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Toxicological evaluation of ammonium perfluorobutyrate in rats: Twenty-eight-day and ninety-day oral gavage studies☆

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ABSTRACT

Sequential 28-day and 90-day oral toxicity studies were performed in male and female rats with ammonium perfluorobutyrate (NH_4^+PFBA) at doses up to 150 and 30 mg/kg-d, respectively. Ammonium perfluorooctanoate was used as a comparator at a dose of 30 mg/kg-d in the 28-day study. Female rats were unaffected by NH_4^+PFBA. Effects in males included: increased liver weight, slight to minimal hepatocellular hypertrophy; decreased serum total cholesterol; and reduced serum thyroxin with no change in serum thyrotropin. During recovery, liver weight, histological, and cholesterol effects were resolved. Results of RT-qPCR were consistent with increased transcriptional expression of the xenosensor nuclear receptors PPARα and CAR as well as the thyroid receptor, and decreased expression of Cyp1A1 (Ah receptor-regulated). No observable adverse effect levels (NOAELs) were 6 and >150 mg/kg-d for male and female rats in the 28-day study and 6 and >30 mg/kg-d in the 90-dat study, respectively.

1. Introduction

Perfluorobutyrate ($C_3F_7CO_2^-$, PFBA) is a perfluorinated carboxylate formed by industrial synthesis and by the metabolism [1] and environmental degradation [2–4] of certain fluorinated chemicals. PFBA has recently been detected in precipitation, surface waters, and water treatment facility effluent in low ng/L concentrations [5–7]. In addition, PFBA has been measured in drinking water sources at low ng/L concentrations in certain communities of the Ruhr area of Germany [7] and low μg/L concentrations certain counties of Minnesota in the United States (http://health.state.mn.us/divs./eh/hazardous/topics/pfbasemetro.html). Recent biomonitoring studies have demonstrated that PFBA can be detected at low ng/mL concentrations in the serum of individuals with potential exposure to PFBA through drinking water or via occupational exposure sources [1] as well as in the general population [8].

A number of studies published in the 1980s and 1990s provided insight into the potential biological responses of exposure to PFBA [9–18]. PFBA is a structural homologue of the perfluorooctanoate ($C_7F_{15}CO_2^-$, PFOA), a molecule which has been the subject of much recent health research due to its widespread presence in the general population [19–21] and its long residence time in the human body [22]. The recent recognition of the potential for human exposure to PFBA via environmental sources and its structural similarity to PFOA have led to new inquiry into the potential health hazards of PFBA [1,23–26]. Many of these studies allow for a direct comparison to PFOA, either because PFOA was included in the study or because the study is of a similar design to a study of PFOA. Findings from these studies provide evidence that the biological activity of PFBA has only a few similarities to PFOA, and, where these are similar, PFBA is typically less active than PFOA.

Using gene array analysis, Rosen et al. [27] have recently shown that approximately 85% of the genes for which expression is altered in livers of mice treated with PFOA for 1 week are

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regulated by the peroxisome proliferator activated receptor α (PPARα). Indeed, many of the previously published studies have demonstrated the potential of PFOA and/or PFBA to activate PPARα in liver [9–13,15,17,24,26,28,29]. In general, both PFBA and PFOA were effective in producing responses consistent with activation of PPARα; however, the potency of PFBA was lower. *In vitro*, at the molecular level, PFBA and PFOA were shown to activate both the human and mouse isoforms of PPARα in a transfection assay system in which these nuclear receptors were expressed in COS-1 cells [28]. PFBA showed less response than PFOA, and the response of the human isoform to PFBA was less than that of the mouse isoform. In evaluating the potential of PFBA to activate both human and mouse PPARα isoforms *in vivo*, Foreman et al. [24] gave 28 daily gavage doses of ammonium PFBA (NH_4^+PFBA) or clofibrate to wild-type mice [30], PPARα-knock-out mice (PPARα-KO), and humanized PPARα mice (hPPARα). NH_4^+PFBA treatment resulted in hepatomegaly and hepatocellular hypertrophy in both WT and hPPARα mice; whereas, these effects were not observed in PPARα-KO mice. Wild-type mice also responded with focal hepatocellular necrosis and inflammatory cell infiltrate; however, these effects were not present in PPARα-KO and hPPARα mice. In exposing primary rat and human hepatocytes to PFBA and PFOA in media at concentrations up to 200 μM and observing mRNA transcription signals by quantitative RT-qPCR for PPARα activation (Cyp4A1 (rats), Cyp4A11 (humans), and Acox), Bjork and Wallace [26] demonstrated that human primary hepatocytes were unresponsive to PFBA with respect to PPARα activation; however, PFOA exposure resulted in a response similar in magnitude to that observed in rat primary hepatocytes. The transcriptional response to PFBA in rat primary hepatocytes was greatly attenuated in comparison to PFOA in rat primary hepatocytes at equimolar concentrations in media, consistent with the primary rat hepatocyte data previously reported by Intrasuksri and Feller [15]. The more recent studies support the findings of the older literature in identifying PFBA as an agonist or PPARα in rodents that is less potent than PFOA.

Because PFOA has been found to produce certain developmental effects in rats [31] and mice [32,33], Das et al. have conducted a study designed to compare potential PFBA-induced developmental outcomes in mice dosed with NH_4^+PFBA during gestation [23] with those in mice dosed similarly with ammonium PFOA (NH_4^+PFOA) [32]. In the study with NH_4^+PFBA, doses were adjusted using available pharmacokinetic data to achieve AUCs for PFBA that were similar to the AUCs (area under curve) for PFOA at the doses used in the study with NH_4^+PFOA. Treatment with NH_4^+PFBA during gestation in mice did not result in developmental effects that were comparable to those observed with NH_4^+PFOA. Abbott et al. [33] have found that a majority of the developmental effects observed in mice given NH_4^+PFOA by Lau et al. [32] did not occur in PPARα-KO mice given NH_4^+PFOA, suggesting a role of PPARα in mediating the developmental effects of PFOA in mice. Therefore, the lack of developmental effects in mice with NH_4^+PFBA as compared to NH_4^+PFOA may result from differences in activation potential for PPARα or other differences, for example, in pharmacokinetics [1,34].

Ikeda et al. [9] noted that the apparent reduced effect of PFBA *in vivo* as compared to PFOA may be the result of a more rapid elimination of PFBA; although pharmacokinetic data for PFBA were not available until recently. In contrast to its eight-carbon structural homolog, PFOA, it is now established that PFBA is eliminated much more rapidly in mice, rats, monkeys, and humans [1]. The estimated terminal serum elimination half-lives for PFBA and PFOA, respectively, are 1.7 days and 20–30 days in cynomolgus monkeys and 2–4 days and 1400 days in humans. Although the elimination half-lives of PFBA and PFOA in female rats is similar (about 2 h), the elimination half-life of PFBA in male rats is approximately 6–9 h as compared to approximately 6 days for PFOA. Male and female mice eliminate PFBA with half-lives of about 12 and 3 h, respectively, while male and female mice excrete PFOA with a serum elimination half-life of about 2–3 weeks [21]. Weaver et al. [34] have found that PFBA is not a substrate for rat renal organic anion transporters associated with PFOA transport. Although pharmacokinetic differences in elimination rates may explain the observed lesser biological activity of PFBA as compared to PFOA, the *in vitro* studies noted above have demonstrated that PFBA is inherently less potent than PFOA in producing responses associated with activation of PPARα.

With the finding of PFBA in surface waters and public water supplies, there has been a renewed interest in the potential toxicity of this compound and its potential risk based on human exposure. Because studies of 1–3 months duration that evaluated the broad range of potential responses that are typically included in safety pharmacology studies were not available, we undertook to conduct the 28- and 90-day repeat-dose studies with NH_4^+PFBA that are reported herein. For comparative purposes, the NH_4^+PFBA homologue, NH_4^+PFOA, which has been extensively studied in rats and is also of current public health interest based in part on demonstration of exposure to general population, exposure via various point sources, and extended human half-life [21,35], was included in the 28-day study design. Based on the available pharmacokinetic and toxicity profiles of PFBA relative to PFOA, it was hypothesized that PFBA would be considerably less active than PFOA in repeat-dose studies in rats.

2. Materials and methods

2.1. Materials

All chemicals used in this study were reagent-grade and were purchased from Sigma–Aldrich (St. Louis, MO) or VWR (West Chester, PA). Ammonium perfluorobutyrate (NH_4^+PFBA, as 28.9% solution in distilled water) and ammonium perfluorooctanoate (NH_4^+PFOA, FC-143, Lot 332, 97.99% purity) were provided by 3M Company (St. Paul, MN, USA). Stable isotope $^{13}C_4$-labelled PFBA ($^{13}C_4F_7O_2H$) was supplied as the free acid in methanol (50 μg/mL, Wellington Laboratories, distributed by TerraChem, Shawnee Mission, KS, USA) and used as internal standard for liquid chromatography/tandem mass spectrometric (LC–MS/MS) analysis of PFBA in samples. Stable isotope $^{13}C_2$-labelled PFOA ($CF_3(CF_2)_5(^{13}CF_2)^{13}CO_2H$) was a gift from E.I. DuPont and used as an internal standard for analysis of PFOA.

2.2. Laboratory rats and husbandry

For 28-day and 90-day studies, male and female Sprague Dawley rats (Crl:CD, outbred, SPF quality) were obtained from Charles River Deutschland, Sulzfeld, Germany. Housing room conditions provided approximately 15 air changes/h, a controlled temperature of 21.0 ± 3.0 °C, a 12-h light/12-h dark cycle, and a relative humidity of 30–70%. Rats were group-housed (5/sex) in standard solid-bottom cages. Pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany) and tap water were provided *ad libitum*. Rats were held for an acclimatization period of at least 5 days prior to study initiation and were approximately 6 weeks old at the start of treatment. Prior to beginning of treatment, rats were randomized according to body weight as 10 rats/treatment group/sex. All rats were within ±20% of the sex mean. Protocols were reviewed by the Laboratory Animal Welfare Officer and the Ethical Committee of NOTOX B.V. in accordance with the Dutch Act on Animal Experimentation (February, 1997).

2.3. Experimental design

Two sequential studies were conducted, the first with a 28-day dosing period and the second with a 90-day dosing period (referred to hereafter as the 28-day study and 90-day study, respectively). All dosing solutions were prepared in either Milli-Q or Milli-U water, which was also used as the vehicle control, and daily doses were given in a volume of 5 mL per kg body weight, with dose volumes adjusted based on the most recent body-weight data. NH_4^+PFBA dose groups were 0, 6, 30, and 150 mg/kg-d for the 28-day study (N = 10 per sex per group) and 0, 1.2, 6, and 30 mg/kg-d for the 90-day study (N = 10 per sex per group). In addition to the main-study groups, each study also included recovery groups that were sacrificed 3 weeks after the end of the dosing period. For the 28-day study, recovery groups were included for control and each treatment (N = 10 per sex per group); whereas, only control and high-dose recovery groups were included in the 90-day study (N = 10 per sex per group). At the termination of the treatment period in the 90-day study, 3 rats per sex in the recovery groups were assigned for evaluation of ocular parameters, reducing the size of the recovery groups to 7 per sex per group. In addition to NH_4^+PFBA groups, the 28-day study included male and female NH_4^+PFOA

main-study and recovery groups at a single dose level of 30 mg/kg-d. All rats were dosed once daily for either 28 days (28-day study) or 90 days (90-day study) by oral gastric gavage using a plastic feeding tube. Dosing occurred at approximately the same time each day with a maximum of 4 h difference between the earliest and latest dosing. All rats were dosed up to the day prior to necropsy of the main-group rats.

2.4. Dosing solution preparations and analyses

Dosing preparations (w/w) were prepared daily within 4 h prior to dosing. The solutions were homogenized to visually acceptable levels. Concentrations were adjusted for purity and specific gravity. Solutions were stored at ambient temperature. Samples of dosing preparations were taken during and after treatment periods to be analyzed for accuracy and homogeneity (samples taken from top, middle, and bottom). Immediately after aliquoting dosing preparation samples, the samples were stored at ≤−15 °C. The dosing solution were analyzed by LC–MS/MS method similar to that described in Chang et al. [1] for PFBA with the exception that NH_4^+PFBA dosing solution was analyzed (instead of serum). Analysis for PFOA in dosing solution was essentially the same as those described in Ehresman et al. [36] with the exception that NH_4^+PFOA dosing solution was analyzed (instead of serum). For both PFBA and PFOA, negative ions and negative ion transitions were monitored as described above for dosing solutions.

2.5. Clinical observations

Rats were observed for morbidity and mortality at least twice daily, and detailed clinical observations were made at least once daily. Rats were examined outside of the cage at the start of treatment and at weekly intervals thereafter.

Body weights were obtained on main-study and recovery day 1 and at weekly intervals thereafter. Food consumption was determined weekly per cage, and water consumption was evaluated subjectively.

2.6. Functional observations

Functional observations were made on recovery-group rats once during dosing weeks 3–4 of the 28-day study. Functional observations were made on all rats during dosing weeks 8 (direct pupillary reflex only) and 12 of the 90-day study. In addition, direct pupillary reflex was evaluated on recovery-group rats on recovery days 11 and 21 of the 90-day study. Functional observations included: motor activity (individual rats, 12 h overnight using a computerized monitoring system; Pearson Technical Services, Debenham, Stowmarket, England); hearing ability, static-righting reflex, grip strength, and direct bilateral-pupillary reflex (evaluated for normal/present and abnormal/absent); and consensual pupillary reflex (90-day study only and only for rats that displayed abnormal direct pupillary reflex in weeks 8 or 12). Pupillary reflexes were evaluated by trained personnel after dark adaptation for at least 5 min using a penlight.

2.7. Hematology

Hematological, clotting, and clinical biochemical analyses were performed on blood-derived samples taken immediately prior to scheduled *post mortem* examinations at the end of the treatment and recovery periods. After an overnight fast with water provided *ad libitum*, blood was collected from the retro-orbital sinus under anesthesia with Ketalin®/Dormitor® subcutaneous injection or isoflurane anesthesia. Blood was collected for hematology in EDTA-treated tubes (0.5 mL), for clotting tests in citrate-treated tubes (0.9 mL), and for clinical biochemistry in Li-heparin-treated tubes (0.5 mL). Samples were obtained between 07:00 and 14:00 h.

Hematological parameters were determined on an ADVIA® 120 (Bayer Diagnostics, Mijdrecht, The Netherlands). Parameters evaluated included: white blood cells (WBC); differential leukocyte count; red blood cells (RBC); reticulocytes (RETICUL); red blood cell distribution width (RDW); hemoglobin (HGB); hematocrit (HCT); mean corpuscular volume (MCV); mean corpuscular hemoglobin [37]; mean corpuscular hemoglobin concentration (MCHC); and platelets (PLAT).

Prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined using a STA Compact analyzer (Roche Diagnostics, manufactured by Diagnostica Stago S.A., Asnières sur Seine, France).

2.8. Clinical biochemistry

Parameters determined were: alanine aminotransferase; aspartate aminotransferase; alkaline phosphatase; total protein; albumin; total bilirubin; urea; creatinine; glucose; total cholesterol; triglycerides; sodium; potassium; chloride; calcium; inorganic phosphate; total thyroxine; free thyroxine; and thyrotropin. Most analyses, with the exception of thyroid-related hormones, were performed using an Olympus AU400 (Olympus Diagnostics). For thyroid-related hormone determinations, serum tubes collected and frozen at necropsy (*see below*) were thawed, and each serum sample was aliquoted into three labelled polypropylene tubes and refrozen at ≤−70 °C pending analysis for total thyroxine (TT4, tube 1), free thyroxine (FT4) and thyrotropin (TSH) (tube 2), and serum concentration of test compound anion (PFBA or PFOA, tube 3). Serum TT4 was determined by chemiluminometric analogue method (ADVIA Centaur® clinical analyzer, Bayer HealthCare LLC, Tarrytown, NY). For samples from the 28-day study, serum FT4 was measured using equilibrium dialysis (ED) followed by radioimmunoassay (ED-RIA) with Nichols Institute Diagnostics Free T4 by Equilibrium Dialysis kits (Nichols Institute Diagnostics, San Clemente, CA). For the 90-day study samples, FT4 was measured by ED followed by LC–MS/MS (Mayo Medical Laboratories, Rochester, MN, Test Code 8859). Serum TSH was determined using a rat TSH radioimmunoassay with components from the National Hormone and Pituitary Program (UCLA Harbor Medical Center, Torrance, CA).

2.9. Necropsy

Rats that survived to the end of the treatment periods (28 or 90 days) were anesthetized with either isoflurane (28-day) or 75 mg/kg-d Ketalin®/0.5 mg/kg-d Dormitor® (90-day) (Ceva Sante Animale BV, Maassluis, The Netherlands/Pfizer Animal Health, Capelle a/d Ijssel, The Netherlands) by subcutaneous injection. Blood was collected by retro-orbital puncture for clinical biochemistry analyses. An additional blood sample for thyroid-related hormone determinations was obtained from the aorta and placed into untreated tubes and allowed to clot for at least 30 min, centrifuged, and the sera were removed and placed into labelled polypropylene tubes which were frozen at ≤−75 °C pending analysis. Following exsanguination, livers were weighed and samples from each liver were taken as follows: one approximately 0.5 g sample that was approximately 0.5 mm^3 that was placed in 6 mL RNALater (Ambion, Catalog No. 7021) at 2–8 °C for at least 16–24 h (maximum of 3 days) under refrigeration, after which they were stored at ≤−75 °C; three approximately 0.5 g samples that were flash frozen in liquid nitrogen and stored at ≤−75 °C; and one approximately 0.1 g sliced sample (approximately 1 mm thick) fixed for approximately 2 h in 3.5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M cacodylate buffer (Merck, Darmstadt, Germany) at ambient temperature, then cut into at least eight approximately 1 mm^3 pieces and fixed in 3.5% glutaraldehyde in 0.1 M cacodylate buffer for an additional 12–24 h under refrigeration, after which they were stored refrigerated in darkness after transferring into fresh 0.1 M cacodylate buffer, pH 7.4.

In addition to liver weight, weights of the following organs were taken: adrenals; brain; epididymides; heart; kidneys; liver; ovaries; spleen; testes; thymus; and thyroid with parathyroid (28-day study only). The thyroid was weighed after fixation for at least 24 h.

After observation for macroscopic abnormalities, the following tissues were fixed in 10% buffered formalin (Klinipath, Duiven, The Netherlands): adrenals; aorta; brain; caecum; cervix; clitoral gland; colon; duodenum; harderian gland; mammary gland area (females); femur and joint; heart; ileum; jejunum; kidneys; larynx; lacrimal gland; liver; lung (infused); mandibular and mesenteric lymph nodes; nasopharynx; esophagus; ovaries; pancreas; Peyer's patches; pituitary; preputial gland; prostate; rectum; mandibular and sublingual salivary glands; sciatic nerve; seminal vesicles; skeletal muscle; skin; spinal cord; spleen; sternum with marrow; stomach; thymus; thyroid including parathyroid; tongue; trachea; urinary bladder; uterus; vagina; any found gross lesions. Epididymides, eyes with optic nerve, and testes were fixed first in modified Davidson's solution for at least 24 h and then transferred to formalin.

Based on an observation of delayed pupillary reflex in some rats of both studies, three rats/sex from the control recovery and 30 mg/kg-d recovery groups in the 90-day study were decapitated by guillotine on the 92nd day of the study. Eyes of these rats were excised and further processed for detailed investigation in the laboratory of Dr. Donald A. Fox (University of Houston, School of Optometry). Results of these analyses are to be reported separately. These 12 rats did not undergo necropsy, organ-weight determination or organ fixation.

2.10. Microscopic histopathology

Most fixed organs taken at necropsy were trimmed, processed, embedded in paraffin wax, cut in 2 to 4 μm sections, and stained with hematoxylin and eosin for histopathological examination. Exceptions were: clitoral gland; mammary gland area (female); femur and joint; larynx; lacrimal gland; nasopharynx; preputial gland; mandibular and sublingual salivary glands (excluded in 28-day study only); skeletal muscle; skin; and tongue. These tissues were not evaluated microscopically as there were no signs of organ toxicity observed.

Histopathological examination was performed on tissues indicated above for the vehicle control and highest dose groups (28-day study: 150 mg/kg-d NH_4^+PFBA and 30 mg/kg-d NH_4^+PFOA; 90-day study: 30 mg/kg-d NH_4^+PFBA) as well as liver for all intermediate NH_4^+PFBA main study group males and females (28-day study only) and control and highest dose recovery group males and females (28-day study only). Thyroids of males and females (28-day study only) in main study intermediate groups and of males in the control and highest dose recovery groups were also examined. In addition, observed gross lesions from all rats were examined.

Findings were scored based on the following grading system: 0 = finding not present; 1 = minimal (very small and/or very few); 2 = slight (small and/or few); 3 = moderate (moderate magnitude and/or moderate number); 4 = marked/severe (large magnitude and/or many); 5 = severe/massive (extensive magnitude and/or number).

Table 1
Genes and primer sequences used for quantitative rt-PCR mRNA transcript analyses.

Gene	Upper primer sequence	Lower primer sequence	Length (bp)	Gene bank reference
18s rRNA	CGC CGC TAG AGG TGA AAT TCT T	CAG TCG GCA TCG TTT ATG GTC	149	M11188
Acaca	AAG CAG CTG ACA GAG GAA GAT GGT	TGG AGT CCA TGG CAA CTT CTG GAT	130	NM_022193
Pcna	GTG AAC CTC ACC AGC ATG TCC AAA	ACA GCT GTA CTC CTG TTC TGG GAT	198	NM_022381
Acox	TGG AGA GCC CTC AGC TAT GG	CGT TTC ACC GCC TCG TAA G	338	NM_017340
Cyp4A1	ACC TCT TTC ACT CCC GTG TG	GTG TGT GGC CAG AGC AGA GA	344	M57718
Cyp2B2	TCC AGA CAC CTT CAA TCC TGA GCA	GAC AAA TGC GCT TTC CTG TGG AGA	98	XM_001062335
Cyp3A1	TTT CGC CCA GAA AGG TTC AGC AAG	AGA GCA AAC CTC ATG CCA ATG CAG	107	NM_173144
Cyp1A1	TGA CCT CTT TGG AGC TGG GTT TGA	ATG TCG GAA GGT CTC CAG GAT GAA	199	NM_012540
Malic	AGG CCT CTT TAT CAG TAT CCA C	CCA TCC CGT TAC AAC CAA	140	AH002199
Por	AAG ACA TGG ACG TAG CCA AGG TGT	TGA GAT GTC CAA CTA CAT GTG CAT	173	NM_031576.1
Thrsp	CTT CTC CAG CCT CCA TCA CAT C	TCG TCT TCC CTC TCG TGT AAA GTG	138	NM_012703
Fasn	TGC AGA GCA TAG CAA GGT GCT AGA	TGT AGT AGA AGG ACA CGG CAG CAA	146	NM_017332.1
Dio1	TCT GCC TGA GAG GCT CTA TGT GAT	TTT CCA GAA CAG CTC GGA CTT CTT	104	NM_021653
Ugt1A	GCC TTC CCA GTG TTA GTC ATT	CTT GGT TTG GGA ACA CAA TAG	200	U20551
Ugt1A1	AGG AAG TAC CCT GTG CCA TTC CAA	TCT TGA TCA AAG ACA CTC CGC CCA	80	U20551
Ugt1A6	AAC GCG GAC ACG ACA TTG T	AAC TGG CAG CAA AGT GGT TGT T	156	U20551
Ugt2B	GTG CAC TGG AGG AAG TCA TAG ACA	TAG GCT GGT CAT GGT GAA TCC TTG	81	XM_0011062335
Cyp7A1	TTT CTA CAT GCC CTT CGG ATC A	TTC GCT TCT TCC AAC CAC GTA T	238	NM_012942
ApoA1	GAT GAA AGC TGC AGT GTT GGC TGT	GTT CAG CTG TTT GCC CAA AGT GGA	199	NM_012738.1

Morphometric analyses were performed on thyroids from male rats from the 28-day and 90-day studies. For the 28-day study, a total of 80 thyroid slides were examined. These included thyroids from all main group male rats (control, 6, 30, and 150 mg/kg-d NH_4^+PFBA and 30 mg/kg-d NH_4^+PFOA groups) as well as all recovery males from the control, 150 mg/kg-d NH_4^+PFBA, and 30 mg/kg-d NH_4^+PFOA groups. For the 90-day study, a total of 54 thyroid slides were examined which included thyroids from all main group male rats (control and 1.2, 6, and 30 mg/kg-d NH_4^+PFBA groups) as well as 7 each of the control and 30 mg/kg-d NH_4^+PFBA recovery-group rats. Morphometric analyses included epithelial cell height of follicular epithelial cells and area. Digital images of thyroids were captured on an Olympus BX-51 photomicroscope equipped with Olympus DP-70 digital camera. Measurements of epithelial cell height and thyroid follicular colloid area were made using Image Pro plus (Media Cybernetics, Silver Spring, MD) software. Cell height measurements were performed on 3 cells from each of 5 follicles per rat (15 total cells measured per thyroid). The cells selected were located approximately at the 12, 4, and 8 o'clock positions in follicles. Measurement extended from deep aspect of nuclear membrane up to the luminal surface of each selected follicular epithelial cell. Measurements were exported to Microsoft Excel, and basic means and standard deviations were determined. For colloidal area, long and short axes measurements were performed on the colloidal mass within the 10 largest follicles that appeared in each digital image. Colloidal dimensions were exported to Microsoft Excel, and means and standard deviations were determined. Measurement lines were placed on each digital image and Image Pro was used to capture the entire images including lines and identification markers. Differences between control and NH_4^+PFBA- or NH_4^+PFOA-treated rats were assessed using ANOVA followed by Dunnett's test. When p was <0.05, differences were considered statistically significant.

2.11. Quantitative RT-qPCR

Transcript levels for specific mRNA (Table 1) were quantified by reverse transcriptase quantitative PCR (RT-qPCR) and normalized based on the expression level of 18s rRNA (house-keeping gene). Procedures were as detailed in Bjork and Wallace [26].

2.12. Analysis of serum and liver samples for PFBA and PFOA concentrations

Analysis of rat serum and liver samples for PFBA was accomplished as presented in Chang et al. [1]. Methods for analysis of rat serum and liver samples for PFOA were essentially the same as those described in Chang et al. [1] with the exception that the $^{13}C_2$-labelled PFOA was used as an internal standard for PFOA analysis. For both PFBA and PFOA, negative ions and negative ion transitions were monitored as described above for dosing solutions.

2.13. Statistical analysis

For normal distributions, Dunnett's test [38] was used for comparison of treated groups to the control group by sex, based on a pooled variance. The Steel test [39] was used when the data could not be assumed to fit a normal distribution. Fisher's exact test [40] was applied to frequency data. All tests were two-sided, and, in all cases, $p < 0.05$ was accepted as the lowest level of significance. No statistical analysis was performed on histopathology incidence data. Group means were calculated for continuous data, and medians were calculated for discrete data. For histomorphometric analyses of male rat thyroids, means and standard deviations were calculated by group, and ANOVA and Dunnett's test were used to assess differences between the control and treated groups ($p < 0.05$).

3. Results

3.1. Dosing solutions analyses

Results of dosing solution analyses are presented in Table 2. In the 28-day study, measured dosing PFBA solution concentrations were 84.5% to 87.9% of target concentrations, which produced estimated actual NH_4^+PFBA doses of 5.3, 25.4, and 130.2 mg/kg-d for the nominal doses of 6, 30, and 150 mg/kg-d, respectively. In the 90-day study, PFBA dosing solutions were 108% to 120% of target concentrations, which produced estimated actual NH_4^+PFBA doses of 1.4, 6.9, and 32.4 mg/kg-d for the nominal doses of 1.2, 6, and 30 mg/kg-d, resepctively. Mean measured PFOA concentration for the 30 mg/kg-d dosing solution was 92.8% of target, which produced an estimated actual NH_4^+PFOA dose of 27.8 mg/kg-d.

3.2. Concentration of PFBA and PFOA in serum and liver

Serum and liver concentrations of PFBA and PFOA achieved at the end of the treatment and recovery periods are presented in Table 3. At the end of the 28-day dosing period, male rats given NH_4^+PFBA had mean serum PFBA concentrations that increased with dose from approximately 25 μg/mL at 6 mg/kg-d to approximately 82 μg/mL at 150 mg/kg-d, while mean serum PFBA in females ranged from 0.3 μg/mL to 10.3 μg/mL at the same respective doses. At the end of the 90-day dosing period, male rats given NH_4^+PFBA had mean serum PFBA concentrations that increased with dose from approximately 6 μg/mL at 1.2 mg/kg-d to approximately 52 μg/mL at 30 mg/kg-d, while mean serum PFBA in females ranged from 0.2 μg/mL to 5.1 μg/mL at the same respective doses. In both studies and for both sexes, serum PFBA concentrations at the end of recovery were a small fraction of those at the end of the dosing period, and mean liver PFBA concentrations were 20–50% of respective (by sex and dose group) serum PFBA concentrations.

At the end of the 28-day dosing period, male and female rats given 30 mg/kg-d NH_4^+PFOA had mean serum PFOA concentrations of 146 and 8 μg/mL, respectively by sex. At the end of recovery, male serum PFOA had declined to 14 μg/mL; whereas, female serum PFOA had declined to 0.03 μg/mL. Mean liver PFOA concentrations were similar to their respective serum PFOA concentrations at the end of treatment and recovery.

Table 2
Results of ammonium perfluorobutyrate (NH_4^+PFBA) and ammonium perfluorooctanoate (NH_4^+PFBA) dosing solutions analyses: 28-day and 90-day studies.

Target dose[a] (mg/kg)	Target dose concentration (mg/mL)[b]	*N*[c]	% of Target concentration (mean ± *SD* (range))[d]	Mean actual dose (mg/kg)[e]
28-Day study: NH_4^+PFBA doses				
6	1.2	3	87.9 ± 2.3 (85.5–90.2)	5.3
30	6.0	1	84.5	25.4
150	30.0	3	86.8 ± 1.8 (84.8–88.1)	130.2
28-Day study: NH_4^+PFOA dose				
30	6.0	3	92.8 ± 2.5 (90.2–95.2)	27.8
90-Day Study: NH_4^+PFBA doses				
1.2	0.24	3	120 ± 5 (117–126)	1.4
6	1.2	1	115	6.9
30	6.0	3	108 ± 0.0 (108)	32.4

[a] Based on study protocol.
[b] Dosing volume was 5 mL/kg.
[c] Number of determinations.
[d] Range represents results of three homogeneity samples taken from the top, middle, and bottom of the dosing solution. Analyses were performed for the anions (perfluorobutyrate (PFBA) and perfluorooctanoate (PFOA)) and corrected for the ammonium salt.
[e] Based on dosing solution analysis, the estimated actual measured dose.

Table 3
Summary of serum and liver concentrations of PFBA or PFOA in male and female rats treated with NH_4^+PFBA or NH_4^+PFOA for either 28 or 90 days.

Dose group[b] (mg/kg-d)	Concentrations of PFBA or PFOA in serum and liver			
	End of treatment period[a]		End of recovery period[a]	
	Serum (μg/mL)	Liver (μg/g)	Serum (μg/mL)	Liver (μg/g)
28-Day Study: Males				
0	*0.04 ± 0.05*[c]	<0.05[d]	<0.01[e]	<0.05[e]
6	24.65 ± 17.63	7.49 ± 4.46	0.06 ± 0.02[f]	<0.05[e,f]
30	38.04 ± 23.15	17.42 ± 8.15	0.20 ± 0.13	*0.06 ± 0.04*[g]
150	82.20 ± 31.83	37.44 ± 18.12	1.07 ± 0.27	0.33 ± 0.10
30 (NH_4^+PFOA)	145.60 ± 28.25	166.10 ± 28.45	14.67 ± 5.30	16.32 ± 6.69
28-Day Study: Females				
0	*0.01 ± 0.01*[h]	*0.05 ± 0.03*[i]	<0.01[e]	<0.05[e]
6	0.34 ± 0.13[f]	0.16 ± 0.04	0.015 ± 0.01[c]	<0.05[e]
30	1.72 ± 0.88	0.434 ± 0.174	0.066 ± 0.058	<0.05[e]
150	10.30 ± 4.50	2.70 ± 1.47	0.234 ± 0.165	*0.06 ± 0.04*[j]
30 (NH_4^+PFOA)	7.98 ± 4.03	11.15 ± 4.91	0.03 ± 0.02	<0.05[e]
90-Day Study: Males				
0	<0.01[e]	<0.05[k]	<0.01[e,l]	<0.05[e,l]
1.2	6.10 ± 5.22	1.34 ± 1.24	–[m]	–[m]
6	13.63 ± 9.12	3.07 ± 2.03	–[m]	–[m]
30	52.22 ± 24.89	16.09 ± 9.06	0.51 ± 0.31[n]	0.19 ± 0.07[n]
90-Day Study: Females				
0	*0.07 ± 0.06*[o]	<0.05[p]	<0.01[e,q]	<0.05[e,q]
1.2	0.23 ± 0.14	*0.05 ± 0.02*[r]	–[m]	–[m]
6	0.92 ± 0.52[f]	0.15 ± 0.08[f]	–[m]	–[m]
30	5.15 ± 3.29	0.91 ± 0.55	0.11 ± 0.08[s]	0.09 ± 0.03[s]

[a] *N* = 10 per group unless otherwise noted due to no sample or insufficient sample for assay.
[b] Ammonium PFBA dose groups except where ammonium PFOA (NH_4^+PFOA) is indicated.
[c] Values in italics are estimates. Three values were below the limit of quantitation (LOQ = 0.010 μg/mL), and these three values were assigned the value of 0.0071 μg/mL (the LOQ divided by the square root of 2) for calculating the mean and standard deviation.
[d] Nine values were less than the limit of quantitation (LOQ = 0.050 μg/g). One sample was quantifiable with a value of 0.068 μg/g.
[e] All samples were below the limit of quantitation (LOQ = 0.010 μg/mL for serum; LOQ = 0.050 μg/g for liver).
[f] *N* = 9 due to non-treatment-related loss of one rat in this group.
[g] Six values were below the limit of quantitation (LOQ = 0.050 μg/g), and these five values were assigned the value of 0.0354 μg/g (the LOQ divided by the square root of 2) for calculating the mean and standard deviation. The four samples were quantifiable and had values of 0.051, 0.083, 0.115, and 0.123 μg/g.
[h] Five values were below the limit of quantitation (LOQ = 0.010 μg/mL), and these five values were assigned the value of 0.0071 μg/mL (the LOQ divided by the square root of 2) for calculating the mean and standard deviation. The five samples that could be quantitated had values of 0.012, 0.014, 0.014, 0.014, and 0.024 μg/mL.
[i] Seven values were less than the limit of quantitation (LOQ = 0.050 μg/g), and these seven values were assigned the value of 0.0354 μg/g (the LOQ divided by the square root of 2) for calculating the mean and standard deviation. The three samples that were quantifiable had values of 0.050, 0.074, and 0.127 μg/g.
[j] Five values were below the limit of quantitation (LOQ = 0.050 μg/g), and these five values were assigned the value of 0.0354 μg/g (the LOQ divided by the square root of 2) for calculating the mean and standard deviation. The five samples that could be quantitated had values of 0.055, 0.070, 0.075, 0.116, and 0.153 μg/g.
[k] Eight values were less than the limit of quantitation (LOQ = 0.050 μg/g). The two samples that were quantifiable had values of 0.057 and 0.089 μg/g.
[l] *N* = 7 due to assignment of three rats in this group for collection of ocular tissues for further examination.
[m] Treatment group not included in study design.
[n] *N* = 6 due to assignment of three rats in this group for collection of ocular tissues for further examination and non-treatment-related loss of one rat in this group.
[o] One value was below the limit of quantitation (LOQ = 0.010 μg/mL), and this sample was assigned the value of 0.0071 μg/mL (the LOQ divided by the square root of 2) for calculating the mean and standard deviation.
[p] Nine values were less than the limit of quantitation (LOQ = 0.050 μg/g). The sample that was quantifiable had a value of 0.126 μg/g.
[q] *N* = 3 due to assignment of three rats in this group for collection of ocular tissues for further examination and non-treatment-related loss of four rats in this group due to complications from anesthesia.
[r] Six values were below the limit of quantitation (LOQ = 0.050 μg/g). These six values were assigned the value of 0.0354 μg/g (the LOQ divided by the square root of 2) for calculating the mean and standard deviation. Four samples were quantifiable and had values of 0.061, 0.067, 0.085, and 0.089 μg/g.
[s] *N* = 7 due to assignment of three rats in this group for collection of ocular tissues for further examination.

Table 4
Mean (±*SD*, *N*) body weights and body weight gain as a percent of starting weight in rats given repeated daily oral doses of NH_4^+PFBA or NH_4^+PFOA for either 28 or 90 days.

Dose group[a], mg/kg-d	Treatment period				Recovery period			
	N	Start (g)	End (g)	Gain[b] (%)	*N*	Start (g)	End (g)	Gain[b] (%)
28-Day Study: Males								
0	20	202 ± 6	357 ± 23	77 ± 11	10	364 ± 26	434 ± 36	115 ± 16
6	20	204 ± 6	357 ± 24	75 ± 9	9	354 ± 20	433 ± 31	113 ± 14
30	20	203 ± 7	354 ± 19	74 ± 8	10	347 ± 15	425 ± 22	109 ± 10
150	20	203 ± 7	346 ± 17	70 ± 8	10	346 ± 16	418 ± 25	107 ± 12
30 (NH_4^+PFOA)	20	204 ± 7	268 ± 26**	31 ± 11**	10	278 ± 20**	370 ± 26**	80 ± 10**
28-Day Study: Females								
0	20	142 ± 4	212 ± 13	49 ± 8	10	214 ± 14	243 ± 14	70 ± 7
6	20	143 ± 5	210 ± 14	46 ± 8	10	197 ± 15	231 ± 15	63 ± 7
30	20	141 ± 7	208 ± 12	47 ± 7	10	191 ± 8*	234 ± 13	66 ± 9
150	20	139 ± 8	214 ± 17	54 ± 8	10	212 ± 19	240 ± 22	72 ± 12
30 (NH_4^+PFOA)	20	143 ± 9	208 ± 18	45 ± 6	10	202 ± 21	236 ± 29	67 ± 11
90-Day Study: Males								
0	20	218 ± 6	523 ± 37	139 ± 16	7	501 ± 20	542 ± 22	148 ± 9
1.2	10	219 ± 9	527 ± 41	140 ± 14	–[c]	–	–	–
6	10	217 ± 7	529 ± 38	144 ± 20	–	–	–	–
30	20	218 ± 6	521 ± 43[d]	139 ± 17	6	502 ± 40	545 ± 41	152 ± 17
90-Day Study: Females								
0	20	167 ± 5	302 ± 17	82 ± 11	3–4	286 ± 19[e]	307 ± 24[f]	87 ± 19[f]
1.2	10	165 ± 4	311 ± 26	89 ± 15	–	–	–	–
6	9–10	168 ± 8[g]	306 ± 21[h]	82 ± 12[h]	–	–	–	–
30	20	163 ± 7	309 ± 25	89 ± 14	7	278 ± 28	311 ± 32	92 ± 14

[a] Ammonium PFBA dose groups except where ammonium PFOA (NH_4^+PFOA) is indicated.
[b] Represents weight gain as a percent of initial body weight at start of the treatment period.
[c] Treatment group not included in study design.
[d] *N* = 19.
[e] *N* = 4.
[f] *N* = 3.
[g] *N* = 10.
[h] *N* = 9.
* Statistically significantly different than control ($p < 0.05$).
** Statistically significantly different than control ($p < 0.01$).

3.3. Mortality and clinical signs

No mortalities related to treatment with either NH_4^+PFBA or NH_4^+PFOA occurred during the conduct of the 28-day and 90-day studies. Two accidental deaths occurred during the 28-day study. A 6 mg/kg-d recovery-group male was found stuck in his cage on dosing day 18, and a 6 mg/kg-d main-study group female died during blood sampling at the end of treatment.

Five deaths and one humane sacrifice, all unrelated to NH_4^+PFBA administration, occurred during the conduct of the 90-day study. A 30 mg/kg-d dose-group male was sacrificed on day 51 and diagnosed with a hemangioma of the brain. One 6 mg/kg-d female was found dead before dosing on day 83 from an apparent gastric puncture. Four control recovery-group females were found dead after blood collection at the end of treatment, and this circumstance was believed to be due to complications from subcutaneous injection of the Ketalin®/Dormitor® anesthetic.

No overt signs of toxicity were noted among the NH_4^+PFBA-treated groups in both the 28-day and 90-day studies. However, all rats given 30 mg/kg-d NH_4^+PFOA presented with hunched posture beginning in the second week of dosing. In addition, lower incidences of occasional abnormal gait, piloerection, and lean appearance were noted in NH_4^+PFOA-treated rats during the dosing period.

3.4. Functional observations

During the course of both studies, no effects on hearing, static righting, and grip strength were observed. Treatment with NH_4^+PFBA did not affect motor activity. In rats given 30 mg/kg-d NH_4^+PFOA, slightly decreased (not statistically significant) motor activity relative to control rat motor activity was noted. Delayed direct bilateral pupillary reflex was noted in all 150 mg/kg-d NH_4^+PFBA males and all 30 mg/kg-d NH_4^+PFOA males in the 28-day study. No abnormal consensual pupillary light reflex was noted. Recovery from this effect was not assessed in the 28-day study.

Because of the delayed direct pupillary reflex noted in the 28-day study males, additional pupillary reflex examinations were included in the 90-day study. Pupillary reflex tests conducted during weeks 8 and 12 of dosing indicated that the pupils of 30 mg/kg-d male rats took slightly longer to enlarge fully under dark conditions. In subjectively comparing pupil size before and during light stimulus, 2 of 40 (5%) observations of control rats and 7 of 39 (18%) observations of 30 mg/kg-d dose-group rats showed no difference in size. These observations were generally unilateral, of low incidence, and not consistently observed. No abnormal consensual pupillary light reflex was noted. Reflexes were normal during the recovery period. Ophthalmoscopic examinations during the 90-day study did not reveal compound-related abnormalities. It is worthwhile noting here that no histological changes in optic nerves from these rats were observed in either the 28-day or 90-day studies.

3.5. Body weights, weight change, and food consumption

Body-weight and weight-gain data are summarized in Table 4. Treatment of male and female rats with NH_4^+PFBA did not affect body weight, weight gain, or food consumption at any dose or dosing period. In the 28-day study, male rats given 30 mg/kg-d NH_4^+PFOA showed significant decreases in weight gain with occasional weight loss throughout the dosing and recovery periods along with decreased food consumption in the first and fourth week of the study (data not shown) which returned to normal during the recovery period.

Table 5
Liver weight (g, mean ± *SD*) at end of treatment period and end of recovery period in male and female rats given 28 or 90 daily doses of NH_4^+PFBA.

	Mean ± *SD* (*N*) liver weight by dose group, mg/kg-d				
	0	1.2	6	30	150
28-Day Study: Males					
Treatment[a]	8.08 ± 0.73 (10)	–[b]	8.76 ± 1.21 (10)	10.26 ± 1.43** (10)	11.69 ± 1.53** (10)
Recovery[c]	9.53 ± 1.42 (10)	–	9.43 ± 0.78 (9)	9.69 ± 0.79 (10)	9.15 ± 0.50 (10)
28-Day Study: Females					
Treatment	4.89 ± 0.53 (9)	–	5.30 ± 0.37 (9)	4.88 ± 0.55 (10)	5.53 ± 0.67 (10)
Recovery	5.57 ± 0.36 (10)	–	5.27 ± 0.65 (10)	5.38 ± 0.55 (10)	5.33 ± 0.71 (10)
90-Day Study: Males					
Treatment	10.92 ± 1.17 (10)	11.40 ± 0.75 (10)	11.39 ± 1.36 (10)	13.41 ± 2.01** (10)	–
Recovery	10.67 ± 0.74 (7)	–	–	11.13 ± 1.79 (6)	–
90-Day Study: Females					
Treatment	6.24 ± 0.39 (10)	6.41 ± 0.71 (10)	6.08 ± 0.39 (9)	6.72 ± 0.75 (10)	–
Recovery	6.06 ± 0.30 (3)	–	–	6.36 ± 0.67 (7)	–

[a] Livers weighed at the end of the treatment period (28 or 90 days).
[b] Treatment group not included in study design.
[c] Livers weighed at the end of the 21-day recovery period.
** Statistically significantly different than control ($p < 0.01$).

3.6. Organ weights

After treatment with NH_4^+PFBA, the only organ weight affected was liver weight and only in male rats (Table 5). Statistically significant ($p < 0.01$) dose-related increases in absolute liver weights occurred in male rats at doses of 30 mg/kg-d (28- and 90-day studies) and 150 mg/kg-d (28-day study). This resulted in increases in mean liver weight of 27% over control at 30 mg/kg-d and 45% over control at 150 mg/kg-d for male rats of the 28-day study, and 23% over control at 30 mg/kg-d for males of the 90-day study. Because body weight was unaffected in males given NH_4^+PFBA, liver weight as a percent of body weight was also increased in male rats in these dose groups (data not shown). In both the 28-day and 90-day studies, absolute and relative organ weights were not statistically significantly different than male control values at the end of the recovery period.

In addition to the increase in liver weight, statistically significant changes occurred in absolute and/or relative weights of a few organs in a non-dose-dependent manner. These additional observed statistically significant changes in absolute and/or relative weights for organs other than liver were considered spurious findings, because they did not show progression with dose, were not consistent between the two studies, and had no histological correlates. Included among these were statistically significantly ($p < 0.01$) elevated absolute thyroid weights in males given 6 and 30 mg/kg-d NH_4^+PFBA. Mean thyroid weights in g for males at the end of treatment were 0.011, 0.023, 0.024, and 0.017 for the 0, 6, 30, and 150 mg/kg-d dose groups, respectively. Mean thyroid weight in males given 30 mg/kg-d NH_4^+PFOA were 0.013 g. At the end of recovery, mean thyroid weights were 0.026, 0.028, 0.024, and 0.025 g for the respective NH_4^+PFBA dose groups and 0.025 g for the NH_4^+PFOA-treated males. Due to lack of dose-response progression, known difficulties in obtaining thyroid weights [41], and lack of consistent histological correlates, the thyroid weight increases observed in the 6 and 30 mg/kg-d NH_4^+PFBA dose group males at the end of the 28-day treatment period were not considered toxicologically significant.

For female rats, NH_4^+PFBA did not result in treatment-related changes in absolute or relative organ weights. The only statistically significant difference compared to control was for increased absolute weight of the thymus at 6 mg/kg-d in the 28-day study (0.434 ± 0.084 g ($N = 9$) versus control value of 0.360 ± 0.048 g ($N = 10$)). Because mean weight of the thymus relative to body weight was not affected and due to lack of dose-response, this finding was not considered toxicologically meaningful.

Treatment of males with NH_4^+PFOA at 30 mg/kg-d for 28-day resulted in statistically significant changes in the absolute and/or relative weights of several organs at the end of treatment and/or recovery periods (Table 6). Absolute and relative liver weights were increased at the end of treatment, and this elevation remained in both parameters at the end of recovery. Absolute and relative liver weight for 30 mg/kg-d NH_4^+PFOA-treated males were 192% and 156% of control values, respectively. These increases were greater than those observed for NH_4^+PFBA-treated rats given 150 mg/kg-d, which were 145% and 149% of control values, respectively. The decrease in absolute weights of the brain, heart, and thymus an the increase in the relative weights of the brain, kidneys, testes, and epididymides likely are the result of the significant reduction in body-weight relative to control males at the end of both the treatment and recovery periods.

For females treated with 30 mg/kg-d NH_4^+PFOA in the 28-day study, only liver weight was changed with statistical significance. At the end of the treatment period, for control and NH_4^+PFOA-treated females, respectively, mean absolute liver weight (expressed as mean ± standard deviation, *SD*) was 4.89 ± 0.53 g vs. 6.13 ± 0.69 g ($p < 0.01$), and mean liver weight as a percent of body weight was 2.44 ± 0.20% vs. 3.07 ± 0.25% ($P < 0.01$). This liver weight difference was not present in NH_4^+PFOA-treated females at the end of the recovery period.

3.7. Hematology

Treatment of rats with NH_4^+PFBA for 28 or 90 days did not result in toxicologically significant changes in hematological parameters. Any statistically significant changes relative to controls at the end of the treatment and recovery periods were of a minor magnitude, within current reference ranges for Sprague Dawley rats of same sex and similar age [42,43], and/or occurred in the absence of a clear dose-related distribution. Compared to controls at the end of the 90-day treatment period, highest dose-group (30 mg/kg-d) males had slight but statistically significant reductions in red blood cell concentration (mean ± *SD* of 9.19 ± 0.26 vs. 8.84 ± 0.41 (units = 10^{12}/L, $p < 0.05$) in control and treated, respectively), hemoglobin concentration (mean ± *SD* of 10.5 ± 0.2 vs. 9.9 ± 0.4 (units = mmol/L, $p < 0.01$)), and hematocrit (mean ± *SD* of 49.1 ± 1.3 vs. 46.9 ± 2.0 (units = %, $p < 0.01$)) along with increased red cell distribution width (mean ± *SD* of 13.3 ± 0.7 vs. 14.0 ± 0.9 (units = %, $p < 0.05$)). The small reduction in hemoglobin concentration and hematocrit in the 30 mg/kg male dose group were still statistically significant at the end of the recovery period, although not outside of published reference ranges.

Table 6
Absolute and relative organ weights in male rats given 30 mg/kg-d NH_4^+PFOA at end of treatment period (28 days) and end of recovery period (21 days).

Organ	Time of weight	Absolute organ weight (g)		Organ weight as % of body weight[a] (%)	
		Control	NH_4^+PFOA-Treated	Control	NH_4^+PFOA-Treated
Brain	Treatment[b]	2.00 ± 0.06	1.90 ± 0.07**	0.60 ± 0.04	0.77 ± 0.09**
	Recovery[c]	2.03 ± 0.08	1.98 ± 0.08	0.05 ± 0.04	0.06 ± 0.04**
Heart	Treatment	1.13 ± 0.12	0.87 ± 0.11**	0.34 ± 0.03	0.31 ± 0.027
	Recovery	1.29 ± 0.17	1.10 ± 0.08**	0.31 ± 0.03	0.32 ± 0.021
Liver	Treatment	8.08 ± 0.73	15.54 ± 2.18**	2.42 ± 0.17	6.19 ± 0.39**
	Recovery	9.57 ± 1.42	9.71 ± 1.16	2.31 ± 0.19	2.77 ± 0.22**
Thymus	Treatment	0.46 ± 0.11	0.30 ± 0.07**	0.14 ± 0.04	0.12 ± 0.02
	Recovery	0.53 ± 0.36	0.36 ± 0.08	0.13 ± 0.10	0.10 ± 0.02
Kidneys	Treatment	2.79 ± 0.34	2.62 ± 0.37	0.84 ± 0.08	1.04 ± 0.08**
	Recovery	3.14 ± 0.40	2.92 ± 0.24	0.76 ± 0.07	0.84 ± 0.07*
Testes	Treatment	3.15 ± 0.21	3.13 ± 0.19	0.94 ± 0.07	1.26 ± 0.13**
	Recovery	3.09 ± 0.85	3.18 ± 0.21	0.75 ± 0.20	0.91 ± 0.06**
Epididymides	Treatment	0.92 ± 0.06	0.84 ± 0.09	0.28 ± 0.02	0.34 ± 0.04**
	Recovery	1.17 ± 0.234	1.12 ± 0.08	0.28 ± 0.06	0.32 ± 0.04

[a] Body weights (mean ± *SD*) for control and NH_4^+PFOA-treated male rats, respectively, were 334 ± 21 g and 251 ± 31** g at end of the 28-day treatment period and 414 ± 36 g and 350 ± 26** g at the end of the 21-day recovery period.
[b] End of treatment (28 days).
[c] End of recovery (21 days).
* Statistically significantly different than control ($p < 0.05$).
** Statistically significantly different than control ($p < 0.01$).

There were no toxicologically significant alterations in hematological parameters in male or female rats treated with 30 mg/kg-d NH_4^+PFOA. Any statistically significant changes relative to controls at the end of the treatment and recovery periods were within reference ranges for Sprague Dawley rats of same sex and similar age. However, compared to controls, NH_4^+PFOA-treated males had changes in several red cell parameters at the end of both the treatment and recovery periods. Statistically significant reductions in red blood cell concentration (mean ± *SD* of 8.19 ± 0.49 vs. 7.58 ± 0.41 (units = 10^{12}/L, $p < 0.01$) in control and treated, respectively), hemoglobin concentration (mean ± *SD* of 9.8 ± 0.6 vs. 8.8 ± 0.5 (units = mmol/L, $p < 0.01$)), hematocrit (mean ± *SD* of 47.9 ± 3.1 vs. 43.2 ± 2.5 (units = %, $p < 0.01$)), and mean corpuscular hemoglobin (mean ± *SD* of 1.20 ± 0.04 vs. 1.16 ± 0.03 (units = fmol, $p < 0.01$)) were noted along with increased red cell distribution width (mean ± *SD* of 12.2 ± 0.3 vs. 13.4 ± 1.0 (units = %, $p < 0.01$)). At the end of recovery, the reductions in red blood cell concentration, hemoglobin, and hematocrit and the increase in red cell distribution width remained statistically significantly different compared to male recovery-group controls ($p < 0.01$).

3.8. Clinical biochemistry

Treatment of male rats with NH_4^+PFBA for 28 days reduced serum total cholesterol in a dose-dependent manner with statistical significance occurring at 30 and 150 mg/kg-d (Table 7). In addition, there were a few statistically significant changes in clinical chemistry parameters that were within recent published reference ranges [42,43], and as such are not toxicologically meaningful. These included increased serum potassium (mean ± *SD* of 3.96 ± 0.27 vs. 4.27 ± 0.54 (units = mmol/L, $p < 0.01$) in control and treated, respectively) and inorganic phosphate (mean ± *SD* of 2.34 ± 0.20 vs. 2.61 ± 0.34 (units = mmol/L, $p < 0.01$)) occurred at the 150 mg/kg-d dose in males. These parameters recovered to be non-statistically significantly different than control levels at the end of the recovery phase (data not shown). In males, at the end of recovery, serum Na^+ was increased in a dose-dependent manner (mean ± *SD* values of 137.8 ± 0.8, 138.8 ± 1.2, 139.0 ± 0.7, and 139.6 ± 1.1 mmol/L for the 0, 6, 30, and 150 mg/kg-d dose groups, respectively), reaching statistical significance at 30 ($p < 0.05$) and 150 mg/kg-d ($p < 0.01$), and serum total protein was increased slightly but with statistical significance ($p < 0.05$) at 150 mg/kg-d (64.5 ± 1.7 g/L vs. 67.3 ± 1.9 g/L in controls vs. treated, respectively). In addition, although not of toxicological significance, at the end of the recovery period following 28 days of treatment, serum total bilirubin was decreased in a dose-dependent manner (mean ± *SD* values of 4.0 ± 0.8, 3.6 ± 0.4, 3.4 ± 0.5, and 3.2 ± 0.6 μmol/L for the 0, 6, 30, and 150 mg/kg-d dose groups, respectively), reaching statistical significance ($p < 0.01$) at 150 mg/kg-d. There were no observations that were considered to be treatment-related or toxicologically significant in females from the 28-day study.

After 90 days of treatment with NH_4^+PFBA, males in the 30 mg/kg-d dose group showed a statistically significant ($p < 0.01$) 32% increase in alkaline phosphatase compared to controls (146 ± 38 IU vs. 193 ± 55 IU, for controls and treated, respectively) as well as slight but statistically significant decreases in serum Ca^{++} (2.73 ± 0.06 mmol/L vs. 2.67 ± 0.05 mmol/L ($p < 0.05$) for controls and treated, respectively) and serum total protein (71.4 ± 3.0 g/L vs. 67.8 ± 3.0 g/L ($p < 0.01$)). In addition, serum total bilirubin was reduced in a dose-dependent manner at the end of the 90-day treatment period in males (mean ± *SD* values of 2.8 ± 0.3, 2.6 ± 0.3, 2.5 ± 0.3, and 2.2 ± 0.3 μmol/L for the 0, 1.2, 6 ($p < 0.05$), and 30 ($p < 0.01$) mg/kg-d dose groups, respectively). Reduction in serum total bilirubin also occurred in females at the 30 mg/kg-d dose (mean ± *SD* values of 3.8 ± 0.6, 3.4 ± 0.5, 3.5 ± 0.5, and 3.1 ± 0.5 ($p < 0.01$) μmol/L for the 0, 1.2, 6, and 30 mg/kg-d dose groups, respectively). All of these findings were within published reference ranges for Sprague Dawley rats [42,43] and were absent at the end of the recovery period (data not shown). Although serum total cholesterol was not reduced with statistical significance in males given NH_4^+PFBA at the end of the 90-day treatment period (Table 7), it is noteworthy that the mean serum total cholesterol in the 30 mg/kg-d dose group was 15% lower than that in the control group, consistent with the statistically significant 20% reduction observed in the 28-day study at the same dose.

A number of statistically significant changes in clinical biochemistry parameters were noted after treatment of rats with 30 mg/kg-d of NH_4^+PFOA for 28 days. Serum alanine aminotransferase was increased by approximately 20% in males (55.8 ± 22.1 IU vs. 66.5 ± 16.2 IU ($p < 0.05$) for control and treated, respectively) and females (38.4 ± 8.7 IU vs. 47.4 ± 11.7 IU ($p < 0.05$)). In males, there were approximately 40% increases in alkaline phosphatase activity (234 ± 51 IU vs. 320 ± 67 IU ($p < 0.01$)) and serum urea concentration (6.3 ± 1.5 mmol/L vs. 9.0 ± 1.5 mmol/L ($p < 0.01$)), as well as a small but statistically significant increase in serum Cl^- concentration (100 ± 1 mmol/L vs. 102 ± 2 mmol/L ($p < 0.01$)) and a small

Table 7
Serum total cholesterol (CHOL) in male rats given daily oral doses of NH_4^+PFBA or NH_4^+PFOA for 28 days or NH_4^+PFBA for 90 days.

	End of treatment					End of recovery				
	NH_4^+PFBA				NH_4^+PFOA	NH_4^+PFBA				NH_4^+PFOA
Dose, mg/kg-d	0	6	30	150	30	0	6	30	150	30
28-Day Study										
CHOL, mmol/L	1.37	1.20	1.09**	1.00**	1.53	1.57	1.60	1.70	1.70	1.53
±*SD*	0.27	0.16	0.20	0.25	0.46	0.34	0.17	0.22	0.28	0.19
(*N*)	(20)	(19)	(20)	(20)	(20)	(10)	(9)	(10)	(10)	(10)

	NH_4^+PFBA, end of treatment				End of recovery	
Dose, mg/kg-d	0	1.2	6	30	0	30
90-Day Study						
CHOL, mmol/L	1.69	1.67	1.74	1.44	1.57	1.90
±*SD*	0.28	0.27	0.37	0.38	0.32	0.30
(*N*)	(17)	(10)	(10)	(16)	(7)	(6)

** Statistically significantly different than control ($p < 0.01$).

but statistically significant reduction in total protein (60.3 ± 3.5 g/L vs. 56.2 ± 3.2 g/L ($p < 0.01$)). Increased serum albumin concentrations were also observed in males (31.3 ± 1.9 g/L vs. 33.1 ± 1.7 g/L ($p < 0.05$)) and females (34.4 ± 2.2 g/L vs. 36.2 ± 2.0 g/L ($p < 0.01$)). At the end of the recovery period, reduced total protein and increased Cl^- persisted in males and increased alanine aminotransferase persisted in females (data not shown). Three statistically significant differences occurred between values for control and NH_4^+PFOA-treated males at the end of the recovery period that were not present at the end of treatment. These included slightly increased serum Na^+ (137.8 ± 0.8 mmol/L vs. 139.1 ± 1.1 mmol/L ($p < 0.05$)) and PO_4^{++} (2.19 ± 0.22 mmol/L vs. 2.35 ± 0.12 mmol/L ($p < 0.05$)) and decreased serum total bilirubin (4.0 ± 0.8 μmol/L vs. 2.8 ± 0.05 μmol/L ($p < 0.01$)).

3.9. Thyroid hormone status

Results of determinations for serum concentrations of TSH, TT4, and FT4 are presented in Table 8. Equilibrium dialysis (ED) was used to measure FT4 in serum samples containing PFBA and PFOA based on prior observations of a negative bias in determining FT4 by analogue methods in the presence of perfluorooctanesulfonate (PFOS) [44] and PFOA (unpublished). However, we have observed that a significant proportion of the serum PFBA in the equilibrium dialysis system migrates into the dialysate resulting in negative bias [45]. As suggested in the literature, a FT4 method based on ultrafiltration or ultracentrifugation may have given more accurate results than the equilibrium dialysis method in the presence of a weak competitor for T4 binding sites which is capable of migrating across the dialysis membrane [46,47]. Therefore, although we provide the equilibrium dialysis results for FT4 in Table 8, these results should be interpreted with caution. Under these circumstances, the TSH results should be considered of greatest clinical significance.

At the end of 28-day oral treatment, administration of NH_4^+PFBA up to 150 mg/kg-d/day did not affect serum TSH levels in either male or female rats compared to the respective controls. There were statistically significant dose-dependent reductions in serum TT4 and FT4 levels observed with NH_4^+PFBA exposure in male rats, however, such alteration was absent at the end of recovery period with the exception of male rats from 150 mg/kg-d dose group for which serum TT4 was still reduced compared to the respective control.

At the end of the treatment and recovery periods in the 90-day study, NH_4^+PFBA did not affect serum TSH in either male or female rats, nor did it affect TT4 or FT4 in females. In males, serum TT4 was decreased at the end of treatment with statistical significance compared to controls and was increased at the end of the recovery period. The ED-based analysis for FT4 required the largest amount of serum, and, unfortunately, adequate serum for measurement of FT4 was not available for control males at the end of the treatment period. However, a reduction in mean FT4 was evident in 30 mg/kg-d dose group males at the end of treatment, based on a statistically significant reduction when compared to the mean value for the 1.2 mg/kg-d dose-group males.

Compared to controls, male rats that received NH_4^+PFOA had statistically significantly reduced serum TSH, TT4, and FT4 levels at the end of treatment period. Female rats, on the other hand, had normal serum TSH but decreased TT4 and FT4. Serum TSH concentrations in both male and female NH_4^+PFOA-treated rats were similar to the controls at the end of the recovery period. While female serum TT4 and FT4 levels had also returned to values similar to those of controls at the end of recovery, male serum TT4 and FT4 remained reduced.

3.10. Microscopic pathology

The incidence and severity of liver and thyroid microscopic histological findings for NH_4^+PFBA-treated male rats from the 28-day and 90-day studies are shown in Tables 9 and 10, respectively. Microscopic findings related to treatment with NH_4^+PFBA and NH_4^+PFOA were noted in liver and thyroid sections. Table 9 also presents the incidence and severity of liver and thyroid microscopic histological findings in NH_4^+PFOA-treated male rats from the 28-day study.

Liver findings associated with NH_4^+PFBA treatment for 28 days (Table 9) consisted of an increased incidence of grade 1 (minimal) hepatocellular hypertrophy in 6 of 10 males (60%) at the highest dose of 150 mg/kg-d. This NH_4^+PFBA-related finding was not present in females and was no longer present in males at the end of the recovery period. One recovery group female in the 150 mg/kg-d NH_4^+PFBA dose group had grade 1 (minimal) hepatocellular necrosis, and one recovery-group control female had a grade 2 (slight) finding of hepatocellular coagulative necrosis. Therefore, the finding of necrosis in the 150 mg/kg-d recovery female was not given toxicological significance. No other notable liver findings were present among the control and the 6 mg/kg-d and 30 mg/kg-d NH_4^+PFBA groups either at the end of treatment or recovery.

In male rats, NH_4^+PFBA treatment for 28 days increased the incidence of thyroid follicular epithelial hypertrophy/hyperplasia at the end of the treatment period in the 30 mg/kg-d dose group (9 of 10, all grade 1) and 150 mg/kg-d dose group (7 of 10, 4 grade 1 and 3 grade 2) as compared to 3 of 10 grade 1 findings in each of the control and 6 mg/kg-d dose groups. Recovery male rats in the control group and 150 mg/kg-d dose group each had 4 of 10 grade 1 findings

Table 8
Summary of serum thyrotropin, serum total thyroxine, and serum free thyroxine concentrations in male and female rats treated with NH_4^+PFBA or NH_4^+PFOA for either 28 or 90 days.

	End of treatment period[a]			End of recovery period[a]		
Dose group[b], mg/kg-d	TSH[c] (ng/dL)	TT4[d] (ug/dL)	dFT4[e] (ng/dL)	TSH (ng/dL)	TT4 (ug/dL)	dFT4 (ng/dL)
28-Day Study: Males						
0	4.88 ± 3.30	3.09 ± 0.82	1.83 ± 0.53	3.15 ± 0.66[f]	3.52 ± 0.56[f]	1.98 ± 0.43[f]
6	3.08 ± 0.82	1.26 ± 0.26[*]	0.98 ± 0.34[*]	3.51 ± 1.59	3.64 ± 0.83	2.02 ± 0.38
30	4.20 ± 1.46	*1.04 ± 0.37*[*,g]	0.91 ± 0.31[*]	4.31 ± 2.15	3.38 ± 0.67	1.84 ± 0.31
150	4.04 ± 1.13	*0.66 ± 0.33*[*,g]	0.63 ± 0.19[*]	4.76 ± 2.22	2.71 ± 0.40[*]	1.93 ± 0.29[f]
30 (NH_4^+PFOA)	2.59 ± 0.96[*]	0.52 ± 0.15[*]	0.94 ± 0.34[*,f]	5.89 ± 4.27	1.40 ± 0.39[*]	1.26 ± 0.29[*]
28-Day Study: Females[e]						
0	2.57 ± 1.85	2.06 ± 0.74	2.00 ± 0.68[f]	3.92 ± 1.73	2.22 ± 0.64	2.09 ± 0.62
6	3.43 ± 1.41[f]	1.90 ± 1.12[f]	1.99 ± 0.87[f]	3.50 ± 1.64	2.16 ± 0.78	2.00 ± 0.81
30	2.22 ± 2.06	2.62 ± 0.76	2.36 ± 0.66	3.11 ± 1.18	1.94 ± 0.65	1.95 ± 0.47
150	2.93 ± 1.05	1.43 ± 0.60	1.50 ± 0.35	3.63 ± 2.92	2.18 ± 0.43	2.26 ± 0.36
30 (NH_4^+PFOA)	2.45 ± 0.82	*0.62 ± 0.42*[*,h]	1.14 ± 0.35[*]	2.92 ± 1.17	1.77 ± 0.74	2.14 ± 0.73
90-Day Study: Males						
0	2.14 ± 1.18	5.27 ± 0.71	–[i]	2.20 ± 1.07[j]	5.14 ± 0.33[j]	1.47 ± 0.12[k]
1.2	2.62 ± 1.96	5.97 ± 1.08	1.38 ± 0.27[l,m]	–[n]	–[n]	–[n]
6	2.87 ± 2.14	4.46 ± 0.88[f]	1.25 ± 0.24[o,m]	–[n]	–[n]	–[n]
30	1.63 ± 1.42	3.23 ± 0.55[*,f]	0.97 ± 0.11[+,j,m]	3.40 ± 1.86[p]	6.37 ± 0.76[*,p]	1.42 ± 0.28[l]
90-Day Study: Females						
0	0.94 ± 0.37	4.97 ± 1.24	1.57 ± 0.32[k]	1.07 ± 0.12[k]	5.90 ± 0.85[k]	1.50[q]
1.2	1.08 ± 0.44	5.54 ± 1.71	1.47 ± 0.12[k]	–[n]	–[n]	–[n]
6	1.46 ± 0.89[f]	5.65 ± 0.82[r]	2.00 ± 0.28[s]	–[n]	–[n]	–[n]
30	1.07 ± 0.58	3.94 ± 0.42[f]	1.33 ± 0.22[o]	2.00 ± 2.07[j]	5.37 ± 1.32[j]	1.98 ± 0.33[l]

[a] $N = 10$ per group unless otherwise noted due to no sample or insufficient sample for assay.
[b] Ammonium PFBA dose groups except where ammonium PFOA (NH_4^+PFOA) is indicated.
[c] Serum thyrotropin (thyroid stimulating hormone) concentration.
[d] Serum total thyroxine concentration.
[e] Serum free thyroxine concentration as determined by equilibrium dialysis followed by radioimmunoassay.
[f] $N = 9$.
[g] Values in italics are estimates. Three values were below the limit of quantitation (LOQ = 0.50 μg/dL); these three values were assigned a value 0.35 (LOQ dived by the square root of 2) for calculation of statistical parameters.
[h] Six values were below the quantitation level of 0.5 μg/dL; these six values were assigned a value 0.35 (LOQ dived by the square root of 2) for calculation of statistical parameters. The four samples that were quantifiable had values of 0.50, 1.00, 1.10, and 1.50.
[i] No sample available due to insufficient sample volume for assays.
[j] $N = 7$.
[k] $N = 3$.
[l] $N = 5$.
[m] Control value not available for comparison due to insufficient volume for assay.
[n] Recovery group not included in study design.
[o] $N = 4$.
[p] $N = 6$.
[q] $N = 1$.
[r] $N = 8$.
[s] $N = 2$.
[*] Statistically significantly different than control ($p < 0.05$).
[+] Statistically significantly different than 1.2 mg/kg dose group ($p < 0.05$).

for thyroid follicular epithelial hypertrophy/hyperplasia, indicating resolution of the finding observed at the end of the treatment period.

In female rats treated for 28 days, treatment with NH_4^+PFBA did not increase the incidence or grade of thyroid follicular epithelial hypertrophy/hyperplasia with toxicological significance. Control female incidence was 3 of 10 (30%, all grade 1) versus 4 of 10 (40%, all grade 1) for the 150 mg/kg-d NH_4^+PFBA-treated females.

As in the 28-day study, microscopic findings were noted in the liver and thyroids of male rats given NH_4^+PFBA for 90 days (Table 10). A diffuse panlobular hepatocellular hypertrophy was noted in 9 of 10 (90%) males at the highest dose (30 mg/kg-d). Of the 9 occurrences, 5 were grade 1 (minimal) and 4 were grade 2 (slight). Hepatocellular hypertrophy was not present among females or among recovery males in the 30 mg/kg-d dose group. An increase in incidence and severity of diffuse thyroid follicular epithelial hypertrophy/hyperplasia was also present in 30 mg/kg-d males at the end of the 90-day treatment period. The incidences were 4 of 10 (40%), 6 of 10 (60%), 4 of 10 (40%), and 9 of 10 (90%) in the control, 1.2 mg/kg-d, 6 mg/kg-d, and 30 mg/kg-d male groups, respectively. With the exception of 5 grade 2 (slight) findings in the 30 mg/kg-d dose group, all findings were grade 1 (minimal). At the end of recovery, there was no toxicologically significant difference between the control incidence (3 of 7 grade 1 findings) and the 30 mg/kg-d dose group (3 of 6 grade 1 findings).

Histological findings in male rats treated with NH_4^+PFOA are provided in Table 9. In NH_4^+PFOA-treated rats (28-day study), grade 1 (minimal) hepatocellular hypertrophy was noted in 3 of 10 females (30%), and hepatocellular hypertrophy occurred in all males (10 of 10, 100%), ranging in grade from grade 1 (minimal, 1 of 10) to grade 2 (slight, 6 of 10) and grade 3 (moderate, 3 of 10). In males treated with NH_4^+PFOA, hepatocellular hypertrophy was accompanied by an increased incidence of hepatocellular coagulative necrosis (4 of 10 (40%), 3 of 10 as grade 1 (minimal) and 1 of 10 as grade 2 (slight)). At the end of recovery, grade 1 hepatocellular hypertrophy was present in 4 of 10 (40%) males; however, hepatocellular necrosis was

Table 9
Microscopic histological observations in male rats treated with NH_4^+PFBA or NH_4^+PFOA for 28 days.

	End of treatment					End of recovery		
Perfluoroalkyl	NH_4^+PFBA				NH_4^+PFOA	NH_4^+PFBA		NH_4^+PFOA
Dose (mg/kg-d)	0	6	30	150	30	0	150	30
Number of rats evaluated	10	10	10	10	10	10	10	10
Microscopic histological observation								
Liver								
Hepatocellular hypertrophy								
Total observations, *N* (%)	0 (0)	0 (0)	0 (0)	6 (60)	10 (100)	0 (0)	0 (0)	4 (40)
Grade = minimal	–	–	–	6 (60)	1 (10)	–	–	4 (40)
Grade = slight	–	–	–	–	6 (60)	–	–	–
Grade = moderate	–	–	–	–	3 (30)	–	–	–
Hepatocellular coagulative necrosis								
Total observations, *N* (%)	0 (0)	0 (0)	0 (0)	0 (0)	4 (40)	0 (0)	0 (0)	0 (0)
Grade = minimal	–	–	–	–	3 (10)	–	–	–
Grade = slight	–	–	–	–	1 (60)	–	–	–
Thyroid								
Follicular hypertrophy/hyperplasia								
Total observations, *N* (%)	3 (30)	3 (30)	9 (90)	7 (70)	10 (100)	4 (40)	4 (40)	8 (80)
Grade = minimal	3 (30)	3 (30)	9 (90)	4 (40)	3 (30)	4 (40)	4 (40)	8 (80)
Grade = slight	–	–	–	3 (30)	6 (60)	–	–	–
Grade = moderate	–	–	–	–	1 (10)	–	–	–

Table 10
Microscopic histological observations in male rats treated with NH_4^+PFBA for 90 days.

	End of treatment				End of recovery	
Dose (mg/kg-d)	0	1.2	6	30	0	30
Number of rats evaluated	10	10	10	10	7	6
Microscopic histological observation						
Liver						
Hepatocellular hypertrophy						
Total observations, *N* (%)	0 (0)	0 (0)	0 (0)	9 (90)	0 (0)	0 (0)
Grade = minimal	–	–	–	5 (50)	–	–
Grade = slight	–	–	–	4 (40)	–	–
Thyroid						
Follicular hypertrophy/hyperplasia						
Total observations, *N* (%)	4 (40)	6 (60)	4 (40)	9 (90)	3 (43)	3 (50)
Grade = minimal	4	6	4 (40)	4 (40)	3 (43)	3 (50)
Grade = slight	–	–	–	5 (50)	–	–

not evident. Female rats given 30 mg/kg-d NH_4^+PFOA had an incidence of 1 of 10 (grade 1) for thyroid follicular hypertrophy/hyperplasia.

Although Tables 9 and 10 provide sound subjective histological observations of the thyroids in male rats from both the 28-day and 90-day studies, the histomorphometric analyses of thyroid follicles provide a more quantitative indication of thyroid response [41]. Results of male rat thyroids histomorphometric analyses are provided in Table 11. In male rats from the 28-day study, mean thyroid follicular epithelial cell height of male

Table 11
Summary of thyroid gland histomorphometric analyses from male rats treated with NH_4^+PFBA or NH_4^+PFOA for either 28 or 90 days.

Dose group[b], mg/kg-d	End of treatment period[a]		End of recovery period[a]	
	Cell height[c] (μm)	Colloidal area[d] (μm^2)	Cell height (μm)	Colloidal area (μm^2)
28-Day Study: Males				
0	10.6 ± 2.3	6629.4 ± 1281.0	14.5 ± 2.1	7132.8 ± 3878.5
6	10.1 ± 1.3	7271.3 ± 2761.1	–[e]	–
30	10.5 ± 1.2	6111.5 ± 2435.6	–	–
150	12.7 ± 2.3	7270.3 ± 2016.1	15.3 ± 2.4	5980.1 ± 2157.0
30 (NH_4^+PFOA)	14.8 ± 2.4*	6039.4 ± 2754.9	16.1 ± 1.9	4950.3 ± 2215.6
90-Day Study: Males				
0	9.4 ± 1.7	5029.4 ± 1958.0	8.3 ± 0.8	5592.1 ± 1636.2
1.2	8.8 ± 1.5	5106.9 ± 2050.1	–	–
6	9.1 ± 1.8	5939.2 ± 2016.4	–	–
30	9.8 ± 1.8	4379.6 ± 1738.5	8.8 ± 1.5	5172.2 ± 2731.1

[a] *N* = 10 per group unless otherwise noted due to no sample or insufficient sample for assay.
[b] Ammonium PFBA dose groups except where ammonium PFOA (NH_4^+PFOA) is indicated.
[c] Thyroid follicular epithelial cell height.
[d] Thyroid follicular colloidal area.
[e] Treatment group not included in study design.
* Statistically significantly different than control ($p < 0.05$).

rats from the main NH_4^+PFBA-treated group at 150 mg/kg-d was greater than concurrent control values; although, the difference was not statistically significant and was not as pronounced as the statistically significant difference observed in male rats treated with NH_4^+PFOA. The increased thyroid follicular cell height persisted in the 150 mg/kg-d recovery group, but not to the extent observed in the NH_4^+PFOA-treated rats, and in neither case were these statistically significantly different from the recovery control rats. Thyroid follicular epithelial cell height was unaffected in the 6 and 30 mg/kg-d NH_4^+PFBA groups. There were no statistically significant effects on follicular colloid cross-sectional area for either NH_4^+PFBA or NH_4^+PFOA, and, although mean follicular colloid cross-sectional area was greater than control values in main group NH_4^+PFBA-treated rats at 6 and 150 mg/kg-d, it was smaller in those given 30 mg/kg-d NH_4^+PFBA or NH_4^+PFOA.

In male thyroids from the 90-day study, mean thyroid follicular epithelial cell heights of male rats from the 30 mg/kg-d main and recovery groups were slightly higher than concurrent control values but within expected variation of the mean values and not statistically significant. Compared to controls, mean thyroid follicular colloidal cross-sectional area was slightly higher in the low- and mid-dose groups and slightly less in the 30 mg/kg-d dose group males, but all differences from control were without statistical significance. Reduced cross-sectional colloidal area would be expected with increased resorption of colloid due to increased activity of follicular epithelial cells, but the degree of variability observed in these parameters suggests that any apparent differences in epithelial cell height and follicular colloidal area were a result of normal biological variation in mean values rather than an effect of NH_4^+PFBA.

3.11. Quantitative RT-qPCR mRNA transcriptional analysis

Results for hepatic quantitative RT-qPCR analyses, expressed as times control for each mRNA transcript, are presented graphically in Figs. 1–3. Fig. 1 provides data for male rats at the end of dosing with NH_4^+PFBA for 28 days. A statistically significant increase in mRNA for proliferating cell nuclear antigen (Pcna), a marker for cell proliferation, occurred at the 150 mg/kg-d dose. Major responses were observed for acyl CoA oxidase (Acox) and arachidonic acid monooxygenase (Cyp4A1) that were statistically significant at 30 and 150 mg/kg-d, suggesting activation of the peroxisome proliferator activated receptor α (PPARα). Another transcript responsive to PPARα activation as well as thyroid receptor activation, malic enzyme, also appeared to be increased in a dose-dependent manner, however, variability precluded a statistically significant response. UDP-glucuronyltransferase 1A1 (Ugt1A1), an isoform known to conjugate thyroxine in rats, was increased with statistical significance at 30 and 150 mg/kg-d, and UDP-glucuronyltransferase 1A (common, Ugt1A) was also increased at the highest dose. However, decreased expression of UDP-glucuronyltransferase 1A6 (Ugt1A6), another isoform known to conjugate thyroxine, and UDP-glucuronyltransferase 2B (Ugt2B), an isoform known to conjugate triiodothyronine, were decreased with statistical significance at the 150 mg/kg-d dose. Three transcripts were decreased with statistical significance in all NH_4^+PFBA dose groups: (1) deiodinase 1 (Dio1), responsible for conversion of thyroxine to triiodothyronine and reverse triiodothyronine; (2) apolipoprotein A1 (ApoA1), the major component of high density lipoprotein; (3) aryl hydrocarbon hydroxylase (Cyp1A1). After 3 weeks of recovery in male groups, all mRNA transcript concentrations were not statistically significantly different than control values, except for: ApoA1 which was increased with statistical significance at all dose levels (1.73, 1.72, and 2.02 times control for the 6, 30, and 150 mg/kg-d dose levels, respectively); Ugt1A which remained increased (1.41 times control) at 150 mg/kg-d; and, fatty acid synthase (Fasn) which was decreased (0.58 times control) with statistical significance at 30 mg/kg-d.

In contrast to males, none of the mRNA transcripts were altered in females with statistical significance relative to control after 28 days of treatment with NH_4^+PFBA (data not shown). However, at the end of 3 weeks of recovery, Acox, Ugt1A1, Cyp2B2, and Cyp4A1 were elevated with statistical significance (1.42, 1.79, 2.19, and 2.06 times control, respectively) in the 150 mg/kg-d group, and ApoA1 was significantly increased in all NH_4^+PFBA groups relative to control (1.67, 1.53, and 1.89 times control for the 6, 30, and 150 mg/kg-d control groups, respectively).

Several transcripts were elevated with statistical significance in males after 90 days of treatment with 30 mg/kg-d NH_4^+PFBA (Fig. 2). Similar to the response in males after 28 days at 30 mg/kg-d, Acox, Cyp4A1, and Ugt1A1 mRNA transcripts were increased. Additionally, malic enzyme and Cyp450 oxidoreductase (Por), which are responsive to activation of PPARα and the thyroid receptor, and Cyp2B2, a marker for activation of the constitutive androstane receptor (CAR), were elevated at 30 mg/kg-d. Also consistent with the 28-day male response, Cyp1A1 mRNA was decreased at all doses, and Dio1 was decreased at 30 mg/kg-d but not 6 mg/kg-d. All of the foregoing transcripts in the 30 mg/kg-d dose group recovery males were in concentrations similar to controls by the end of the recovery period; however, Cyp1A1 was decreased (0.72 times control) and Ugt2B was increased (2.4 times control).

In females from the 90-day study, with the exception of a statistically significant decrease in Por (0.79 times control) at 6 mg/kg-d, there were no altered levels of mRNA transcripts after treatment or recovery (data not shown).

Data for rats given 30 mg/kg-d NH_4^+PFOA for 28 days are presented in Fig. 3. Most of the transcripts evaluated were altered with statistical significance relative to control in the male rats, with the exception of Pcna, Cyp3A1, Ugt1A common, and cholesterol-7α-hydroxylase (Cyp7A1). Transcripts for Acaca, Acox, Cyp4A1, Cyp2B2, malic enzyme, Por, thyroid hormone responsive protein, or Spot14 (Thrsp), Fasn, and Ugt1A1 were all elevated. The response for PPARα-related transcripts (Acox, Cyp4A1, and malic enzyme) was greater than that observed for the same transcripts in males given NH_4^+PFBA for 28 days. In addition, several thyroid hormone responsive gene transcripts were elevated, including malic enzyme, Por, Thrsp, and Fasn. As in rats given NH_4^+PFBA, Dio1, Ugt1a6, Ugt2B, Cyp1A1, and ApoA1 were decreased relative to control. At the end of recovery, most of these changed values in transcripts had resolved to control levels, with the exception of Cyp4a1, Cyp2B2, and Thrsp, all of which had subsided but remained elevated with statistical significance.

In females given 30 mg/kg-d NH_4^+PFOA for 28 days, modest statistically significant increases were observed in Acox, Cyp2B2, Cyp3A1, malic enzyme, Thrsp, and Cyp7A1 (Fig. 3). All recovery values with the exception of Cyp2B2 and malic enzyme were similar to control values (Fig. 3).

4. Discussion

The administration of NH_4^+PFBA appeared to be well tolerated by male and female rats in both the 28-day and 90-day studies. No NH_4^+PFBA treatment-related deaths or overt clinical signs of toxicity were noted, and food consumption and body weights were not significantly different than those in the control groups. Non-treatment-related deaths did not significantly affect the ability to interpret study data for the 28-day and 90-day treatment periods as well as the recovery periods for males and females from the 28-day study and males from the 90-day study. However, the loss of four control recovery-group females at the end of the treatment period due to a complication with Ketalin®/Dormitor® administration had

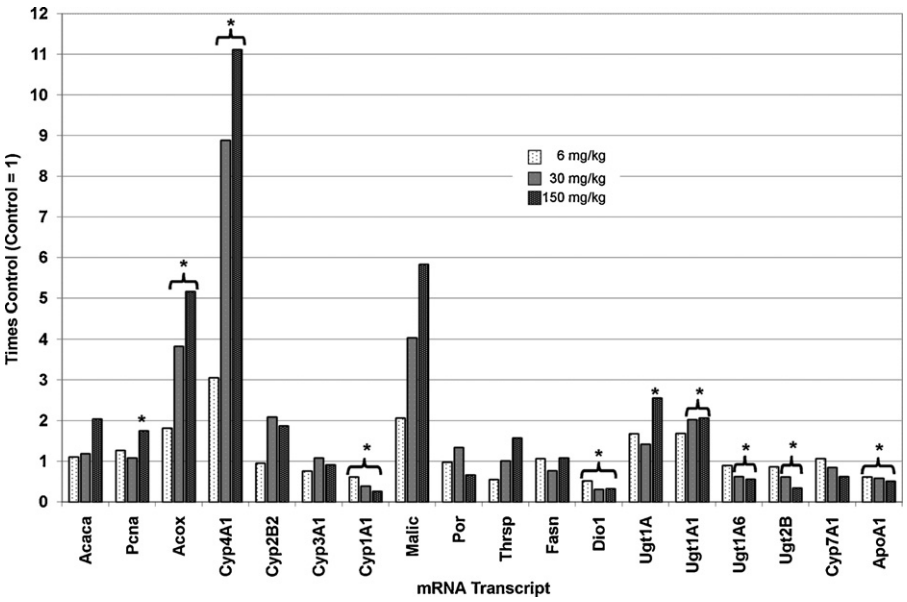

Fig. 1. Mean liver tissue mRNA transcript levels by dose group as measured by quantitaitive rt-PCR for male rats after 28 daily oral doses of NH_4^+PFBA. Quantitative rt-PCR data were normalized to 18s mRNA, and expressed in the figure as times the control transcript level for the individual transcript. Statistically significant results are indicated by an asterisk.

the effect of reducing the *N* in the female recovery control group from 7 to 3, which limited the statistical power for certain comparisons. Because observed statistically significant treatment-related effects in female rats from the 30 mg/kg-d dose group at the end of the 90-day treatment period were limited to slightly reduced MCH and MCHC as well as reduced serum bilirubin, both of which effects were not considered toxicologically meaningful, the reduction in the size of the recovery control group had minimal effect on overall interpretation of study outcome.

With respect to treatment with NH_4^+PFBA, male rats appeared more sensitive than female rats in both the 28-day and 90-day studies. The observed reduced sensitivity of females likely is a result, in part, of the greater elimination rate of PFBA in female rats as compared to males [1]. This inference is supported by the lower within dose group serum and liver PFBA concentrations measured in females as opposed to males (Table 3). Effects attributed to NH_4^+PFBA treatment in males were generally mild, reversible on cessation of treatment, and included: hepatic hypertrophy with minimal to slight hepatocellular hypertrophy; hypothyroxinemia without evidence of a thyroid follicular response; reduced serum total cholesterol; mild reductions in red blood cell parameters without evidence of an effect on red blood cell turnover; and, delayed bilateral pupillary light reflex.

Unlike the experience with NH_4^+PFBA, dosing with 30 mg/kg-d NH_4^+PFOA resulted in increased incidence of clinical signs of toxicity (e.g., hunched posture), increased liver weight in females as well as males, and a major reduction in body weight of males. Mean male body weight in NH_4^+PFOA-treated rats was reduced to 75%

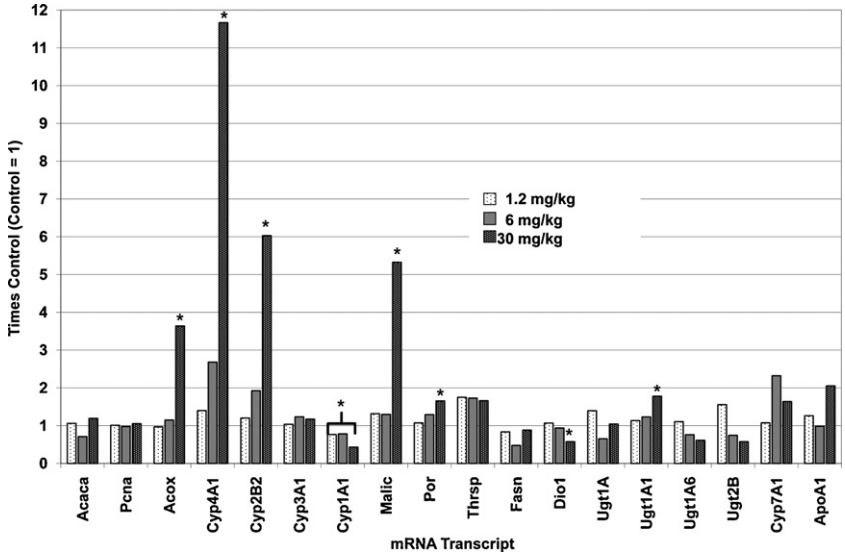

Fig. 2. Mean liver tissue mRNA transcript levels by dose group as measured by quantitaitive rt-PCR for male rats after 90 daily oral doses of NH_4^+PFBA. Quantitative rt-PCR data were normalized to 18s mRNA, and expressed in the figure as times the control transcript level for the individual transcript. Statistically significant results are indicated by an asterisk.

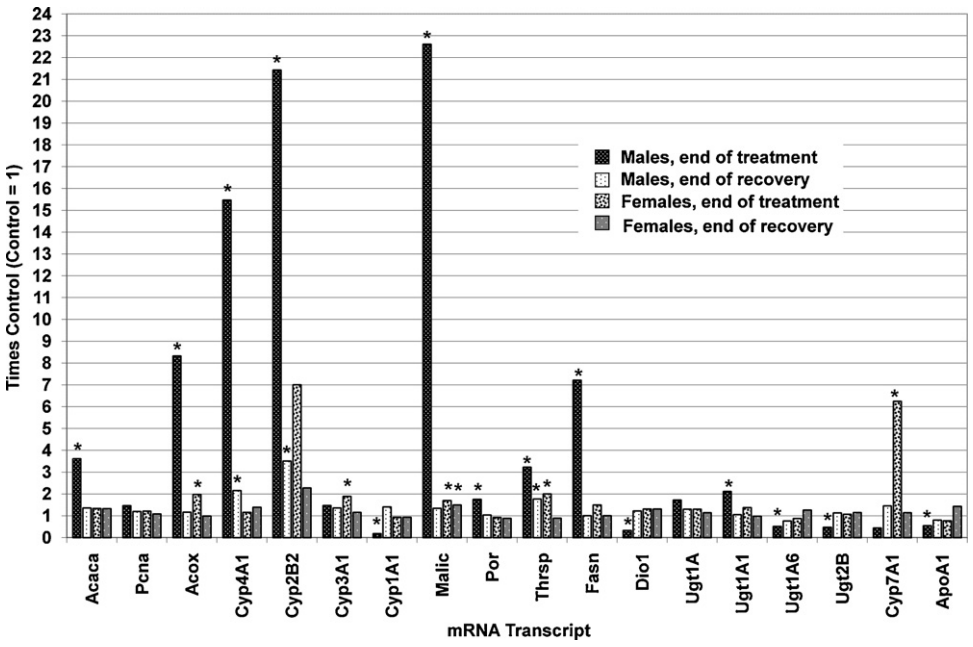

Fig. 3. Mean liver tissue mRNA transcript levels by sex as measured by quantitaitive rt-PCR for male and female rats after 28 daily oral doses of 30 mg/kg-d NH_4^+PFOA and after 21 days of recovery. Quantitative rt-PCR data were normalized to 18s mRNA, and expressed in the figure as times the control transcript level for the individual transcript. Statistically significant results are indicated by an asterisk.

of mean control body weight at the end of treatment. At the end of the recovery period, mean male body weight was still reduced to 85% of the control mean. This reduction in mean body weight likely affected the increases in relative organ weight observed for brain, kidneys, testes, epididymides, and liver in NH_4^+PFOA-treated males. Mean heart and thymus weights were reduced in NH_4^+PFOA-treated males on an absolute basis, but not relative to body weight. However, liver weight was increased on both an absolute and relative basis, with absolute and relative liver weights liver being approximately 1.9 and 2.6 times that of controls at the end of treatment. Microscopic effects in the liver of NH_4^+PFOA-treated males included slight-to-moderate hepatocellular hypertrophy and minimal coagulative necrosis.

For NH_4^+PFBA-treated males, liver hypertrophy was observed in the absence of either clinical or microscopic evidence of liver injury and was fully reversible on cessation of treatment. PPARα activation is a known mode of action for hepatic hypertrophy in rodents [48,49], and the quantitative RT-qPCR mRNA transcript data for Acox and Cyp4A1 clearly show evidence for activation of PPARα at doses associated with hepatic hypertrophy. In addition, increased Cyp2B2 mRNA transcript levels at the 30 mg/kg-d dose in the 90-day study provide evidence for increased activation of CAR, which may have also contributed to the hepatic hypertrophic response [49]. The reversibility of the increased liver size correlated with reversibility in the expression level of transcripts associated with PPARα and CAR. Comparing liver responses between the 28-day and 90-day studies, increasing length of treatment by a factor of approximately 3 did not decrease the dose at which significant liver-weight effects were observed, nor did signs of overt liver toxicity appear on extending the dosing period.

The greater liver weight and hepatocellular hypertrophic response observed in rats treated with NH_4^+PFOA as compared to those treated with NH_4^+PFBA correlated with a somewhat greater increase in liver concentrations of mRNA transcripts regulated by PPARα (Acox and Cyp4A1) and an increase in the CAR-regulated Cyp2B2 that was more than 20 times control. Although decreased by the end of the recovery period, Cyp4A1 and Cyp2B2 mRNA transcript levels remained elevated in NH_4^+PFOA-treated males as compared to controls, corresponding to the 20% increase in relative liver weight and 40% incidence of slight hepatocellular hypertrophy observed at the end of recovery.

The hypothyroxinemia observed in males on dosing with NH_4^+PFBA and in males and females dosed with NH_4^+PFOA likely resulted from a combination of competitive displacement of T4 as well as increased metabolism and elimination of T4. Hypothyroxinemia was not accompanied by an elevation of TSH. In fact, male rats dosed with NH_4^+PFOA had a statistically significant reduction in TSH compared to controls. Nor was dosing with NH_4^+PFBA accompanied by evidence of a hypertrophic or hyperplastic response of the thyroid follicles, based on morphometric endpoints. Morphometric analysis of the thyroids of male rats given NH_4^+PFOA suggested a response with regard to follicular epithelial cell height, but the colloidal area was normal. Elevations of liver concentrations of mRNA transcripts for Ugt1A1, known to conjugate T4, were observed with both NH_4^+PFBA and NH_4^+PFOA. These observations suggest that the observed hypothyroxinemia may be due to a combination of increased displacement, tissue uptake, conjugation, and elimination of T4, as has been described for PFOS [50–52]. Indeed, in subsequent work, we have found evidence for displacement of T4 by PFBA in rat sera [45].

A FT4 reference method employing equilibrium dialysis (ED) was used due to prior observations that analogue assays for FT4 were biased to lower values in the presence of μg/mL serum concentrations of PFOS [44]. Despite the use of the ED method, decreases in measured values of FT4 were observed in male rats treated with NH_4^+PFBA and NH_4^+PFOA. In the case of treatment with NH_4^+PFBA, there was no effect on serum TSH. However, with NH_4^+PFOA treatment, TSH was decreased with statistical significance in male rats, suggesting abundant T4 signaling in the hypothalamic-pituitary-thyroid (HPT) axis. In males given NH_4^+PFOA, liver concentrations of transcripts known to be regulated by the thyroid receptor were also increased. These included malic enzyme (malic) [53], NADPH Cyp450 oxidreductase (Por) [54,55], thyroid hormone responsive protein (Thrsp) [56], and fatty acid synthase (Fasn) [57]. Malic and Por may also be regulated by

PPARα [58–61], and a contribution by PPARα cannot be ruled out. The responses were not consistent with a reduction in FT4.

It has been well documented that an underestimation of FT4 by ED is known to occur when water-soluble (low affinity) competitors of T4 are presented in the serum [46,47]. During dialysis, these water-soluble (low affinity) T4 competitors are capable of migrating across the dialysis membrane from serum compartment. As a result, T4 are often drawn away from the serum compartment into the dialysis buffer due to its affinity with these competitors. Overall, this causes an artificially lowered T4 equilibrium in serum. Since the ED data were obtained, we have determined that ED gave an underestimate of serum FT4 in the presence of PFBA due to its high solubility in aqueous solution. Therefore, it is not possible to accurately interpret the FT4 data from our study. Fortunately, the TSH determinations are diagnostic for thyroid status, and we have determined that the HPT axis remains functional in the presence of PFBA [45].

Of the few alterations in clinical chemistry values in male rats treated with NH_4^+PFBA, the reduction in serum total cholesterol in the 28-day study is perhaps of most interest from a toxicological point of view. The lowering of serum total cholesterol observed in 28-day study at 30 and 150 mg/kg-d likely resulted, at least in part, from events tied to activation of PPARα based on the observation of increased transcription levels of Acox and Cyp4a1 at these dose levels. Although these transcripts were also elevated in the 90-day study males at the 30 mg/kg-d dose level, there was not a statistically significant reduction in total serum cholesterol; although, a non-statistically significant 15% reduction in mean serum total cholesterol was present in males of the 30 mg/kg-d dose group at the end of the treatment period, consistent with the 20% reduction observed at the same dose in the 28-day study. PPARα agonists, such as the fibrate class of drugs, increase HDL and decrease non-HDL. It is perhaps of note that NH_4^+PFBA treatment in the 28-day study appeared to decrease ApoA1 mRNA transcript levels, a finding that was not present in the 90-day study when measured at termination of the treatment period. This observation may suggest that the formation of HDL cholesterol is down-regulated, as has been recently observed with perfluorooctanesulfonate (PFOS) and perfluorohexanesulfonate (PFHxS) [62]. This reduction in HDL may be a result of activation of the xenosensor nuclear receptor, pregnane X receptor (PXR), resulting in reduced synthesis, maturation, and clearance of HDL [62,63]. However, in the 28-day study, increased transcription of Cyp3A1, a PXR-regulated gene, was not observed when measured at the end of the treatment period. It is noteworthy that Loveless et al. [64] have observed decreased non-HDL and HDL cholesterol after gastric lavage treatment of rats and mice with NH_4^+PFOA for 14 days; although, in the study reported herein, treatment of rats with NH_4^+PFOA did not result in statistically significant changes in total serum cholesterol. In addition, in the 90-day study, there was evidence for increased transcriptional expression of the xenosensor nuclear receptor CAR based on a several-fold, statistically significant elevation of Cyp2B2 mRNA transcript levels. Activation of CAR can increase lipogenesis and repress fatty acid β-oxidation [65]. In the male rats of the current study treated for 28-day with NH_4^+PFOA, a strong induction of transcript levels for Cyp2B2 was observed (Fig. 3). While it is not possible to understand fully the biochemical basis for the observed reduction in serum total cholesterol in NH_4^+PFBA-treated males in the 28-day study, the above observations may provide some leads for further research.

Minor but statistically significant hematological changes in red blood cell parameters occurred in male rats given 30 mg/kg-d $NH4^+$PFBA and $NH4^+$PFOA for 90-day and 28-day, respectively. These included reduced red blood cell counts, hemoglobin and hematocrit level and increased red cell distribution width. These observations occurred in the absence of evidence for compensatory increased hematopoiesis, because neither increased circulating reticulocytes nor splenic extramedullary hematopoiesis were observed. Furthermore, spleen weights were not increased, and there was no evidence for increased red blood cell degradation such as increased splenic hemosiderosis or serum bilirubin. Therefore, these minor changes in red blood cell parameters, although statistically significant, are of unlikely toxicological significance due to the small magnitude of change and lack of evidence for a direct effect on red blood cell turnover.

Even though the changes in red blood cell parameters observed were minor in magnitude and of no real toxicological consequence, it is of interest that a similar pattern in red cell parameter changes has been observed in toxicological studies of other perfluoroalkyls with rats. This pattern has been observed in male rats dosed by gastric lavage with the potassium salts of perfluorobutanesulfonate (PFBS) [66] and perfluorohexanesulfonate (PFHxS) [67], in female (but not male) rats fed potassium salt of perfluorooctanesulfonate (PFOS) [68], and in male and female rats treated by gavage with the sodium salt of perfluorohexanoate (PFHxA) [69]. Curran et al. [68] have suggested that the observation of a decrease in RBC, HGB, and HCT in females at the highest dietary dose (100 ppm) of PFOS in their study was consistent with their observation of increased RBC membrane rigidity (decreased membrane deformability), increased unconjugated bilirubin, and increased spleen to body weight ratios in female rats. For the male rats of the study reported herein that were treated for 90 days with NH_4^+PFBA, splenic weights were unaffected, reticulocytes were normal, and total bilirubin was actually decreased by the end of the treatment period in a dose-dependent manner and with statistical significance occurring in the 6 and 30 mg/kg-d groups. In the study by Loveless et al. [64] with sodium PFHxA, a close analogue of NH_4^+PFBA, spleen weights relative to body weights were unaffected by treatment of either sex and total bilirubin was reduced in both sexes; however, reticulocytes were elevated in both sexes at the dose at which the red cell parameters were affected. Further, targeted evaluations may shed additional light on the etiology of these observations.

The delayed pupillary reflex response noted in males given either NH_4^+PFBA (150 mg/kg-d and 30 mg/kg-d in 28-day and 90-day studies, respectively) or NH_4^+PFOA (30 mg/kg-d in 28-day study) was not accompanied by histological changes in neuronal tissues, including the optic nerve, or changes in other neurotoxicological parameters. In order to obtain ocular tissues for more detailed analysis, three recovery rats in the control and high-dose group of 90-day study were reassigned to obtain tissues at the end of the dosing period for additional study of the potential etiology of this effect. Additional work, to be reported separately, suggests that the delayed pupillary response results from effects on retinal cell populations at higher levels of exposure (Dr. Donald Fox, personal communication).

The 30 mg/kg-d NH_4^+PFOA dose was included in the design of the 28-day study in order to provide context back to the rather large toxicological database for PFOA. (Most toxicological testing with PFOA has been undertaken using the NH_4^+ salt, principally because the NH_4^+ salt was the form most used in the fluoropolymer industry.) In the 28-day study reported herein, any effects attributable to treatment with NH_4^+PFBA were also observed with NH_4^+PFOA, typically in exaggerated form. In addition, the NH_4^+PFOA-treated rats showed decreased food consumption, weight loss, and physical signs of malaise. Also, several effects were noted in females given NH_4^+PFOA; whereas, female rats given NH_4^+PFBA were largely unaffected.

Serum and liver measurements of PFBA and PFOA from samples taken at terminal sacrifice support a role for differences in pharmacokinetic handling of PFBA and PFOA in causing the observed differences in potency of effects between rats treated with

NH_4^+PFBA and NH_4^+PFOA. On a molar-dose equivalent, 30 mg/kg-d of NH_4^+PFBA and NH_4^+PFOA convert to 130 and 70 μmol/kg-d, respectively. Thus, rats dosed at 30 mg/kg-d NH_4^+PFBA were receiving nearly twice the molar dose of PFBA as compared to the molar dose of PFOA in rats dosed with NH_4^+PFOA. Even so, mean μM serum PFBA concentrations in male rats given 30 mg/kg-d NH_4^+PFBA were approximately one-half the mean μM serum PFOA concentrations in male rats given 30 mg/kg-d NH_4^+PFOA (178 μM vs. 354 μM, respectively). An even more pronounced difference was found between mean liver PFBA and mean PFOA μmol/kg concentrations for male rats given 30 mg/kg-d of NH_4^+PFBA and NH_4^+PFOA, respectively. Liver PFBA concentration was 80 μmol/kg as compared to liver PFOA concentrations of 402 μmol/kg, a five-fold difference. Mean male serum and liver PFBA concentrations at the 150 mg/kg-d dose of NH_4^+PFBA were 385 μM and 173 μmol/kg, respectively, showing that mean serum PFBA in this dose group was roughly equivalent to mean serum PFOA in the 30 mg/kg-d NH_4^+PFOA dose-group males, yet liver PFBA μmol/kg concentration was roughly half of that for the NH_4^+PFOA-treated males. These differences are not unexpected based on the known differences in pharmacokinetics between PFBA and PFOA in the rat [1,35], which may be influenced by the observation that PFBA does not appear to be a substrate for renal tubular reabsorption, as is the case for PFOA [34,70]. Even so, in assays with primary rat and human hepatocytes in culture, PFBA has been found to be considerably less potent than PFOA on a μmolar basis in increasing the transcriptional expression or activity of enzymes associated with PPARα activation [15,26]. Thus, the attenuated relative response of rats to dosing with NH_4^+PFBA as compared to NH_4^+PFOA is likely the result of both the more rapid pharmacokinetic clearance and lesser pharmacodynamic potency of PFBA.

Based on observations from both the 28-day and 90-day studies, a definitive no observed adverse effect level (NOAEL) can be established for oral exposure of male and female rats to NH_4^+PFBA for each exposure duration. For female rats, NOAEL values of 150 and 30 mg/kg-d can be established for the 28-day and 90-day studies, respectively, both dose levels being the highest dose in their respective study. Based on analysis of dosing solutions, these NOAELs correspond to actual doses of approximately 130 and 32 mg/kg-d. For males, a NOAEL of 6 mg/kg-d, corresponding to actual doses of approximately 5 and 7 mg/kg-d for the 28-day and 90-day studies respectively, can be established; although, the recorded changes in males at 30 mg/kg-d (including minimal-to-slight hypertrophy/hyperplasia of the follicular epithelium of the thyroids that were not confirmed by histomorphometric analysis, hepatocellular hypertrophy, increased liver weights, and slight hematological and clinical biochemistry changes) did not constitute clear functional or morphological deficits. These changes were therefore not considered to be of primary toxicological importance. However, based on the number of variations in parameters of males at 30 mg/kg-d, a male rat NOAEL of 6 mg/kg-d (5–7 mg/kg-d based on analysis of dosing solutions) would be considered appropriately conservative for both studies.

The 6 mg/kg-d nominal dose level in male rats led to mean serum PFBA concentrations of approximately 25 and 14 μg/mL at the end of 28 and 90 days of treatment, respectively. Based on the analysis of 600 (301 male and 299 female) American Red Cross adult blood donor serum samples obtained in 2006 from six regional blood donation centers for concentration of PFBA, Olsen et al. [8] have recently reported a geometric mean serum PFBA concentration of 0.00033 μg/mL (95% CI 0.00030–0.00036 μg/mL). This geometric mean serum PFBA is approximately 42,000 times lower than the 14 μg/mL serum PFBA concentration associated with the male rat NOAEL. In that the serum elimination half-life of PFBA in humans has been estimated at approximately 3 days versus approximately 0.4 days in the male rat [1], demonstrating relatively good elimination in humans compared to PFOA, there appears to be a large margin of exposure for the general population, even when considering potential pharmacokinetic differences.

The pattern of effects observed in the liver of male rats treated with NH_4^+PFBA is consistent with the known pleiotrophic effects of activation of the xenosensor nuclear receptors PPARα and CAR in rodents. In view of established differences between rodents and humans in response to PPARα [48,49,71] and CAR [49,72] activation, human exposure to PFBA would be expected to be of less consequence than the exposure of rodents to PFBA. In fact, a study by Bjork and Wallace [26], showed a relative lack of response of human hepatocytes in primary culture to PFBA as compared to rat hepatocytes. In addition, Foreman et al. [24] have shown that, in comparing the hepatic response to PFBA in mice expressing the human form of PPARα with that in wild-type mice, a PPARα-dependent increase in PFBA-induced hepatocyte focal necrosis with inflammatory cell infiltrate was mediated by the mouse PPARα but not the human PPARα. Thus, there is direct evidence for a reduced human response to the hepatic effects of PFBA exposure.

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