19,20] was used to determine which dose groups, if any, were significantly different from the negative control.

2.4. Cell transformation assay (Sample B, Study 2)

The potential of PFNA to transform mouse embryo-derived C3H 10T1/2 cells was studied \textit{in vitro} based on methods originally described by Reznikoff et al. [21,22]. Prior to performance of the transformation assay, cytotoxicity data were obtained and expressed as plating (cloning) efficiency. APFO (0.1–20 μg/mL) was added to the cultures that were seeded the day before at 300 cells per 60 mm diameter culture dish. After 24-h exposure (6 replicates per dose) in 5 mL Eagle’s basal medium supplemented with 10% FBS, APFO was removed with a complete change of media. Media was replenished every 3 days. After 7 days in culture, the plates were washed in PBS, fixed with absolute methanol, and stained with Giemsa. Solvent control (10 μg/mL DMSO) and serum baseline controls were included. The number of colonies per plate was counted and the plating efficiency (PE) was determined by the formula:

\[
\text{PE} = \frac{\text{average number of colonies per plate}}{\text{number of cells seeded per plate}} \times 100
\]

The study was conducted in two phases in that colony transformation was evaluated in the first phase while foci transformation was evaluated in the second phase. For the colony transformation assay, six replicates plates seeded with 300 cells per plate were treated with APFO (0.1–200 μg/mL), a positive control (benzo(a)pyrene, 0.1–10 μg/mL), and a solvent control (10 μg/mL DMSO) for 24 h. After removal of the chemicals, culture media was replenished every 3 days. After 14 days, the plates were washed in PBS, fixed with absolute methanol, and stained with Giemsa. Each colony was examined macroscopically and microscopically and scored for transformation according to criteria of Reznikoff et al. [21].
For the foci transformation assay, six replicates plates seeded with 300 cells per plate were treated with APFO (0.1–100 μg/mL), a positive control (butadiene diepoxide, 0.0001–0.01 μg/mL), and a solvent control (10 μg/mL DMSO). Following exposure for up to 38 days, the plates were washed in PBS to remove the chemical, fixed with absolute methanol, and stained with Giemsa. Focal area was examined macroscopically and microscopically and scored for transformation according to criteria of Reznikoff et al. [21].

3. Results

3.1. Assays to assess potential to cause point mutations

3.1.1. Prokaryotic assay systems

3.1.1.1. S. typhimurium reverse mutation assays (Sample A, Study 1; Sample D, Study 8; Sample E, Study 12). For these three separate assays, samples tested included linear APFO (Sample D, tested up to 1500 μg/plate), linear/branched APFO (Sample A, tested up to 500 μg/plate), and linear/branched Na*PFOA (Sample E, tested up to 100 μg/plate). No evidence for a significant increase in the number revertant colonies was obtained in these assays utilizing several sensitive histidine auxotrophic strains of S. typhimurium at test doses of up to 1500 μg/plate, both in the presence and in the absence of a metabolic activation system (Table 4). It should be noted that with Sample D in Trial 2 (see Study 8), when tested in the absence of the metabolic activation system, a non-dose-responsive fivefold increase in mean revertant colonies was observed with S. typhimurium TA1537 at 1000 μg/plate when compared to Trial 1. This could be due to the fact the concurrent vehicle control value for mean revertant in Trial 2 was 2 ± 2 while the vehicle control value was higher in Trial 1, which was reported at 5 ± 2. To clarify, a third trial with the TA1537 strain in the absence of the metabolic activation system was conducted and found a lack of mutagenic potential. In addition, these assays evaluated samples with linear APFO (Sample D), linear/branched APFO (Sample A), and linear/branched Na*PFOA (Sample E), it is reasonable to conclude that from these data, PFOA was not mutagenic under the test conditions, regardless of salt form or isomeric composition.

3.1.1.2. E. coli reverse mutation assay (Sample D, Study 8; Sample E, Study 12). No evidence for a significant increase in the number revertant colonies was obtained in two separate assays utilizing a sensitive tryptophan auxotrophic strains of E. coli at test doses of up to 1500 μg/plate, both in the presence and in the absence of a metabolic activation system (Table 4). Similar to above, the samples tested included both linear APFO (Sample D, tested up to 1500 μg/plate) and linear/branched Na*PFOA (Sample E, tested up to 100 μg/plate). Again, from these data, PFOA was not mutagenic under the test conditions regardless of salt form or isomeric composition.

3.1.2. Eukaryotic assay systems

3.1.2.1. S. cerevisiae D4 mitotic recombination (gene conversion) assay (Sample A, Study 1). In the D4 strain of S. cerevisiae, no increase in convertant colonies were seen with APFO when tested in the absence or presence of a metabolic activation at concentrations up to 500 μg/plate (Table 4). Thus, APFO did not induce non-specific DNA damage leading to mitotic recombination measured as gene conversion in this assay.

3.1.2.2. CHO/HGPRT forward mutation assay (Sample C, Study 5). On the basis of a preliminary range finding assay, dose levels of APFO at 9.75 μg/mL, 19.5 μg/mL, and 39 μg/mL were selected for this assay. While exposure to the test article at these concentrations did not affect the growth with the regular culture medium, they did result in cytotoxicity with 50% cell survival in selective medium with 6-thioguanine (Table 5). None of the three selected dose levels showed a statistically significant increase in the number of mutant frequencies. Positive controls for non-activation (EMS) or activation (DMBA) showed the expected significant increased frequency in the number of mutant colonies proving the sensitivity of the test system. Under these experimental conditions, APFO is considered non-mutagenic in this forward mutation assay in CHO cells.

3.2. Assays to assess potential for cytogenetic damage

3.2.1. In vitro chromosomal aberration studies

3.2.1.1. Chromosomal aberrations in Chinese hamster ovary (CHO) cells (Sample C, Study 4; Sample D, Study 7; Sample E, Study 11). Table 3 summarizes the results of the three in vitro chromosomal aberration assays conducted in CHO cells: one with linear/branched APFO (Sample C, Study 4); one with linear APFO (Sample D, Study 7); and one with linear/branched Na*PFOA (Sample E, Study 11).

In cultures with APFO exposure (Samples C and D) that lacked the metabolic activation system, no significant increases in cells with chromosomal aberrations, polyploidy, or endoreduplication were observed at APFO concentrations up to the maximum for which data could be reasonably assessed, 300 μg/mL. Cytotoxicity, based on reduced mitotic index, was evident at concentrations ranging from 100 to 300 μg/mL, dependent on assay trial. For Na*PFOA (Sample E), five assays were conducted in the absence of the metabolic activation system for exposure periods of 3–41.8 h, and harvest times of 20–44.1 h. Maximum concentrations for which data could be analyzed varied from 198 μg/mL (41.8-h exposure, 44.1 h harvest) to 748 μg/mL (3-h exposure, 44 h harvest). No chromosomal aberrations were noted for cultures that could reasonably be assessed in the absence of significant cytotoxicity. In the assay that incorporated a 3-h exposure period and 44 h harvest, one of the two duplicate cultures at the cytotoxic 748 μg/mL concentration was observed to have 11% of cells with aberrations, while the parallel duplicate had 4% of cells with aberrations (a value similar to the negative and solvent control values) yielding an average of 7.5% of metaphase cells with aberrations. These cultures also had 24.5% of polyplloid metaphase cells. Evidence for cytotoxicity at the 748 μg/mL concentration in these cultures included a 50% reduction in monolayer confluency and a 57% reduction in mitotic index. In the assay incorporating a 3 h exposure and 20 h harvest, it should be
Table 4
Salmonella typhimurium, Escherichia coli, Saccharomyces cerevisiae mutation data summary (Studies 1, 8 and 12).

| S. typhimurium (mean revertants ± SD when applicable) | S. cerevisiae (mean convertants) | E. coli (mean revertants ± SD) |
|------------------------------------------------------|----------------------------------|--------------------------------|
| (µg/plate)                                           | TA98 | TA100 | TA1535 | TA1537 | TA1538 | D4 | WP2uvrA |
| Without S9 activation                                 |      |       |        |        |        |    |         |
| Controls                                             |      |       |        |        |        |    |         |
| Vehicle                                              |      |       |        |        |        |    |         |
| Positive                                              |      |       |        |        |        |    |         |
| 1198                                                 | 180  | 12    | 19     |        | 924   |    | 1555    |
| APFO (Sample A)                                      |      |       |        |        |        |    |         |
| 0.1                                                  | 54   | 183   | 12     | 23     | 50    |    |         |
| 1                                                    | 36   | 171   | 11     | 19     | 83    |    |         |
| 10                                                   | 43   | 193   | 5      | 29     | 80    |    |         |
| 100                                                  | 32   | 143   | 13     | 28     | 86    |    |         |
| 500                                                  | 39   | 211   | 6      | 23     | 86    |    |         |
| Study 1                                               |      |       |        |        |        |    |         |
| Controls                                             |      |       |        |        |        |    |         |
| Vehicle                                              |      |       |        |        |        |    |         |
| Positive                                              |      |       |        |        |        |    |         |
| 97 ± 10                                               | 83 ± 9| 10 ± 4| 5 ± 2  | –      | –     | 13 ± 3|         |
| APFO (Sample D)                                      |      |       |        |        |        |    |         |
| Study 8 (Trial 1)                                    |      |       |        |        |        |    |         |
| Controls                                             |      |       |        |        |        |    |         |
| Vehicle                                              |      |       |        |        |        |    |         |
| Positive                                              |      |       |        |        |        |    |         |
| 196 ± 21                                             | 79 ± 10| 15 ± 5| 2 ± 2  | –      | –     | 17 ± 5|         |
| APFO (Sample D)                                      |      |       |        |        |        |    |         |
| Study 8 (Trial 2)                                    |      |       |        |        |        |    |         |
| Controls                                             |      |       |        |        |        |    |         |
| Vehicle                                              |      |       |        |        |        |    |         |
| Positive                                              |      |       |        |        |        |    |         |
| 15 ± 3                                               | 79 ± 10| 15 ± 5| 2 ± 2  | –      | –     | 17 ± 5|         |
| APFO (Sample D)                                      |      |       |        |        |        |    |         |
| Study 8 (Trial 3)                                    |      |       |        |        |        |    |         |
| Controls                                             |      |       |        |        |        |    |         |
| Vehicle                                              |      |       |        |        |        |    |         |
| Positive                                              |      |       |        |        |        |    |         |
| 8 ± 1                                                | 7 ± 4    | 6 ± 2 | 6 ± 2  | –      | –     | 14 ± 4|         |
| APFO (Sample E)                                      |      |       |        |        |        |    |         |
| Study 12                                              |      |       |        |        |        |    |         |
| Control                                              |      |       |        |        |        |    |         |
| Vehicle                                              |      |       |        |        |        |    |         |
| Positive                                              |      |       |        |        |        |    |         |
| 146 ± 15                                             | 87 ± 2 | 6 ± 1 | 5 ± 4  | –      | –     | 14 ± 4|         |
| NaFPOA (Sample E)                                    |      |       |        |        |        |    |         |
| Study 12                                              |      |       |        |        |        |    |         |

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| Study 1                  | S. typhimurium (mean revertants ± SD when applicable) | S. cerevisiae (mean revertants) | E. coli (mean revertants ± SD) |
|--------------------------|------------------------------------------------------|---------------------------------|--------------------------------|
|                          | (µg/plate)                                           |                                 |                                |
|                          | TA98       | TA100     | TA1535    | TA1537    | TA1538    | D4       | WP2uvrA  |
| Controls                 |            |           |           |           |           |          |          |
| Vehicle<sup>a</sup>      | 46         | 277       | 20        | 23        | 24        | 138      | –        |
| Positive<sup>c</sup>     | 872        | 1426      | 262       | 233       | 963       | 189      | –        |
| APFO (Sample A)          |            |           |           |           |           |          |          |
| Study 8 (Trial 1)        |            |           |           |           |           |          |          |
| Controls                 |            |           |           |           |           |          |          |
| Vehicle<sup>a</sup>      | 20 ± 6     | 109 ± 8   | 14 ± 4    | 8 ± 3     |           |          | 13 ± 2   |
| Positive<sup>c</sup>     | 894 ± 66   | 879 ± 64  | 133 ± 9   | 211 ± 26  |           |          | 288 ± 23 |
| APFO (Sample D)          |            |           |           |           |           |          |          |
| Study 8 (Trial 2)        |            |           |           |           |           |          |          |
| Controls                 |            |           |           |           |           |          |          |
| Vehicle<sup>a</sup>      | 23 ± 4     | 91 ± 14   | 16 ± 2    | 6 ± 2     |           |          | 15 ± 3   |
| Positive<sup>c</sup>     | 736 ± 100  | 1041 ± 82 | 160 ± 32  | 103 ± 23  |           |          | 116 ± 31 |
| APFO (Sample D)          |            |           |           |           |           |          |          |
| Study 12                 |            |           |           |           |           |          |          |
| Controls                 |            |           |           |           |           |          |          |
| Vehicle<sup>a</sup>      | 20 ± 8     | 117 ± 2   | 12 ± 4    | 9 ± 2     |           |          | 19 ± 1   |
| Positive<sup>c</sup>     | 884 ± 98   | 923 ± 32  | 113 ± 8   | 112 ± 8   |           |          | 178 ± 20 |
| NaPPFOA (Sample E)       |            |           |           |           |           |          |          |
| Study 12                 |            |           |           |           |           |          |          |
| Vehicle<sup>a</sup>      | 35 ± 4     | 112 ± 3   | 13 ± 3    | 9 ± 3     |           |          | 20 ± 9   |
| Positive<sup>c</sup>     | 28 ± 2     | 118 ± 4   | 11 ± 3    | 7 ± 3     |           |          | 17 ± 3   |
| Controls                 |            |           |           |           |           |          |          |
| Vehicle<sup>a</sup>      | 30 ± 4     | 114 ± 4   | 13 ± 4    | 7 ± 4     |           |          | 13 ± 1   |
| Positive<sup>c</sup>     | 34 ± 4     | 123 ± 5   | 13 ± 2    | 9 ± 3     |           |          | 16 ± 1   |
| Controls                 |            |           |           |           |           |          |          |
| Vehicle<sup>a</sup>      | 20 ± 3     | 118 ± 9   | 12 ± 3    | 8 ± 2     |           |          | 24 ± 5   |
| Positive<sup>c</sup>     |            |           |           |           |           |          |          |

<sup>a</sup> Vehicle control = 50 µL deionized water.

<sup>b</sup> Not evaluated.

<sup>c</sup> See Table 2 for the corresponding positive controls for each strain.
noted that the combined duplicate cultures exposed to the 748 µg/mL concentration had only 38 metaphase cells available for analysis as opposed to the 200 required by protocol. Although seven metaphase cells were observed to have chromosomal aberrations, the 748 µg/mL cultures were excluded from analysis due to severe toxicity, and the chromosomal aberrations were attributed to the obvious high toxicity at 748 µg/mL. No increase in polyploid cells was evident in the assays conducted in the absence of metabolic activation, with the exception of one of the two replicate cultures at 748 µg/mL. Therefore, Na⁺PFOA was not considered clastogenic in the absence of severe cytotoxicity.

When tested in the presence of the metabolic activation system, no increase in chromosomal aberration was observed after linear/branched PFO exposure (Sample C) at concentrations up to the maximum concentration for which data could be assessed, 750 µg/mL. Similarly, 3-h exposure to linear PFO (Sample D) in the presence of the metabolic activation system with a 20 h harvest time did not produce cytogenetic effects in the initial trial at concentrations up to the maximum analyzed, 750 µg/mL. However, increases over control in percent cells with aberrations were observed in two confirmatory trials with Sample D (3-h exposures and 20.1 h and 44.2 h harvest times) at the highest concentrations for which data could be assessed in each trial (12.5% and 13% at 750 µg/mL and 825 µg/mL, respectively, for the 20.1 h and 44.2 h assays, respectively). These findings were accompanied by cytotoxicity as evidenced by an increase in the percent of cells with endoreduplication at 675 µg/mL and higher concentrations and effects on monolayer confluence and mitotic index. In the 20.1 h assay, for the 600, 675, and 750 µg/mL concentrations, monolayer confluence was reduced by 15, 30, and 45%, respectively, and mitotic index by 15, 11, and 30%, respectively. Similarly, in the 44.2 h assay, for the 675, 750, and 825 µg/mL concentrations, monolayer confluence was reduced 15, 45, and 55%, respectively, with mitotic index reductions of 17, 56, and 53%, respectively. Floating dead cells and debris were also noted at 750 and 825 µg/mL.

When Na⁺PFOA was tested in the presence of the metabolic activation system in three assay trials, no increase in chromosomal aberrations or polyploid cells was noted at concentrations up to 500 µg/mL; however, contradictory results were observed at 748 µg/mL in two replicate assay trials in which cells were exposed 3 h with a 20 h harvest time. At that concentration, a statistically significant percent of cells with chromosomal aberrations (14.5%) and percent polyploid cells (7%) occurred in one of the two replicate assay trials but not in the other. The highest concentration tested in these two replicate assay trials, 1000 µg/mL, was clearly cytotoxic, and increased percent cells with aberrations (31.5% and 42.7%) and increased percent polyploid cells (15% and 26%) were statistically significant over control in both replicate assay trials at this concentration. Cytotoxicity at 1000 µg/mL was severe, consisting of large reductions in monolayer confluency (70% and 95%) and mitotic index (14% and 47%). In the single assay trial with 3-h exposure period and 44.1 h harvest time, no increase in the percent of cells with either chromosomal aberrations or polyploid was observed at 748 µg/mL, the highest concentration that allowed for analysis. At the highest concentration tested under these exposure and harvest conditions, 1000 µg/mL, mitotic index at was reduced by 70%, disallowing analysis.

The observation of an increase in chromosomal aberration and polyploidy with Sample D at cytotoxic concentrations in the presence of a metabolic activation system are of questionable relevance for the assessment of the clastogenicity of PFO. These effects were not noted in the absence of the metabolic activation system or in the absence of cytotoxicity. The physical/chemical properties of PFO preclude metabolic activation, DNA binding, and intercalation. Therefore, PFO was not considered to be a selective genotoxic agent either in the presence or in the absence of a metabolic activation system. Similarly, the observed chromosomal aberrations and polyploidy at the highest concentrations of Na⁺PFOA (Sample E) was considered to be the likely consequence of severe cytotoxicity leading to disruption of normal cellular processes and not a specific genotoxic effect of PFOA [23,24].
Table 6
Chromosomal aberrations assay summary data with human whole blood lymphocytes (Study 10).

|                  | 22.0–22.1 h | 46.0 h |
|------------------|-------------|--------|
|                  | #Cel | #Ab | %Cel | %>1< | %Poly | % End | %Mit | #Cel | #Ab | %Cel | %>1< | %Poly | % End | %Mit |
| **Without S9 activation** |      |     |      |      |       |       |      |      |      |     |      |      |       |       |      |
| Controls         |      |     |      |      |       |       |      |      |      |     |      |      |       |       |      |
| Media            | 200  | 0.01| 0.5  | 0     | 0     | 0     | 2.9  |      | 200  | 0.03| 2    | 0.5  | 0     | 0     | 4.9  |
| Water            | 200  | 0   | 0    | 0     | 0     | 0     | 4.3  |      |      |      |      |      |      |      |      |
| MMC              | 50   | 0.40| 38.0 | 2.0   | 0     | 0     | 2.8  |      |      |      |      |      |      |      |      |
| [Na^+PFOA], μg/mL (Sample E) |      |     |      |      |       |       |      |      |      |     |      |      |       |       |      |
| 12.4             | 200  | 0   | 0    | 0     | 0     | 0     | 4.1  |      | 200  | 0.01| 0.5  | 0     | 0     | 0     | 5.6  |
| 25               | 50   | 0.01| 0.5  | 0     | 0     | 0     | 4.2  |      |      |      |      |      |      |      |      |
| 50.6             | 100  | 0   | 0    | 0     | 0     | 0     | 3.6  |      |      |      |      |      |      |      |      |
| 101              | 112  | 0.01| 0.5  | 0     | 0     | 0     | 1.5  |      |      |      |      |      |      |      |      |
| 202              | 240  | 0   | 0    | 0     | 0     | 0     | 2.0  |      |      |      |      |      |      |      |      |
| 320              | 302  | 0.02| 2.0  | 0     | 0     | 0     | 1.8  |      |      |      |      |      |      |      |      |
| 400              | 402  | 0   | 0    | 0     | 0     | 0     | 0.3  |      |      |      |      |      |      |      |      |
| **With S9 activation** |      |     |      |      |       |       |      |      |      |     |      |      |       |       |      |
| Controls         |      |     |      |      |       |       |      |      |      |     |      |      |       |       |      |
| Media            | 200  | 0   | 0    | 0     | 0     | 0     | 4.9  |      |      |      |      |      |      |      |      |
| Water            | 200  | 0.01| 0.5  | 0     | 0     | 0     | 4.6  |      |      |      |      |      |      |      |      |
| CI               | 50   | 0.7 | 48.0 | 19.0  | 0     | 0     | 1.5  |      |      |      |      |      |      |      |      |
| [Na^+PFOA], μg/mL (Sample E) |      |     |      |      |       |       |      |      |      |     |      |      |       |       |      |
| 50               | 50.6 | 0   | 0    | 0     | 0     | 0     | 4.3  |      |      |      |      |      |      |      |      |
| 100              | 101  | 0.01| 1    | 0     | 0     | 0     | 4.8  |      |      |      |      |      |      |      |      |
| 200              | 202  | 0.02| 1.5  | 0     | 0     | 0     | 3.7  |      |      |      |      |      |      |      |      |
| 300              | 302  | 0.04| 3.0  | 0.5   | 0     | 0     | 1.2  |      |      |      |      |      |      |      |      |
| 500              | 500  | 0.02| 1.5  | 0.5   | 1     | 0     | 3.4  |      |      |      |      |      |      |      |      |
3.2.1.2. Chromosomal aberrations in human whole blood lymphocytes (Sample E, Study 10). Presented in Table 6 are data from the chromosomal aberration assay using human whole blood lymphocytes. The test compound produced cytotoxicity at concentrations above 100 μg/mL in the absence of the metabolic activation system and above 200 μg/mL with the metabolic activation system. Regardless of S9 activation, Na⁺PFOA treatments caused a dose-dependent reduction in mitotic index indicative of toxicity. The extent of % reduction was very pronounced at higher Na⁺PFOA doses. Even so, when chromosomal aberrations were analyzed from cultures treated with higher concentrations of Na⁺PFOA doses, there were no statistically significant increases in the percent of cells with chromosomal aberrations or polyploidy, when compared to the cells treated with vehicle control. Endoreductions were generally not observed following treatment with Na⁺PFOA with the exception of one instance at highly toxic dose level at which the mitotic index was reduced by about 80%. This effect was considered to be the consequence of generalized cell damage as opposed to selective genotoxicity, because it occurred at dose levels for which it was not possible to assess a sufficient number of surviving metaphase cells in one of the duplicate cell cultures. Cells treated with positive controls had statistically significant increases in the percent cells with aberrations in the absence or presence of metabolic activation.

3.2.2. In vivo mouse micronucleus assay (Sample C, Study 3; Sample D, Study 6; Sample E, Study 9)

Presented in Table 7 are data for percent micronucleated PCEs and the ratio between PCE and NCE from three separate studies, one with Na⁺PFOA (Sample E, Study 9) and two with APFO (Sample C, Study 3 and Sample D, Study 6). No significant increases in bone marrow PCE micronuclei occurred in mice treated with the test compounds in any of the three studies. On one occasion, in females treated with 800 mg/kg APFO at the harvest time 48 h post dosing, the rate of micronucleated PCE was slightly increased over vehicle control (0.3%). This singular observation is considered not biologically relevant because: (1) it was within the historical control range, (2) it was not observed at a later harvest time (72 h post dose) in the same experiment, (3) it was restricted to one sex, and (4) it was not observed at an even higher dose in females treated with 1000 mg/kg of Na⁺PFOA.

In mice treated with cyclophosphamide, the positive control, both sexes had a statistically significantly increase in the percentage of micronucleated cells. Male and female mice dosed with 1000 mg/kg of Na⁺PFOA had a statistically significant decrease in PCE/NCE ratio, indicating bone marrow toxicity and demonstrating that the test compound had reached the target organ.

3.3. Cell transformation (Sample B, Study 2)

The data for the C3H 10T1/2 cell transformation assay are presented in Table 8. While APFO showed a dose-dependent decrease in colony formation and plating efficiency, there was no morphologic evidence of
transformation in the colonies treated with APFO. A longer term foci transformation regimen was also evaluated for APFO and there was no evidence of foci or transformed foci. The response to positive controls (benzo(a)pyrene and butadiene epoxide) confirmed the sensitivity of the assay as expected, and all findings observed in positive controls were absent in solvent controls. In summary, APFO did not induce cell transformation in this in vitro system.

4. Discussion

The potential genotoxicity of PFOA was tested in fifteen assays covering several genotoxicological endpoints. These assays included: seven mutation assays (three in vitro reverse mutation assays with histidine auxotrophic strains of S. typhimurium, two in vitro reverse mutation assays with the tryptophan auxotrophic E. coli WP2uvr strain, one in vitro mitotic recombination (gene conversion) assay with S. cerevisiae D4, and an in vitro Chinese hamster ovary (CHO)/HPRT forward mutation assay); seven cytogenetic aberration studies (three in vitro Chinese Hamster Ovary chromosomal aberration studies, an in vitro human whole blood lymphocyte chromosomal aberration study, and three in vivo mouse micronucleus assays); and an in vitro C3H 10T1/2 cell transformation assay. Although these studies have been available as laboratory reports via the United States Environmental Protection Agency Administrative Record 226 (AR-226, see Table 1) and briefly summarized in previous reviews [5,6], the details of these assays have not been published. The results of these assays demonstrate no significant potential for genotoxicity, regardless of PFOA salt form (ammonium or sodium) or isomeric composition (combination of linear and branched or pure linear).

Many of the assays reported incorporated a metabolic activation system. It should be noted that metabolism of PFOA by microsomal systems has never been demonstrated and would not be expected based on the exceptional strength of the carbon-fluorine bonds in the alkyl chain.

Table 7
In vivo mouse micronucleus data summary table, data are presented as mean ± standard error. Adult male and female Crl:CD-1® (ICR) BR mice were treated with a single oral dose of test compound with n = 5/sex/time point unless specified otherwise (Studies 3, 6, and 9).

| Compound  | Dose (mg/kg) | Time | % Micronucleated PCEs (mean ± SE) | Ratio PCE:NE (mean ± SE) |
|-----------|--------------|------|----------------------------------|-------------------------|
| Na’PFOA   | 0 (vehicle control) | 24 h PD<sup>a</sup> | 0.14 ± 0.02 0.02 ± 0.02 | 0.51 ± 0.06 0.69 ± 0.11 |
| (Sample E) | 250 | | 0.28 ± 0.05 0.00 ± 0.00 | 0.69 ± 0.09 0.75 ± 0.17 |
| (Study 9) | | | 0.00 ± 0.00 0.02 ± 0.02 | 0.68 ± 0.08 0.74 ± 0.06 |
| 500 | 24 h PD | 0.12 ± 0.04 0.04 ± 0.02 | 0.69 ± 0.07 0.64 ± 0.05 |
| 1000 | 24 h PD | 0.06 ± 0.04 0.10 ± 0.04 | 0.80 ± 0.08 0.79 ± 0.09 |
| Cyclophosphamide | 80 (positive control) | 24 h PD | 5.44 ± 0.37<sup>b</sup> 2.50 ± 0.33<sup>c</sup> | 0.64 ± 0.07 0.63 ± 0.05 |
| APFO      | 0 (vehicle control) | 24 h PD<sup>a</sup> | 0.08 ± 0.04 0.08 ± 0.04 | 0.64 ± 0.11 0.60 ± 0.07 |
| (Sample E) | 200 | | 0.14 ± 0.05 0.04 ± 0.02 | 0.59 ± 0.05 0.66 ± 0.06 |
| (Study 3) | | | 0.16 ± 0.05 0.08 ± 0.04 | 0.56 ± 0.08 0.52 ± 0.07 |
| 400 | 24 h PD | 0.16 ± 0.05 0.06 ± 0.04 | 0.72 ± 0.08 0.65 ± 0.09 |
| 800 | 24 h PD | 0.12 ± 0.06 0.04 ± 0.02 | 0.59 ± 0.13 0.72 ± 0.17 |
| Cyclophosphamide | 80 (positive control) | 24 h PD | 3.06 ± 0.50<sup>c</sup> 4.24 ± 0.47<sup>c</sup> | 0.63 ± 0.10 0.81 ± 0.07 |

<sup>a</sup> Mean of 1000 micronucleated PCEs per mouse.
<sup>b</sup> PD = post dose.
<sup>c</sup> Mortality occurred during exposure in one of the five mice.
<sup>d</sup> Mortality occurred during exposure in two of the five mice.
<sup>e</sup> Statistically significantly different than vehicle control, p < 0.05.
Table 8
Cell transformation assay using C3H 10T1/2 cells (Study 2).

| Compound | Dose (μg/mL) | Mean # of colony | Mean plating efficiency | Combined type II and type III colonies |
|----------|--------------|------------------|-------------------------|---------------------------------------|
|          |              |                  |                         | Transformed colonies/cell | Transformed colonies (%) |
| Controls | 0 (solvent control) | 43 | 93.65 | 0 | 0 |
|          | 0 (serum baseline control) | 45.8 | 100 | 0 | 0 |
| Benzo(a)pyrene | 0.1 | 44.33 | 96.79 | $4.43 \times 10^{-3}$ | 3.0 |
|          | 1.0 | 40.5 | 88.41 | $7.2 \times 10^{-3}$ | 5.33 |
|          | 2.5 | 37.83 | 82.51 | $7.7 \times 10^{-3}$ | 6.16 |
|          | 5 | 29.3 | 63.52 | $8.86 \times 10^{-3}$ | 9.08 |
|          | 10 | 26.0 | 56.78 | $1.11 \times 10^{-3}$ | 12.81 |
| Controls | 0 (solvent control) | 41.33 | 96.9 | 0 | 0 |
|          | 0 (serum baseline control) | 43.0 | 100 | 0 | 0 |
| APFO (Sample B) | 0.0001 | 1 | 41.0 | 95.39 | 0 | 0 |
|          | 0.005 | 2.5 | 40.5 | 94.21 | 0 | 0 |
|          | 0.001 | 5 | 38.67 | 89.95 | 0 | 0 |
|          | 10 | 37.5 | 87.23 | 0 | 0 |
|          | 50 | 35.0 | 81.44 | 0 | 0 |
|          | 100 | 32.67 | 79.99 | 0 | 0 |
|          | 200 | 27.61 | 63.15 | 0 | 0 |

Foci transformation potential determination (continuous compound exposure in culture)

| Compound | Dose (μg/mL) | Days in culture | Combined type II and type III foci | % transformation per cell per plate (based on 300 cells/plate) |
|----------|--------------|----------------|------------------------------------|-------------------------------------------------------------|
|          |              |                | Total # of foci | # of plates | Average # of foci per plate |                                     |
| Butadiene | 0.0001 | 36 | 64 | 3 | 21.3 | 7.1 |
|          | 0.005 | 36 | 84 | 3 | 28.0 | 9.33 |
|          | 0.001 | 36 | 129 | 3 | 43.0 | 14.33 |
| Controls | 0 (solvent control) | 38 | 0 | 6 | 0 | 0 |
|          | 0 (serum baseline control) | 38 | 0 | 6 | 0 | 0 |
| APFO (Sample B) | 1 | 38 | 0 | 6 | 0 | 0 |
|          | 10 | 38 | 0 | 6 | 0 | 0 |
|          | 100 | 38 | 0 | 6 | 0 | 0 |

Therefore, formation of an active metabolite by exposure of PFOA to the microsomal activation system is precluded. However, it is worth noting that the S9 proteins included in the metabolic activation system may sequester PFOA through ionic binding.

PFOA has not shown the potential to induce DNA point mutations or mitotic recombinations in a series of assay systems. These included the bacterial reverse mutation assays utilizing S. typhimurium histidine auxotrophic strains (TA98, TA100, TA1535, TA1537, and TA1538) and E. coli tryptophan auxotroph (WP2uvr), the S. cerevisiae D4 mitotic gene conversion/recombination assay, and the Chinese hamster ovary cell (CHO) forward mutation assay. Three independently published studies utilizing various S. typhimurium strains exposed to PFOA in the presence or absence of a metabolic activation system were consistent with the results reported herein. In one published study, PFOA was not mutagenic in four strains of S. typhimurium (TA98, TA100, TA102, TA104) when tested at concentrations up to 500 μM (206 μg/mL) [31]. In another study by Endo et al. [32], S. typhimurium strains TA 98 and TA100 exposed to PFOA at unspecified concentrations did not show an increase in revertant colonies. Using the assay for umuc gene expression assay with S. typhimurium TA1535/pSK1002 (hisG46, rfa, uvrB) to analyze for β-galactosidase activity, no significant increased activity was seen at concentrations of PFOA up to 1000 μM (413 μg/mL), indicating a lack of mutagenicity in the assay system.

The studies reported herein also demonstrated that PFOA lacked the potential to produce cytogenetic changes, including chromosomal damage, aberrations and...
polyploidy under non-cytotoxic exposure conditions. These studies included in vitro assays to assess the potential of PFOA to produce chromosomal aberrations in cultured human lymphocytes and CHO cells, as well as the in vivo assessment of micronucleated polychromatic erythrocytes in PFOA-treated mice. In CHO cells, aberrations were observed only in the presence of significant cytotoxicity in three of 16 assay trials that were conducted. PFOA does not possess the chemical/physical characteristics of a genotoxic agent, is incapable of metabolism [25–29], is not expected, based on its structure, to bind covalently to DNA or to intercalate into the double helix, and lacks the planar characteristics of an intercalating agent. Therefore, it is reasonable to assume that the positive findings at high and cytotoxic concentrations in some CHO chromosomal aberration assay trials were the consequence of cytotoxic disruption of normal cellular processes and not a specific genotoxic effect. This is supported by the general lack of genotoxicity in the in vitro chromosomal aberration study in human lymphocytes, in which no evidence of an increase in chromosomal aberrations, polyploidy, or endoreduplication was observed. Previously published reports in which HepG2 cells were exposed in vitro to PFOA have provided mixed results with respect to the production of DNA strand breaks and micronuclei [34–36]. Florentin et al. [35] observed no increases in HepG2 cell DNA strand breaks or micronuclei at PFOA concentrations up to 800 μM (330 μg/mL), and Eriksen et al. [34] also found no increase in PFOA-induced DNA strand breaks in this cell line at concentrations up to 400 μM (165 μg/mL). By contrast, Yao and Zhong [36] observed increases in micronuclei in HepG2 cells exposed to 100–400 μM (41–165 μg/mL) PFOA and increases in DNA strand breaks at concentrations of 50–400 μM (21–165 μg/mL). Among the assays reported here, the lack of increases in mouse bone marrow micronucleated polychromatic erythrocytes found in the three separate in vivo mouse micronucleus assays with respective oral doses of 600, 800, and 1000 mg/kg provides compelling evidence for a lack of potential for PFOA to induce cytogenetic alteration. Taken together, these assays demonstrate a lack of potential for chromosomal effects as a result of PFOA exposure.

Using the C3H 10T1/2 cell culture system in vivo, APFO did not produce cell transformation at concentrations up to the maximum tested, 200 μg/mL. Cell transformation, whether mutational or epigenetic, alters the normal properties, organization, and propagation of cells in culture with emphasis on the acquisition of properties typical of cancer cells. One additional report [37], published previously, has examined to potential of PFOA to produce cell transformation in Syrian hamster embryo (SHE) cells. PFOA tested up to 300 μM (124 μg/mL) did not induce SHE cell transformation frequency. No genotoxicity in SHE cells was seen via the COMET assay, nor was DNA breakage increased in benzo-α-pyrene initiated cells. The data reported herein and that reported by Jacquet et al. [38] demonstrate a lack of cell transformation potential in vitro to be associated with PFOA.

The data reported herein are designed to observe for genotoxic effects under non-cytotoxic exposure conditions to PFOA; however, there are several published articles that have reported cytotoxic disruption of cellular homeostasis, modified gene expression, and increased intracellular ROS production as a consequence of in vitro exposure to PFOA. The potential for PFOA to induce oxidative stress, which may relate to potential cytotoxicity and genetic alteration, has been investigated in several in vitro studies and at least one in vivo study. Freire et al. [31] reported an increase in PFOA-induced reactive oxidative species (ROS), as measured by oxidation of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to 2′,7′-dichlorofluorescin (DCF) in the monkey kidney-derived Vero cell line at the highest concentration tested 500 μM (210 μg/mL); however, cytotoxicity in these cells was evident at concentrations greater than 50 μM (21 μg/mL) with dose-related mitochondrial changes as fission and reduced cell density and increased apoptosis. Using HepG2 cells exposed to PFOA, Yao and Zhong [36] reported an observed increase in ROS (measured as DCF fluorescence) as well as 8-hydroxydeoxyguanosine (8-OHdG) DNA adducts. They reported micronuclei breaks occurred with PFOA concentrations at 100–400 μM (42–168 μg/mL) and DNA strand breaks at 50–400 μM (21–168 μg/mL). Also using HepG2 cells, Hu and Hu [39] found that PFOA concentrations from 50 to 200 μM (20–82 μg/mL) induced production of ROS, dissipation of mitochondria membrane potential, and apoptosis. Cellular antioxidant defenses were reduced, including glutathione content, as well as activities of catalase, superoxide dismutase, and glutathione reductase. Differential expression of genes such as p53, Bcl-2, caspase-9 was evident. The authors suggested that these effects occurred via a mechanism in which the homeostasis of antioxidant systems was overwhelmed, thus modifying expression of genes involved with the apoptotic regulation.

In another experiment with HepG2 cells, Eriksen et al. [34] found that PFOA increased the intracellular ROS production by 1.52 fold in a non-concentration-dependent manner and did not cause DNA strand breakage and acid-labile sites or an increase in formamidopyrimidine-DNA-glycosylase sites; thus, the authors concluded that PFOA, along with other perfluorinated chemicals, induced only modest effects in terms of ROS production and produced no DNA damage in HepG2 cells. Florentin et al. [35] also exposed HepG2 cells to PFOA for 24 h and observed cytotoxicity at concentrations of 200–800 μM (83–330 μg/mL); however, no increases in ROS, DNA strand breaks, or micronuclei were observed. Following a single intraperitoneal injection of 100 mg/kg PFOA, Takagi et al. [40] found increased liver weights and increased liver 8-OHdG adducts of male Fisher 344 rats. It should be noted that the level of 8-OHdG adducts reported was a marker for oxidative DNA damage only and not a measurement of PFOA–DNA adduct.

Because PFOA is a strong organic acid with excellent surfactant and surface tension-lowering properties, it is likely that some of the biochemical changes observed in vitro, as reported by others above, were mediated by cytotoxicity and surfactant-induced membrane disruption. For perspective on cytotoxicity of PFOA, PFOA has been shown to be similar to naturally occurring fatty acids as well as other perfluorinated carboxylates with respect to having low cytotoxicity in human colon carcinoma (HCT116) cells. For PFOA, the 24-h EC50 and 72-h EC50 were 937 μM
(387 μg/mL) and 313 μM (130 μg/mL), respectively, while the 72-h EC50’s for the naturally occurring fatty acids decanoic, docosanoic, and octadecanoic acids ranged from 677 μM (279 μg/mL) to 2907 μM (1200 μg/mL) [41]. Therefore, these inconclusive observations are difficult to translate to in vivo conditions and they do not contradict the conclusion that PFOA has not shown the potential for genotoxicity under non-cytotoxic conditions.

When considering the structural and chemical characteristics of PFOA relative to what is known about direct mutagens and clastogens, it is evident that PFOA does not possess structural or chemical properties that would be consistent with those known to be important in identifying potential genotoxic agents. The complete fluorination of the seven carbons of the chain that supports the carboxyl group precludes chemical reactivity under physiological conditions. Neither is PFOA a planar molecule capable of intercalation into DNA. Interactions of PFOA with DNA using circular dichroism indicated a change in DNA conformation with the binding to DNA being non-covalent in which PFOA aligned along the backbones and interacted with the homolateral bases via hydrophobic interactions [42]. DNA binding was not found when tested at either 50 (21 μg/mL) or 500 μM (210 μg/mL) concentrations [43].

In addition, no significant oxidative DNA damage was produced in primary rat testicular cells [44] and Ddit3 gene expression (marker for DNA damage) was unaltered by PFOA in HepG2/C3A human hepatoma cells at concentrations below 200 μM (83 μg/mL) [45].

In vivo and in vitro mutagenicity/genotoxicity tests are useful predictive tools for evaluating potential genotoxic carcinogens and mutagenicity of chemicals. The results reported in this paper are consistent with the carcinogenicity data obtained from two chronic dietary studies, in which daily dietary treatment with branched/linear PFPO up to 300ppm in the diet of Sprague Dawley rats up to 104 weeks did not produce any excess malignant (invasive) tumors of any type when compared to the control [46,47]. From a mechanistic perspective, the benign tumors observed in these cancer bioassays have been proposed to be most likely the result of non-genotoxic processes related to activation of the xenosensor nuclear receptors in rats such as NR1IC1 (PPARα) [48–51].

Therefore, with the exception of limited positive findings at high and cytotoxic concentrations in some CHO chromosomal aberration assay trials which reflected the likely consequence of cytotoxic disruption of normal cellular processes and not a specific genotoxic effect, at non-cytotoxic conditions, the results of the studies presented in this paper and other published results clearly demonstrate the absence of direct mutagenic or genotoxic risk associated with PFOA.

**Conflict of interest**

John Butenhoff and Shu-Ching Chang are employees of the 3M Company, a former manufacturer of PFOA. Gerald Kennedy is a consultant to DuPont Company, a previous user and manufacturer of PFOA. Reinhard Jung is a consultant to Dyneon, a subsidiary of 3M Company. 3M Company funded the studies reported herein.

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